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Current status of natural products targeting Alzheimer's disease

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Current status of natural products targeting Alzheimer's disease

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2-Mercaptobenzimidazole clubbed hydrazone for Alzheimer's therapy: *In vitro*, kinetic, *in silico*, and *in vivo* potentials

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Alzheimer's is a type of dementia that affects the affected person's thinking, memory, and behavior. It is a multifactorial disease, developed by the breakdown of the neurotransmitter acetylcholine via acetylcholinesterase (AChE). The present study was designed to evaluate potential inhibitors of acetylcholinesterase that could be used as a therapeutic agent against Alzheimer's disease (AD). For this course, synthetic compounds of the Schiff bases class of 2-mercaptobenzimidazole hydrazone derivatives (**9–14**) were determined to be potent acetylcholinesterase inhibitors with IC_{50} values varying between 37.64 ± 0.2 and 74.76 ± 0.3 μM. The kinetic studies showed that these are non-competitive inhibitors of AChE. Molecular docking studies revealed that all compounds accommodate well in the active site and are stabilized by hydrophobic interactions and hydrogen bonding. Molecular dynamics (MD) simulations of selected potent inhibitors confirm their stability in the active site of the enzyme. Moreover, all compounds showed antispasmodic and Ca^{2+} antagonistic activities. Among the selected compounds of 2-mercaptobenzimidazole hydrazone derivatives, compound **11** exhibited the highest activity on spontaneous and K^+-induced contractions, followed by compound **13**. Therefore, the Ca^{2+} antagonistic, AChE inhibition potential, and safety profile of these compounds in the human neutrophil viability assay make them potential drug candidates against AD in the future.

Abbreviations: Ach, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; DTNB, 5-dithiobis 2-nitrobenzoic acid; ED_{50}, effective dose; ELISA, enzyme-linked immune-sorbent assay; ES, enzyme substrate; hb, hydrogen bonding; I-TASSER, iterative threading assembly refinement; MD, molecular dynamics; mhz, Megahertz; MOE, molecular operating environment; NMR, nuclear magnetic resonance; PAS, peripheral anionic site; PDB, protein data bank; RMSD, root mean square deviation; RMSF, root mean square fluctuations; TLC, thin layer chromatography

KEYWORDS

acetylcholinesterase, Alzheimer's disease, 2-mercaptobenzimidazole derivatives, molecular docking, molecular dynamic (MD) simulation

1 Introduction

Alzheimer's is a neurodegenerative disease characterized by memory loss, behavioral disturbances, and cognitive problems (Deture and Dickson, 2019; Kaur et al., 2021). Alzheimer's disease (AD) is related to insufficiency of functions in the basal forebrain and cortex (Shekari and Fahnestock, 2019; Liu et al., 2022). Cholinergic neurotransmission impairment negatively affects learning and memory loss in AD patients. It has been reported that inhibition of these cholinesterases, which leads to the activation of cholinergic function, may be an effective method for AD treatment (Marucci et al., 2021; Rong et al., 2021). Cholinesterases like acetylcholinesterase (AChE) are the key enzymes for regulating neurotransmission by hydrolysing acetylcholine (ACh) in cholinergic neurons (Massoulié et al., 1993; Aramjoo et al., 2021). AChE is a membrane-bound multi-subunit enzyme mainly present in cholinergic neurons, the brain and muscles. In the human brain, the majority of the AChE is found in the membrane-bounded tetrameric G4 form, and its level decreases as the degeneration of the neurons occurs (Manna et al., 2008). It plays a significant role in the management of various physiological reactions by hydrolysing ACh in cholinergic synapses (Schetinger et al., 2000; Kaur et al., 2021). Usually, in the brains of AD patients, the activity of AChE remains unchanged or declines (Kumar et al., 2018; Karthika et al., 2022). Currently available synthetic acetylcholinesterase inhibitors, including galanthamine, rivastigmine, donepezil, and tacrine, have been clinically used for AD treatment (Moreira et al., 2022). However, these drugs have therapeutic activity along with side effects such as short duration of biological action, gastrointestinal disturbance, low bioavailability, and hepatotoxicity (Reza et al., 2018). Due to the adverse effects of previously approved drugs, new AChE inhibitors are of great interest for the treatment of AD (Kabir et al., 2021). Schiff bases are significant organic compounds involved in several biological activities like α-glucosidase, urease, β-glucuronidase, and antiglycation (Khan et al., 2011; Taha et al., 2019a; Taha et al., 2019b).

The study of new biologically important Schiff bases has been drawing the attention of pharmacists and chemists (Matela, 2020). Previous studies indicate that the lone pair of nitrogen atoms of the azomethine group of Schiff bases is chemically and biologically significant (Ashraf et al., 2011). The nitrogen atom also participates in the creation of hydrogen bonds with active cell constituent centers and interferes with cell functions. The azomethine or imine (–C=N–) group in Schiff bases is also found to be a versatile pharmacophore for the development of new drugs (Hameed et al., 2017; Vanitha et al., 2022). Gallic hydrazide-derived Schiff bases are ketone derivatives with antioxidant activity that also show AChE inhibition and are considered as a potential treatment for AD. Schiff bases are also involved in biological activities like antimicrobial (Da Silva et al., 2011), anticancer (Tadele and Tsega, 2019) and herbicidal activities (Zhu et al., 2016). Moreover, it has been investigated that Schiff base compounds have antiviral (Kumar et al., 2010), anthelmintic (Husain et al., 2018), antiprotozoal (Kordestani et al., 2021) and anticonvulsant activities (Nilkanth et al., 2020).

The current study was designed to evaluate AChE inhibitory activity and the kinetics of compounds (**9–14**). Moreover, binding interactions and stability of these potent inhibitors were determined using molecular docking and molecular dynamic (MD) simulation.

2 Materials and methods

During this experimental study, analytical grade solvents were used. The purity of products was monitored through alumina plates, and the melting point was determined using a hot-stage Gallenkamp melting point apparatus (Loughborough, United Kingdom). Generally, n-hexane and ethyl acetate solvent medium were used to check reaction through plates. The progress of the reaction was monitored by thin-layer chromatography. An ultraviolet lamp was used as a visualizing agent.

The whole reaction was carried out in clean glassware with specific catalysts in basic or acidic conditions. All synthesized compounds were characterized by using different spectroscopic techniques such as 13C NMR 1H NMR replace with 13C NMR 1H NMR were performed on the Advance Bruker AM at 300, 400, and 500 MHz. Thin Layer Chromatography (TLC) was performed on pre-coated silica gel aluminum plates with dimensions of 3 × 8 cm (Kieselgel 60, 254, E. Merck, Germany). The chromatogram was visualized with dual wavelengths of UV at 254 and 365 nm. The melting point was found on the Gallon kemp apparatus.

2.1 General procedure for the synthesis of novel hydrazone derivatives on 2-mercaptobenzimidazole (9–14)

2-Mercaptobenzimidazole based hydrazone derivatives synthesis were carried out through multistep reactions. First, 2-mercaptobenzimidazole was refluxed with bromoethane in

basic conditions (KOH) in ethanol with equimolar amounts for about 10 h. After completion of the reaction, the reaction mixture was filtered. The filtrate so obtained was kept until the whole ethanol was evaporated and got shiny white needle-like crystals of 2-ethylthio benzimidazole. In the second step, 2-ethylthio benzimidazole was taken in a round bottom flask and refluxed with ethyl chloroacetate (dropwise) using anhydrous potassium carbonate in DMF (solvent) for about 15 h. After completion of the reaction, the product (2-(2-(ethylthio)benzimidazolyl) acetate) was obtained and got through a separating funnel in semisolid form. In the third step, 2-(2-(ethylthio)benzimidazolyl) acetate was refluxed in methanol with hydrazine hydrate for about 10 h. The product, 2-((ethylthio)benzimidazolyl) acetohyrazide get was poured into ice-cold water until a precipitate was formed. The precipitate was filtered and then dried in an open atmosphere. In a fourth step, (ethylthio) benzimidazolyl) acetohyrazide was dissolved in methanol with 2-3 drops of acetic acid (catalyst) on a hotplate. After 10 min, aldehyde was added and refluxed into the whole mixture for about 5–6 h. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was poured into ice-cold water until a precipitate was formed. The precipitate was collected by filtration, washed with water, and then dried in an open atmosphere.

2.2 General

All chemicals used in the current study were of analytical grade. The acetylcholine iodide (Cat. No. A7000-25G), acetylcholinesterase EC 3.1.1.7 (Cat. No. C3389-2KU), DTNB (5-dithiobis 2-nitrobenzoic acid) (Cat. No. D8130-10G), Galanthamine (Cat. No. 69353-21-5) (MO, United States). Di-Sodium hydrogen phosphate (Cat. No. 558-79-4), sodium phosphate monobasic dehydrate buffer (Cat. No. 1342-35-0) and ethanol (Cat. No. 64-17-5) were purchased from Sigma Aldrich. All compounds of 2-mercaptobenzimidazole hydrazone derivatives (**9–14**) were synthesized as described earlier (Yousaf et al., 2020).

2.3 *In vitro* acetylcholinesterase inhibition assay

AChE inhibitory activity was evaluated as described previously with few modifications using a microtiter plate reader (Molecular Device, CA, United States) (Hasan et al., 2005). Initially, 100 mM of sodium phosphate buffer (140 µl) pH 8, 0.25 mM DTNB (10 µl), 0.5 mM of synthetic compounds dissolved in 20 µl of ethanol and AChE (20 µl) were mixed and incubated for 15–20 min at room temperature in a microtiter plate. Finally, 10 µl of substrate ACh (0.4 mM) was added and incubated at room temperature for 4–6 min. The reaction started after the addition of ACh was hydrolyzed by AChE in the presence of DTNB; a yellow-colored 5-thio-2-nitrobenzoate anion was formed, which indicates the reaction completion and was read at a wavelength of 412 nm. All experiments were performed in triplicate and were analyzed using the SoftMax Pro6.3 program (Molecular Device, CA, United States).

Percentage inhibition was calculated by

$$\%\ \text{inhibition} = (\text{O.D control} - \text{O.D test well}) / \text{O.D control}) \times 100.$$

2.4 Evaluation of kinetics parameters

The IC_{50} is the quantity of a test compound required for 50% inhibition. IC_{50} was determined at various concentrations, and EZ-Fit EK was employed to calculate the IC_{50} values of the test compounds (Perrella Scientific Inc., Amherst, MA, United States).

Enzyme-substrate (ES) is the complex of AChE and acetylcholine, while P represents the product formed after the reaction's completion. Dissociation constant values were calculated through Dixon plots, their secondary replots, and the Lineweaver-Burk plot (Khan et al., 2021).

The values of K_m, K_i, and V_{max} were determined from Dixon and Lineweaver-Burk plots using no linear regression equation. K_i values were determined by using the Lineweaver-Burk plot; first, values of $1/V_{maxapp}$ were found at every intersection point of the lines of each test compound concentration on the y-axis. On the Lineweaver-Burk plot, the slope of each line obtained as a result of the compound was plotted against different concentrations of the test compounds.

2.5 Statistical analysis

GraFit software (Khan et al., 2021) was utilized for plotting graphs. The values of the correlation coefficients, slopes, intercepts, and their standard errors were determined by the linear regression equation using the same program.

2.6 Homology modelling

In the current study, electric eel AChE was used in the laboratory to perform an AChE inhibitory assay, but for computational analysis, we used hAChE (4EY6). Therefore, homology modeling was performed to check the similarity between the electric eel and hAChE.

The eel 3D crystal structure is not available. So, the 3D structure of electrophorus electric eel AChE was predicted using the Iterative Threading Assembly Refinement (I-TASSER) (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). I-TASSER prioritized the five best models from which the model with the best C-Score of −0.15 was selected with an accuracy value

of 0.69 and a root mean square deviation (RMSD) value of 8.2 Å. C-score is a confidence score for assessing the quality of prioritized protein structure models by I-TASSER. The 3D model of electrophorus electric eel was then superposed on the 4EY6 retrieved from PDB (Figure 1) to check the similarity between both structures. Both the model and 4EY6 structures were exactly aligned with each other. Therefore, (4EY6) was selected for further molecular docking and MD simulation study.

2.7 Molecular docking

Molecular docking was conducted to predict the binding interactions between AChE and tested compounds using the MOE-Dock (Molecular Operating Environment-docking) (www.chemcomp.com) program. The results of docking studies were correlated with the experimental outcomes. The ligands and receptors were prepared before being subjected to molecular docking.

2.8 Preparation of protein

The 3D structure of 4EY6 at a resolution of 2.40 Å was retrieved from the protein databank (PDB) (https://www.rcsb.org/) and was used in further analysis. Before docking, the AChE was prepared by playing the molecule to add hydrogen atoms, correct bonds, and complete chains (Martínez-Rosell et al., 2017). All crystallographic molecules such as water were removed from AChE, then energy was minimized after the 3D protonation using the default parameters of the MOE energy minimization algorithm.

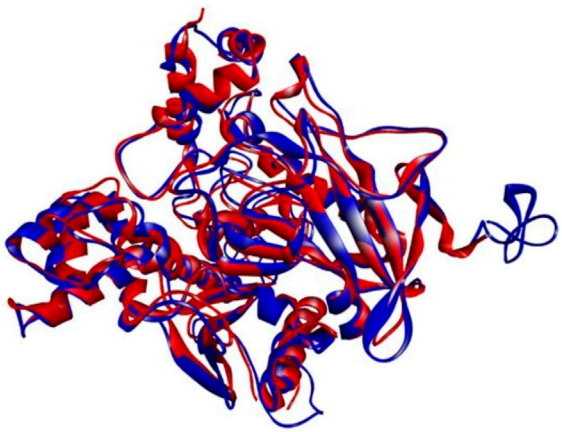


FIGURE 1
Structural alignment of electric eel AChE (red) and hAChE (blue).

2.9 Preparation of ligands

The 3D structures of all tested compounds were generated using a molecular building program in MOE. A database was designed and the energy of the tested compounds was minimized up to a 0.05 gradient by employing the MMFF94x force field. All the AChE interacting compounds were docked into the AChE binding pocket as described earlier (Fang et al., 2014). Molecular docking protocols were validated by re-docking a co-crystallized ligand (galanthamine) in the active pocket of AChE 4EY6 (Supplementary Figure S1).

2.10 MD simulation of the inhibitor with AChE

Top docking score poses of compound **11** and **13** complexes with AChE were subjected to MD simulations using the GROMACS 5.1.2 software package (Berendsen et al., 1995). The protein-ligand complex system has been prepared by using the pdb2gmx module in the GROMACS package. The GROMACS OPLS-AA/L force field (Berendsen et al., 1995) was utilized for parameterization and the generation of the topology of the native protein. Ligand topologies were separately prepared using the swissparam web server (Zoete et al., 2011). Each complex was solvated in a cubic box with a size of (10 ×10 ×10), encompassing approximately 33436 TiP3P water molecules. To neutralize the whole system with a salt ion environment, the Genion module in GROMACS was utilized under physiological conditions (NaCl 0.15 M). Finally, nine sodium ions were added to neutralize the ligand enzyme complex. The steepest descent minimization algorithm with PR (position restrain) of ligands and protein was used to minimize the complex until the maximal force of 10 kJ/mol (Nath et al., 2017) by eradicating errors in atomic position and structural disputes such as bond angle, bond length, and also the structural clashes between the ions, the position of water molecules, and the protein complex (Yang et al., 2019). Two additional equilibration steps were performed in a sequential process: 1) at constant temperature (300 K) by utilizing isothermal-isochoric ensemble (NVT) [No. of Particles (N) system volume (V) and temperature (T)] programs and 2) under fixed stabilized 1 bar pressure through NPT [No. of particles (N), system Pressure (P), temperature (T)] ensemble system. The ensemble would run 50,000 steps for 0.1 ns. After equilibration steps, the temperature and pressure stabilized complex system was subjected to position restraining where the molecules of solvent in the cubic box were fully dissolved with the protein-ligand complex system. Finally, the position restrained protein-ligand complex was used to simulate for 10,000,000 ps at 300 K (V-rescale thermostat) temperature, atmospheric NPT ensemble pressure (Parrinello-Rahman barostat), and periodic boundary conditions for 0.002 ps using

leap-frog algorithms. In order to constrain all the hydrogen bonds, the LINC algorithm was applied during the whole equilibration (Yuan et al., 2012), whereas the Particle Mesh Ewald (PME) module with 0.16 Å Fourier grid spacing has been functionalized (Petersen, 1995). The entire trajectories have been saved at a frequency of 2 fs time step rate during the simulation for further analysis.

2.11 Cytotoxicity testing

A cytotoxicity test was performed to determine the adverse effect of tested compounds on humans using neutrophil cells.

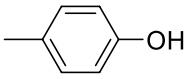
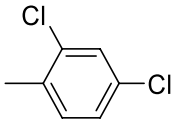
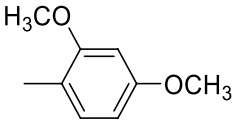
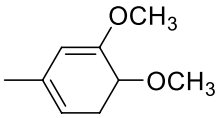
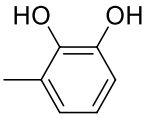
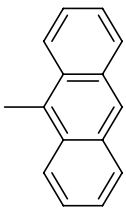
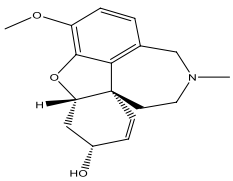
2.11.1 Viability of human neutrophil cells

Heparinized whole blood of healthy volunteers was obtained from a local blood bank, and neutrophils were separated using the Choudhary et al. protocol with slight modifications as previously described (Choudhary et al., 2005).

2.11.2 Assay procedure

The isolated neutrophils (1×10^7 cells/ml) were incubated for 30 min with inhibitors; after that, 0.25 mM WST-1 (water-soluble tetrazolium salt) was added, the microtiter plate was incubated at 37°C in a water bath shaker for 3 h and absorbance was calculated at 450 nm using a microplate reader (Spectra-MAX, CA, United States). The absorbance is the mean of five experimental replicates.

TABLE 1 Inhibitory activities of 2-mercaptobenzimidazole hydrazone derivatives (9–14) against AChE.

Compound	R	Chemical formula	IC_{50} ± SEM (μM)	Docking score
9		$C_{18}H_{18}N_4O_2S$	74.76 ± 0.3	−9.816
10		$C_{18}H_{16}Cl_2N_4OS$	51.23 ± 0.2	−12.950
11		$C_{20}H_{22}N_4O_3S$	37.64 ± 0.2	−15.455
12		$C_{20}H_{22}N_4O_3S$	51.07 ± 0.5	−11.019
13		$C_{18}H_{18}N_4O_3S$	50.58 ± 0.3	−14.560
14		$C_{26}H_{22}N_4OS$	51.23 ± 0.2	−12.572
Galantamine			26.03 ± 0.4	−17.301

The % viability of neutrophils was measured by the following formula:

$$\text{Percentage viability}: \{(\text{O.D test} \times 100/\text{O.D control}) - 100\} - 100.$$

2.12 Calcium ion channel blocking and spasmolytic activities

The spasmolytic activity of the inhibitors was evaluated using isolated contracting rabbit jejunum (Rahman et al., 2019). Rabbits used in the current study were provided by Aga Khan Medical University, Karachi, Pakistan, weighing between 1.5 and 2.0 kg. The standard operating protocol has already been defined (Choudhary et al., 2005).

The rabbit jejunum displayed spontaneous rhythmic contractions under these experimental conditions, allowing us to observe spasmolytic activity directly without the use of an agonist. Calcium antagonistic activity was also performed and confirmed the relaxation of 80 mM K^+ produced contraction.

3 Results and discussion

The chemistry of these compounds is in the Supplementary Material.

3.1 Biology

The synthetic 2-mercaptobenzimidazole hydrazone derivatives were screened against AChE. All compounds (**9–14**) showed significant inhibitory activities with IC_{50} values between 37.64 ± 0.2 μM to 74.76 ± 0.3 μM in different concentrations (Table 1). The most potent compound **11** of the series, with an IC_{50} value of 37.64 ± 0.2 μM, showed excellent inhibitory activity against AChE as compared to other compounds of the series but showed less potency as compared to standard galanthamine (IC_{50} = 26.03 ± 0.4 μM). The inhibitory potential of this compound may be due to the presence of strong electron-donating $-OCH_3$ groups at positions-2 and 4 in the benzene ring. The methoxy group has more electron-donating capacity at the ortho and para positions as compared to other positions. Compounds **13** and **12** are the second most active compounds in the series with IC_{50} values of 50.58 ± 0.3 and 51.07 ± 0.5 μM exhibit good inhibitory activity. This may be due to the presence of electron-donating groups, two –OH groups in compound **13** and two $-OCH_3$ in compound **12**. As previously reported, that –OH and OCH_3 are strong electron-donating groups and are involved in biological activities (Ullas et al., 2020). Compounds **10** and **14** are the third and fourth most potent compounds in the series, with the same IC_{50} (51.23 ± 0.2 μM) values. The activity of compound **10** could be associated with the electron-donating –Cl group, and the potency of compound **14** might be due to the delocalization of π-electrons in the anthracene ring, which is highly conjugated and show–M effect (Yousaf et al., 2020).

The compound **9,** with an IC_{50} value of 74.76 ± 0.3 μM contains an –OH group at the *ortho* position in the benzene ring, which is mostly associated with enhanced enzymatic activity, while in some cases, decreasing enzyme activity has also been observed. The increased activity of –OH group may be related to the involvement of oxygen and hydrogen interactions with various residues of the enzyme. In conclusion, the enhanced activities of these compounds may be due to the presence of electron-donating groups like $-OCH_3$, –OH, and –Cl.

3.2 Kinetic study

During kinetic studies, the reaction rate was measured and the effect of different concentrations of a substrate on the enzyme was investigated. The kinetic study of enzymes helped to determine the catalytic mechanism of enzymes. The substrate concentrations and enzyme activity relationship was first suggested by Leonor Michaelis and Maud Menten (Johnson and Goody, 2011). The rate of reaction [V] was plotted against various substrate concentrations [S] at constant enzyme concentration. Initially, the rate of reaction [V] increases as substrate concentration increases, and at higher [S], the enzyme becomes saturated, and

TABLE 2 Inhibition and kinetic parameters data of AChE in the presence of compounds (9–14).

Compound	K_i (μM) ± SEM	K_m (mM)	$K_m app$ (mM)	V_{max} (μmol/min)$^{-1}$	V_{maxapp} (μmol/min)$^{-1}$	Type of inhibition
9	24.36 ± 1.2	0.12	0.12	5.0	2.0	Non-competitive
10	17.01 ± 1.3	0.12	0.12	5.0	2.1	Non-competitive
11	14.01 ± 1.6	0.12	0.12	5.0	2.6	Non-competitive
12	19.22 ± 0.9	0.12	0.12	5.0	2.6	Non-competitive
13	19.03 ± 1.7	0.12	0.12	5.0	2.3	Non-competitive
14	19.23 ± 1.9	0.12	0.12	5.0	2.3	Non-competitive
Galanthamine	10.03 ± 0.7	0.12	0.12	5.1	2.6	Non-competitive

the rate of reaction reaches $V_{max,}$ which is referred to as the maximum rate of reaction. These synthetic compounds inhibited the enzyme AChE in a dose-dependent manner with the values of K_i (14.01–24.36 μM) (Table 2).

The dissociation constant K_i values were found using three different methods. Primarily, from the Lineweaver-Burk plot, which is the reciprocal rate of reaction (1/V) versus reciprocal of substrate concentration (1/S), helps to determine the effect of inhibitor on the values of K_m and V_{max}. Secondly, the Dixon plot is the plot between the reciprocal of initial velocities (1/V) versus different concentrations of the compound. Finally, from secondary replot, the slopes of each line versus various concentrations of all compounds. Kinetic studies were carried out to determine the inhibition type, which helps to specify the mechanism of action of enzyme inhibition and the inhibitor binding moieties. All three applied method results indicated non-competitive types of inhibition against AChE by synthetic compounds **9–14**. In all cases, K_m remains constant while V_{max} decreases. The values of K_i, K_m, V_{max}, and V_{maxapp}, along with the type of inhibition, are shown in Table 2. The graphical representation of steady-state inhibition for 2-mercaptobenzimidazole hydrazone derivatives (**9–14**) against AChE enzyme is shown in Figure 2.

3.3 Molecular docking results

Molecular docking was performed to find the binding interactions of the synthetic compounds in the active site of the enzyme AChE. All compounds were well accommodated in the binding pocket of 4EY6. Herein, the selected compounds based on docking score and IC_{50} values were considered.

Compound **11** was the most potent compound of the series (IC_{50} = 37.64 ± 0.2 μM, docking score = −15.455), which formed three hydrogen bonds with Ser-125, Tyr-124, and Asp-74. The amido group of compound **11** formed a strong hydrogen bond with Ser-125 having a bond length of 2.0 Å. Furthermore, one oxygen atom of the 1,3-dimethoxybenzene ring acted as an acceptor involved in hydrogen bonding with Asp-74 having a bond length of 2.4 Å, and the second oxygen atom of the methoxy group formed another hydrogen bond with aromatic amino acid Tyr-124 with a bond length of 2.0 Å of the peripheral anionic binding site (Figures 3A,B). These results were comparable with those of galanthamine (IC_{50} = 26.03 ± 0.4 μM, docking score = −17.301) which was used as a standard inhibitor of AChE. Gly121, Gly 122 of the oxyanion subsite and Tyr-124 of the peripheral anionic site (PAS) were found to be involved in the stabilization of the galanthamine-AChE complex. Similarly, Tyr-124 is involved in hydrogen bonding with the methoxy group of galanthamine with a bond length of 2.8 Å. Furthermore, Gly-121 and Gly-122 formed two hydrogen bonds with the same hydroxyl group of galanthamine with bond lengths of 1.8 and 1.9 Å, respectively (Figures 4A,B). The determined galanthamine inhibitory potential was found to be higher than compound **11**, as it is previously reported that, it may be due to the fact that galanthamine has interactions with all the four subsites (anionic and esteratic subsites) of the active site of the AChE (Choudhary et al., 2005). Whereas compound **11** has interactions mainly with PAS and does not interact with the amino acid residues of the catalytic traid (His-447, Ser-203, Glu-334). Therefore, compound **11** displayed a non-competitive type of AChE inhibition. The kinetic measurements agreed with the docking results, indicating compound **11** as a non-competitive inhibitor of AChE.

From the docking analysis of other members of the series, compound **13** was the second most active compound with a docking score −14.560 and an IC_{50} = 50.58 ± 0.3 μM. This compound also displayed good inhibitory activity but was less potent than the standard galanthamine (Table 1). The compound **13** formed two hydrogen bonds and one arene-arene interaction with Tyr-337, Tyr-341, and Trp-86, respectively. In the case of compound **13**, the pyrocatechol ring formed π-π stacking interaction with Trp-86 of

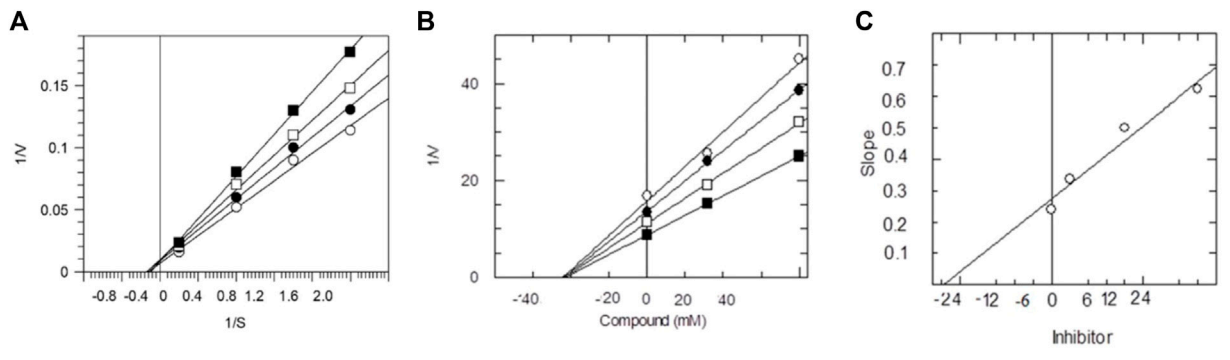


FIGURE 2
The inhibition of AChE by compound **9**, **(A)** is the reciprocal plot (Linewear-Burk) between initial velocities and four fixed ACh conc. in the absence (■) and presence of 100 μm (□), 150 μM (●), 175 μM (○) of compound **9**. **(B)** Dixon plot of reciprocal between initial velocities and different concentrations of compound **9** at constant ACh concentrations, (■) 100 μM, (□) 150 μM, (●) 175 μM and (○) 200 μM. **(C)** Plot between different concentrations of compound **9** and slope.

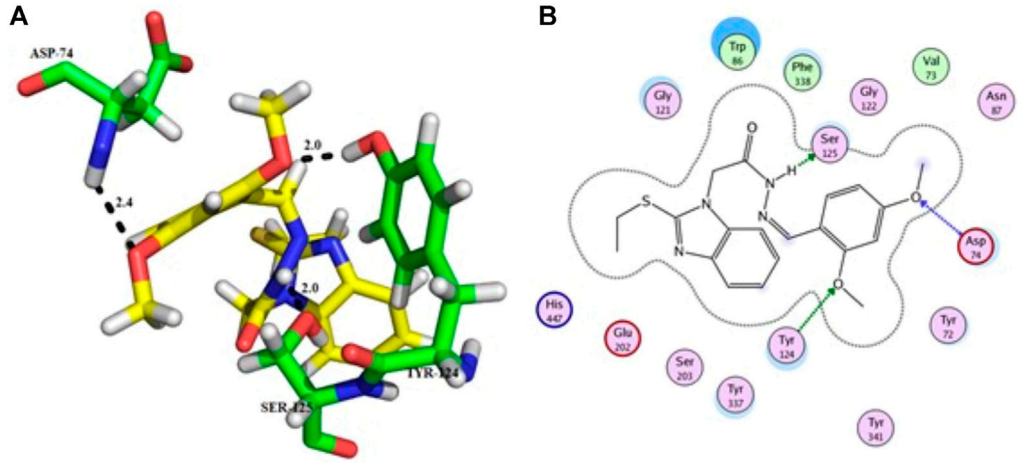


FIGURE 3
Showing the molecular docking conformation of compound **11 (A,B)** in the active site of AChE.

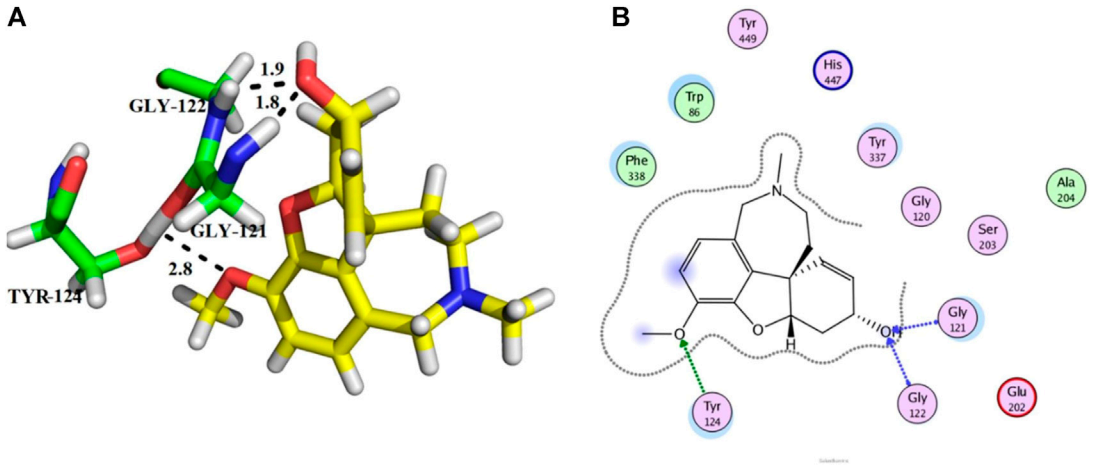


FIGURE 4
Showing the docking conformation of galanthamine (**A,B**) (positive control) in the active site of AChE.

the anionic subsite. While Tyr-337 of the anionic site and Tyr-341 of PAS subsite established strong hydrogen bonds with the imidazole ring of the same compound having bond lengths 2.3 and 2.6 Å, respectively, as shown in Figures 5A,B. Compound **13** is slightly less potent as compared to compound **11**; it may be due to one less hydrogen bonding in this compound. All the interactions of compound **13** are with anionic subsites; therefore, docking results agree with the experimental kinetic data, indicating a non-competitive type of inhibition.

Compounds **10** and **14** were the third most active members of the series, with the same IC_{50} = 51.23 ± 0.2 μM) and docking scores −12.950 and −12.572, respectively. These compounds also showed good inhibitory potential but were less potent as compared to standard galanthamine against AChE. From docking studies, it was noted that compound **10**'s imidazole ring formed two strong hydrogen bonds with Tyr-337 and Tyr-341, having bond lengths of 2.4 and 2.5 Å, respectively (Figures 6A,B). Compound **14** also mediated two polar interactions π-π interactions and hydrogen bonding with Tyr-341 and Ser-125 of the binding pocket. Ser-125 formed a strong H-bond with the carbonyl oxygen of the same compound, having a bond length = 2.9 Å. Tyr-341 also established two π-π linkages with anthracene moiety of compound **14**, as shown in Figures 6C,D. As all the four subsites are not involved in interactions with the ligand, therefore, docking results and kinetic

experiments agree with each other, indicating both compounds are non-competitive inhibitors of AChE.

3.4 MD simulations

Molecular docking gives the representation of the static conformation of the protein-ligand complex (Hassan et al., 2022). However, the ligand continuously moves in the pocket of the enzyme. Therefore, to observe the motion of ligands in the enzyme's active site, we performed MD simulations to explore different conformations and check the stability of our inhibitor-AChE complexes. Out of the six compounds, two potent compounds, **11** and **13,** were selected for MD simulation analysis.

The stability of selected protein-ligand complexes was analyzed using RMSD, root mean square fluctuations (RMSF), and radius of gyration (Rg). The RMSD values of the complex backbone atoms were calculated to analyze the stability of the simulation process. Our results showed that the amplitude of the RMSD deviation curve for compound **11** in the complex with AChE is better in comparison to the stability of the compound 13-AChE complex. The smaller deviation curve indicates high stability and vice versa. Slight fluctuations were observed in the RMSD values for both the compound **11**-AChE and compound 13-AChE complexes during the initial 5 ns. However, after 10 ns, both the complexes began to stabilize, which indicates the stable behavior of the compounds by strongly inhibiting the target and showing explicit binding to the active site (Figures 7A,B).

Afterward, we analyzed the RMSF to investigate the fluctuations of each residue of our enzyme in complex with both compounds **11** and **13** during simulations. For both the complexes, higher fluctuations were observed in the highly pliable regions of the AChE protein, including the –N and –C terminal and loop regions (Gly 256, Cys 25, Arg 274, Thr 275, Arg 276, Pro 277, Asn 283, His 284, Glu 285, Trp 286, and His 287) (Figures 8A,B).

In addition, the Rg was used to measure the structural compactness of ligands and interactions with the tertiary structural volume and is also utilized to determine the stability of the protein in the biological system along the MD trajectories. For the selected complexes, our result shows that the Rg values did not fluctuate considerably, as shown in Figures 9A,B, which indicates that the complexes remained compact upon ligand binding.

The hydrogen bond (HB) analysis was performed to assess the stability of the selected compound-AChE complexes. In general, both complexes retained HB interactions throughout MD simulation. In the case of the compound **11**-AChE complex, 0 to 6 HB were observed throughout the MD, while the compound **13**-AChE complex showed 0 to 4 HB. The current study result revealed that compound **11**-AChE complex possesses a maximum number of HB thus showing better stability in comparison to the compound **13**-AChE complex (Figures 10A,B). Overall, the stability analysis through RMSD, RMSF, Rg, and HB supports the high stability and inhibitory potential of compound **11** as compared to compound **13**.

3.5 Spasmolytic and calcium antagonistic activities

In the current study, all compounds of Schiff bases showed an antispasmodic effect by inhibiting the spontaneous contraction at different concentrations (Table 3). Among all the analogues, compound **11** was the most efficient, with an effective dose (ED_{50}) value, 326.239 ± 0.01 μM (mean ± SEM, n = 3).

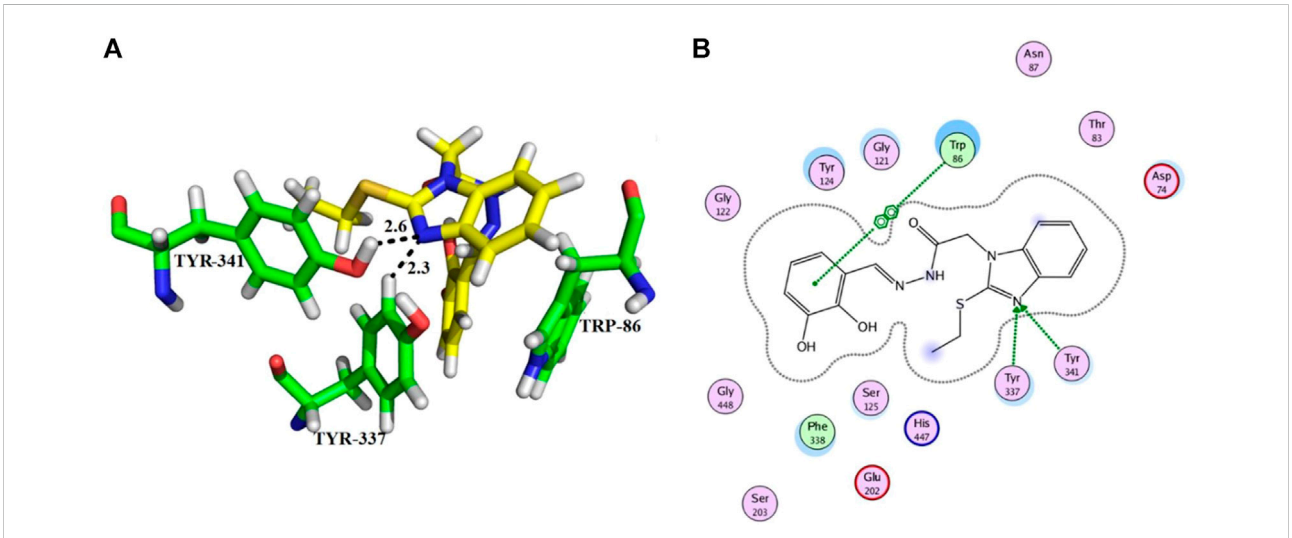


FIGURE 5
Showing the molecular docking conformation of compound **13** (**A,B**) in the active site of AChE.

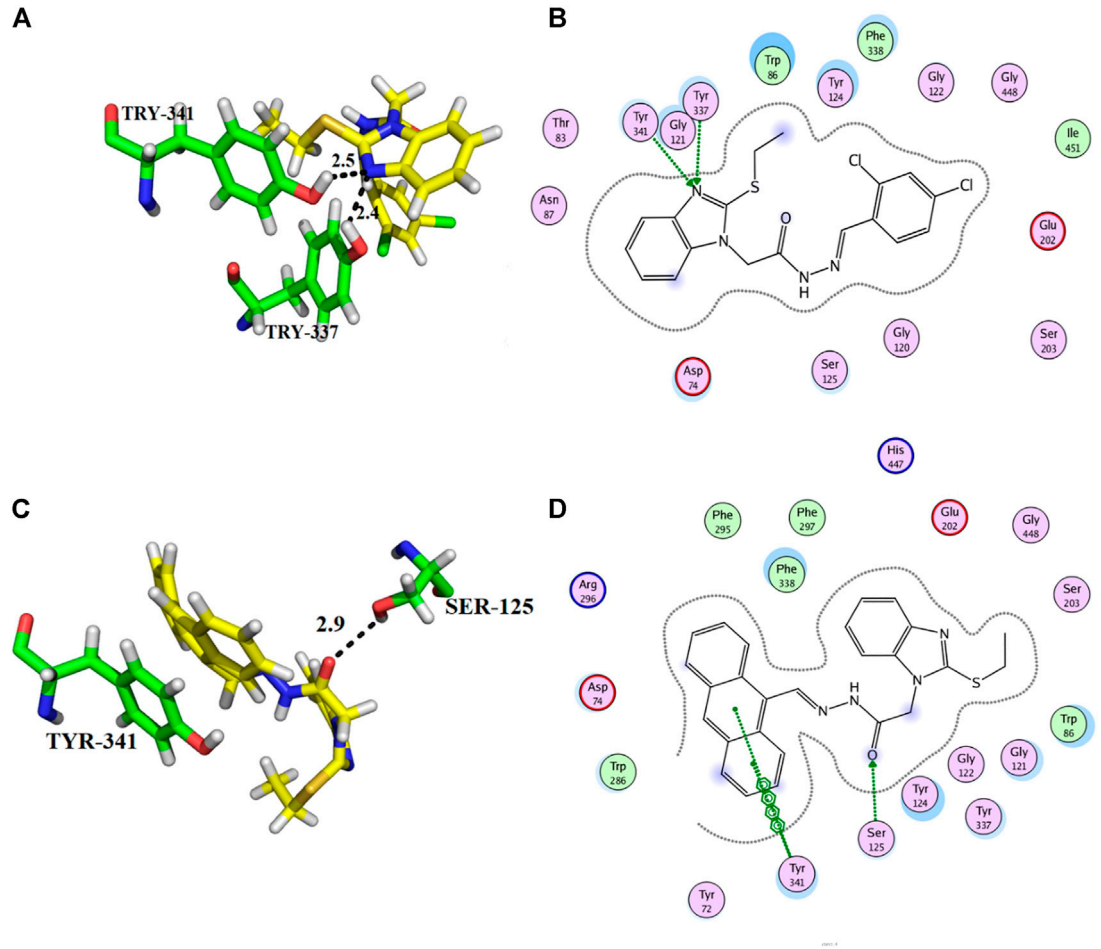


FIGURE 6
Showing the docking conformation of compound **10 (A,B)** and **14 (C,D)** in the active site of AChE.

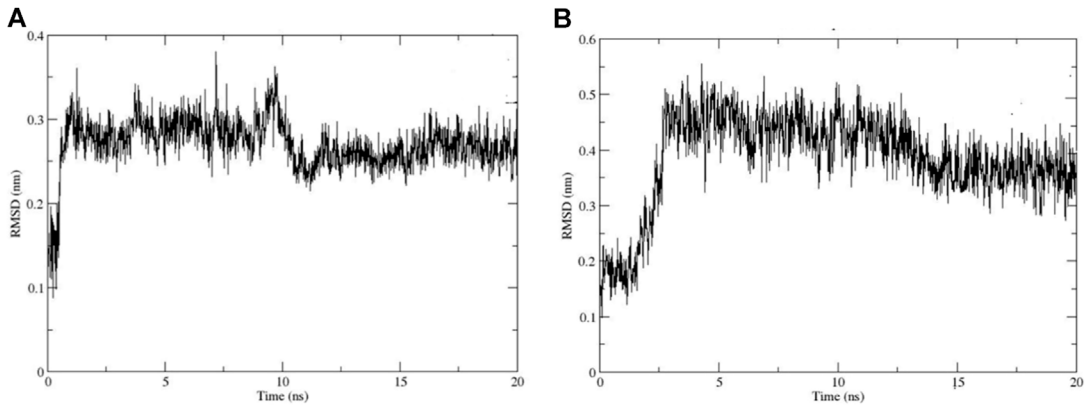


FIGURE 7
RMSD Plots for **(A)** Compound **11**-AChE complex **(B)** Compound **13**-AChE complex.

Schiff bases were also evaluated at high K^+ (80 mM) contracted isolated rabbit jejunum preparations for their spasmolytic effect, which was relaxed by all compounds (Table 3). Compound **11** again exhibited the highest potential with an ED_{50} value of 727.765 ± 0.09 μM (mean ± SEM; n = 3), indicating a Ca^{+2} antagonist effect. Schiff bases represent a class of compounds showing a wide range of bioactivities, such as antimicrobial and anticancer activities. Schiff base compounds are the products of primary amines and condensation of carbonyl compounds, which are extensively investigated owing to their wide range of biomedical applications (Sztanke et al., 2013). Amines have been found to exhibit antiproliferative activity against numerous cancer cell lines (Gama et al., 2011; Abu Bakr et al., 2016), while the presence of an azomethine bond is reported to be vital for bioactivities (Da Silva et al., 2011). The antispasmodic and Ca^{+2} antagonist activity of Schiff base compounds was first reported and could be linked to the azomethine bond.

3.6 Cytotoxicity

The cytotoxicity of 2-mercaptobenzimidazole hydrazone derivatives (**9–14**) on human neutrophils was determined. Galanthamine, a well-known inhibitor of AChE, was used as a

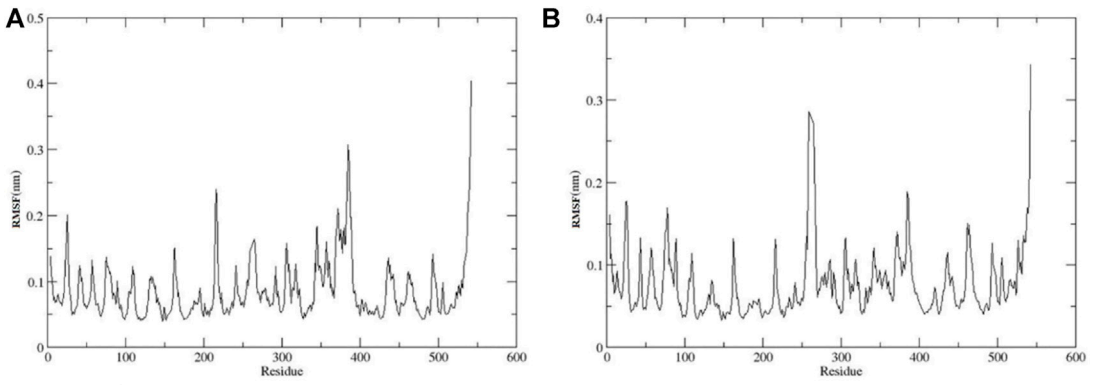


FIGURE 8
RMSF Plots for **(A)** Compound **11**-AChE complex **(B)** Compound **13**-AChE complex.

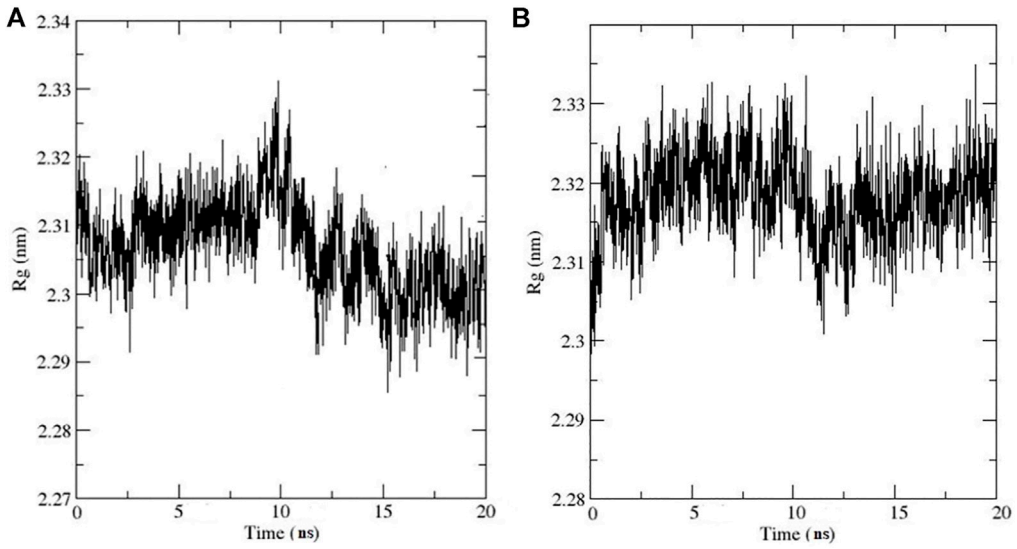


FIGURE 9
Rg Plots for **(A)** Compound **11**-AChE complex **(B)** Compound **13**-AChE complex.

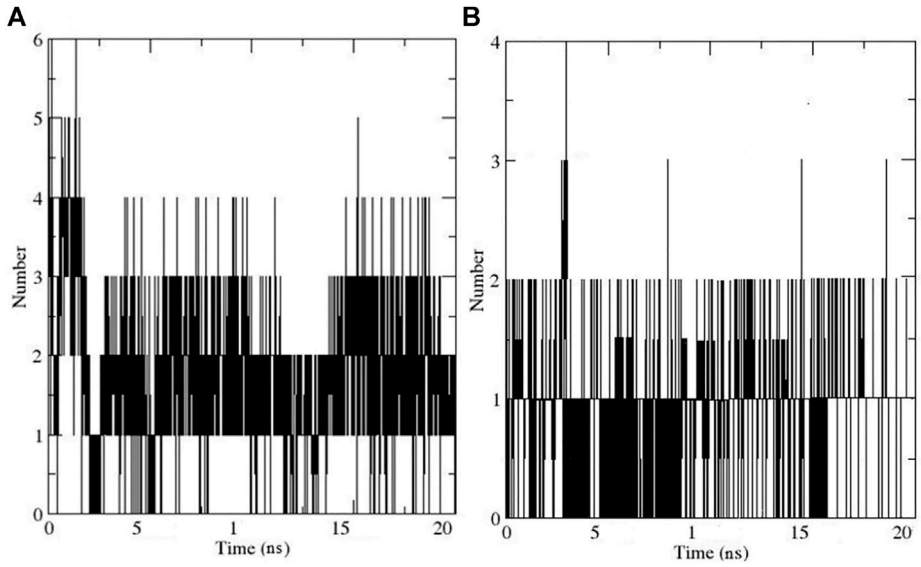


FIGURE 10
Illustrates the Hydrogen bonding for **(A)** Compound **11**-AChE complex **(B)** Compound **11**-AChE complex.

TABLE 3 Effective Dose (ED_{50}) values of the compounds 9–14 for their spasmolytic effect on spontaneously and high K^+ contracted isolated rabbit jejunum preparations.

Compounds	Spontaneous (μM)	High K^+ (μM)
9	733.572 ± 0.01	2,257.145 ± 0.03
10	662.869 ± 0. 00	1,620.347 ± 0.04
11	326.239 ± 0.01	727.765 ± 0.09
12	702.670 ± 0.00	1,932.342 ± 0.06
13	458.926 ± 0.01	836.865 ± 0.05
14	592.876 ± 0.12	980.526 ± 0.05

TABLE 4 Viability of human neutrophils (1×10^7 cells/ml) in the presence of compounds 9–14.

Compounds	Conc. μM	Viability [%]
9	564.286	50.54 ± 3.1
10	491.014	90.07 ± 2.3
11	501.907	98.02 ± 1.0
12	501.907	73.01 ± 3.0
13	539.913	77.22 ± 0.5
14	456.058	66.57 ± 4.0
Galanthamine	696.005	96.03 ± 2.0

standard drug. Results of human neutrophil viability (1×10^7 cells/ml) against Schiff bases were shown in Table 4. Our results showed that all compounds have no toxic effects on human neutrophils like standard galantamine.

4 Conclusion

In the current study, we evaluated the inhibitory potential and kinetic study of newly synthesized Schiff base class of 2-mercaptobenzimidazole hydrazone derivatives (**9–14**) and conformed their non-competitive inhibition. *In vitro* studies revealed the promising inhibitory potential of compound **11** against AChE. However, the potency of this compound was less as compared to standard galanthamine. These compounds also showed antispasmodic, Ca^{2+} antagonistic, and nontoxic effects on human neutrophils. These results suggested that Schiff base compounds could be used as a potential drug candidate against AChE to treat AD. Nevertheless, additional animal model-based studies are required to validate these results, which will help to design a new drug.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization, ML and AK; methodology, FB, SI, and RP; software, FB, ML, and SI; validation, NR; formal analysis, AA-H and RP; resources, AA-H and AK; writing—original draft preparation, FB, ML, and AK; writing—review and editing, ML and AK; supervision, ML and AK; funding acquisition, AA-H.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.946134/full#supplementary-material

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Rosmarinus officinalis and Methylphenidate Exposure Improves Cognition and Depression and Regulates Anxiety-Like Behavior in $AlCl_3$-Induced Mouse Model of Alzheimer's Disease

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Alzheimer's disease (AD) is a neurological illness that causes severe cognitive impairment. AD patients also experience at least one of the neuropsychiatric symptoms including apathy, depression, and anxiety during the course of their life. Acetylcholine esterase inhibitors are the available treatment options to alleviate cognitive deficits, whereas methylphenidate (MPH), a psychostimulant, is considered for the treatment of apathy in AD patients. *Rosmarinus officinalis*, a perennial herb, has been potentially known to have antioxidant and anti-inflammatory properties. The present study investigated the potential effects of MPH and *R. officinalis* in comparison with the standard drug, Donepezil, on cognition, anxiety, and depression in the $AlCl_3$-induced mouse model of AD. The animals were divided into eight groups (n = 8, each). The results revealed that the MPH- and *R. officinalis*-treated groups significantly improved memory impairment, whereas *R. officinalis* substantially reduced depression and anxiety as compared with other treatment groups. MPH treatment induced an antidepressant effect and increased anxiety-like behavior. Moreover, the $AlCl_3$ exposure led to the formation of amyloid beta (Aβ) plaques in mice hippocampus; however, none of the tested drugs caused a significant reduction in amyloid burden at the selected doses. The present study suggested the potential of *R. officinalis* to improve memory as well as neuropsychiatric symptoms in AD. Although *R. officinalis* improved cognitive abilities, it did not reduce the amyloid plaque burden, which indicates that the memory-enhancing effects of *R. officinalis* are due to some alternate mechanism that needs to be explored further.

Keywords: Rosmarinus officinalis, methylphenidate (MPH), anxiety, depression, cognition

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INTRODUCTION

Alzheimer's disease (AD) is a neurological illness that causes neuronal loss and cognitive impairment. The major pathological characteristic of the disease is the successive accumulation of amyloid beta (Aβ) plaques and neurofibrillary tangles (Scheltens et al., 2021). AD has public health burden because of not just cognitive symptoms but also noncognitive neuropsychiatric symptoms

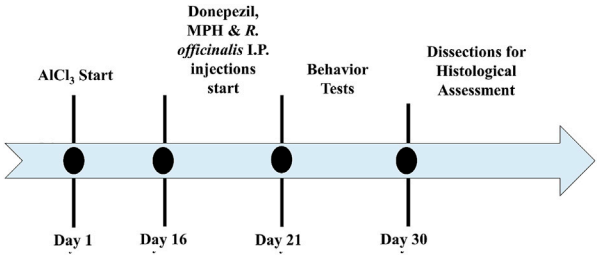

FIGURE 1 | Timeline depicting a period for the induction of Alzheimer's disease (AD), treatment with Donepezil, methylphenidate (MPH), and *R. officinalis* extract; behavior analysis; and decapitation of animals for histological assessment.

including apathy, agitation, depression, aggression, and anxiety. During the course of the disease, most of the AD patients will have at least one of these symptoms (Heilman and Nadeau, 2022). Apathy affects 70% of people with AD, whereas depression and anxiety are also evident in AD patients (Teixeira et al., 2021). The occurrence of depression and anxiety in AD patients has a significant ramification on the patient's quality of life, wellness of the caregivers, chances of hospitalization, and mortality rate (Botto et al., 2022).

A number of pharmaceutical treatments for treating apathy in AD have also been investigated. Various randomized clinical trials of cholinesterase inhibitors have shown minor improvements in apathy-associated symptoms (Sepehry et al., 2017). Furthermore, antidepressants are also unable to improve the apathy-related symptoms in AD patients, and some studies reported the negative consequences of these medications (Magierski et al., 2020).

Methylphenidate (MPH) is a potent psychostimulant that has been utilized to enhance cognition and promote wakefulness for a range of disorders (Wenthur 2016). It has become the standard treatment for attention deficit hyperactivity disorder with time, because of its ability to decrease impulsivity and improve cognition and executive control (Shellenberg et al., 2020).

Based on clinical anecdotal reports, MPH is being considered for the treatment of apathy in AD, and preliminary trials have shown promising outcomes (Padala et al., 2018; Mintzer et al., 2021).

Plant-originated natural compounds having pharmacological properties have appeared as a promising treatment alternative for AD in recent years (Uddin et al., 2020; Akter et al., 2021; Fernandes et al., 2022). These natural compounds or phytochemicals are largely categorized into alkaloids, terpenoids, and polyphenols, where the terpenoids and polyphenols are the major groups of plant's secondary metabolites, targeting several signaling pathways in the biological system (Zhu et al., 2018). The pharmacological properties of these compounds are due to their distinctive structures that enable them to interact with different key enzymes, receptors, antioxidant systems, and signaling cascades including transcription factors as well as cytokines (Safe et al., 2021; Santos et al., 2021; Kamran et al., 2022). For instance, the antioxidant activity of flavonoids, a subgroup of polyphenols, is correlated with the number of phenolic groups (Glevitzky et al., 2019), whereas anti-inflammatory and antidepressant effects are also associated with polyphenols (Hussain et al., 2016; Jiang et al., 2019). Moreover, terpenoids are considered for exhibiting anticholinesterase activity and are a promising source for future AD treatment (Lai Shi Min et al., 2022).

Rosmarinus officinalis (*R. officinalis*), having the common name rosemary, is a member of the Lamiaceae family and is rich in phenolic and terpenoid compounds (Andrade et al., 2018). It is potentially known to have antioxidant, anti-inflammatory, and antidepressant properties (Guo et al., 2018; Dabaghzadeh et al., 2022).

At present, there is no effective medicine that can cure or stop the deterioration of neurons and manage multiple symptoms of AD at a time, although many new drugs are under clinical trials proposed as neuroprotective treatment (Huang et al., 2020). Cholinesterase inhibitors including Donepezil are the only available treatments for managing cognitive symptoms in AD (Haake et al., 2020). However, they are associated with several adverse side effects (Zhang et al., 2022). A recent study demonstrated the potential anticholinesterase activity of *R. officinalis* to combat cognitive decline disorders (Kamli et al., 2022). By contrast, our earlier findings also highlighted the potential of *R. officinalis* to enhance memory and affect synaptic regulation in the $A\beta_{1-42}$-induced AD mouse model (Mirza et al., 2021). Likewise, another study by our group showed the therapeutic potential of *R. officinalis* and MPH to improve cognition and regulate inflammation, synaptic gene expression, and hippocampal neuronal density in mouse models of $AlCl_3$-induced neurotoxicity (Khalid et al., 2020).

Based on the promising results of our previous work and the available literature, the current study focused on the potential effects of MPH and *R. officinalis* on anxiety, depression, and cognition through behavioral analysis in an $AlCl_3$-induced mouse model of AD, to explore a better and comprehensive therapeutic regimen that can manage multiple symptoms including memory loss and psychiatric symptoms. The study highlights the potential effects of *R. officinalis* and MPH on psychological behaviors suggesting it as a better option to treat neuropsychiatric as well cognitive symptoms associated with AD. However, further studies are needed to explore the molecular mechanisms regulated by *R. officinalis* to help understand its mode of action.

MATERIALS AND METHODS

Reagents and Drugs

Aluminum chloride hexahydrate ($AlCl_3{\cdot}6H_2O$, Cat #: AL0770) was procured from Pharmpur® Scharlau, Spain. Donepezil hydrochloride (Donecept) was from ATCO Laboratories, Pakistan, and methylphenidate hydrochloride (Ritalin®) was procured from Novartis specs, Pakistan. All chemicals were obtained from Merck, Germany, unless noted otherwise, and were of molecular biology grade.

Animals

Male BALB/c mice (6–8 weeks old) were chosen for the study. Animals were bred and resided in the Laboratory Animal House of Atta-ur-Rahman School of Applied Biosciences (ASAB),

National University of Science and Technology (NUST). Mice (n = 64) were housed in standard metal cages under standard conditions of constant temperature (25°C ± 2°C) and a regular light–dark cycle (12–12 h). All experimental protocols were conducted in accordance with the rulings of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, United States (Guide for the Care and Use of Laboratory Animal). Internal Review Board of ASAB, NUST approved this study protocol.

Rosmarinus officinalis Extract Preparation

Commonly available dried leaves of *R. officinalis* were collected during the fall of 2020 from a vendor in the local spice market of Islamabad, Pakistan. Verification of the plant was conducted by an experienced botanist prior to the initiation of the procedure. The specimen was stored in Neurobiology Lab, ASAB, NUST, and also submitted at Pakistan Natural History Museum, Islamabad, Pakistan, with voucher no. 42570. *R. officinalis* extract was prepared according to the protocol described by Khalid et al. (2020). *R. officinalis* (500 g) leaves were ground to fine powder form and allowed to pass through 80 mesh sieves. It was followed by taking 10 g of fine powder in a thimble and loading the thimble into Soxhlet extractor with a distillation flask containing 100% ethanol as extraction solvent. The process was run for 24 h before filtrate was collected and concentrated using a rotary evaporator (R200 Rotavapor, Buchii) under the pressure of 68°C to attain a crude extract. The crude extract was incubated at 37°C to remove the remaining solvent. The extract was stored at 4°C until further use.

Animal Treatment

Animals were divided into eight groups, eight mice each. Animals of groups 1, 2, 3, and 4 were given distilled water and normal feed for 15 days. After 15 days, the animals of groups 2, 3, and 4 were administered with intraperitoneal (i.p.) injections (single injection volume of 100 µl) of 2 mg/kg (i.p.) Donepezil (Madani Neishaboori et al., 2021), 10 mg/kg (i.p.) MPH, and 100 mg/kg (i.p.) *R. officinalis* extract (Khalid et al., 2020) for 5 days. Aluminum chloride ($AlCl_3$) (300 mg/kg) (Amber et al., 2018; Khalid et al., 2020) was given in drinking water with normal feed for 15 days to groups 5, 6, 7, and 8 (n = 32). After the development of $AlCl_3$-induced AD models, the animals of groups 5, 6, 7, and 8 were switched to normal drinking water and those of groups 5, 6, and 7 were administered with intraperitoneal (i.p.) injections (single injection volume of 100 µl) of 2 mg/kg (i.p.) Donepezil, 10 mg/kg MPH, and 100 mg/kg *R officinalis* extract, respectively. Behavior tests were conducted in the next 10 days followed by dissection, collection of brain tissues, and histological assessment. The treatment timeline is depicted in **Figure 1**.

Behavior Studies

Morris Water Maze Test

The procedure described by (Bromley-Brits et al., 2011) was used with small modification for the Morris water maze (MWM) test. A circular pool (120 cm × 60 cm), divided equally into four quadrants (east, west, north, and south), was filled with water (21°C ± 2°C). A transparent platform (13 cm × 32 cm) was placed 1 cm below the surface of the water in the north–west quadrant. Five acquisition trials were conducted five times a day for five consecutive days, and before each trial, a minimum of 10 min intertrial interval was kept for each mouse. In each trial, the mice were released in water at one of the four but different quadrant positions with their heads facing the tank. The cut-off time was identified as 90 s, and those who failed to locate the platform in 90 s were manually placed on the platform for 20 s. Those animals who located the platform before 90 s were allowed to sit there for 5 s. The escape latency for all the trials of 5 days was recorded, and the average was calculated. On day 6, the platform was removed and a probe test was conducted. In this test, the mice were allowed to swim in the pool for 90 s and the video was recorded. The animal's reference memory was monitored by calculating the number of entries, time spent in the target quadrant, and number of crossings made by the subjects over the removed platform position.

Forced Swim Test

A forced swim test was conducted to analyze the depressive behavior of the animals. The protocol of the test was followed as described by (Murad et al., 2014) with few modifications. A transparent container with a diameter of 20 cm and a height of 30 cm was filled with water at 25°C ± 2°C. The depth of the water was adjusted according to the size of the mice, to prevent their hind limbs and tail from touching the bottom of the container. The animals were placed into the water, and their activity was recorded using a camera for 6 min. The water in the container was discarded and refilled for each mouse prior to testing. The time spent immobile, number of immobile episodes, and latency to immobility were calculated for each mouse.

Open Field Test

An open field test was conducted to assess the anxiety level and exploratory behavior of the animals. Protocol for the test was followed as described by (Farhat et al., 2017) with slight modifications. A square-shaped arena (40 × 40 × 40) was used, which was divided into center and periphery by drawing a boundary. Each mouse was placed in the center of a wooden box and was allowed to explore the box for 30 min. The behavior of the animals was monitored using a camera and was assessed by calculating the time spent in the center and periphery of the box.

Statistical Analysis

Data were analyzed using GraphPad Prism 8.0.2 by applying a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Two-way ANOVA was applied for analyzing escape latency in the MWM test. The data observed are shown as mean ± SEM having a 95% confidence interval, which is considered statistically significant when $p < 0.05$.

Histological Examination

Heart perfusion was conducted to excise the whole brain according to the procedure described by Gage et al. (2012). Tissue sections (5 µm) of the hippocampus were deparaffinized with xylene, rehydrated, and washed with 70% isopropanol and double-distilled water (ddH_2O) respectively. The Congo red stain

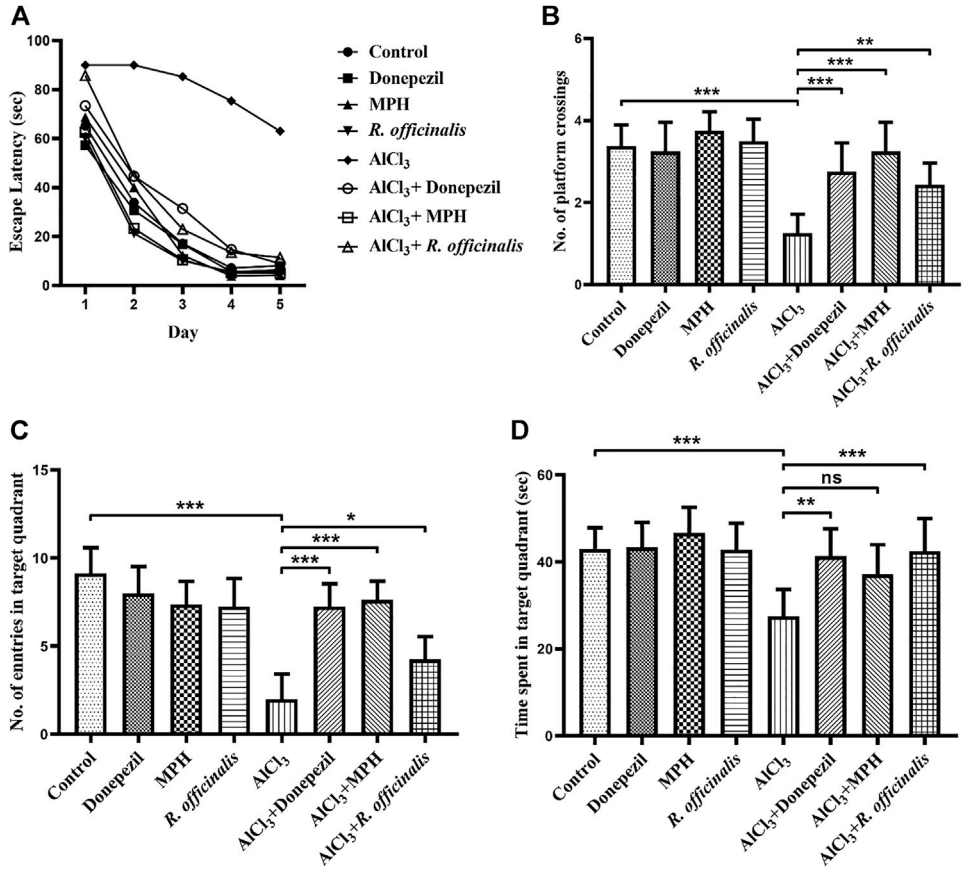

FIGURE 2 | Effects of Donepezil, MPH, and *R. officinalis* on spatial memory in $AlCl_3$-induced mice models using the Morris water maze test. **(A)** Graph demonstrates escape latency (sec) of the experimental groups, **(B)** number of platform crossings by the study groups, **(C)** number of entries in the target quadrant, and **(D)** time (sec) spent in the target quadrant during the probe trial. One-way ANOVA followed by the Bonferroni comparison test (mean ± SEM) was applied to analyze the data using GraphPad Prism. n = 8. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

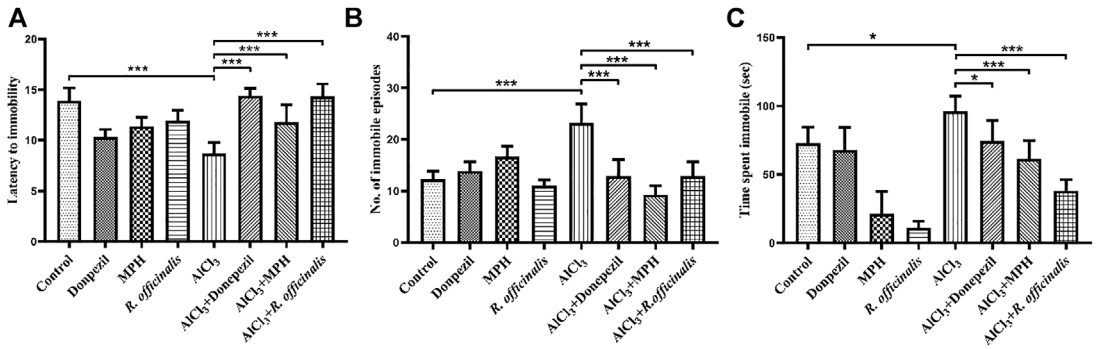

FIGURE 3 | Effects of Donepezil, MPH, and *R. officinalis* on depression-like behavior in the forced swim test. **(A)** Graph depicting latency to immobility, **(B)** number of immobile episodes, and **(C)** time spent immobile. One-way ANOVA followed by the Bonferroni comparison test (mean ± SEM) was applied to analyze the data using GraphPad Prism. n = 8. ***$p < 0.001$, *$p < 0.05$, ns nonsignificant.

(Cat #C6767, Merck Germany; working solution: 49.5 ml Congo red (Stock) and 0.5 ml 1% NaOH) was poured on the deparaffinized brain sections and retained for 20 min. ddH_2O and alkaline alcohol were used to wash sections for 2 min. The sections were then counterstained by hematoxylin for 30 s and further washed with 70% isopropanol for 6 min and then with ddH_2O. After air-drying (1 h), the slides were mounted by coverslips and later visualized using B-150, OPTIKA

microscope (Italy) at 40×, 10×, and 4× resolution. The images were captured using Optika Vision Lite 2.1 image analysis software.

RESULTS

MPH and *R. officinalis* Improved Cognition

The MWM test was used to assess the outcomes of MPH and *R. officinalis* on spatial learning and memory in comparison with Donepezil. Average escape latency to find out the platform directly indicates the effects of the drugs on spatial memory. The $AlCl_3$-treated group showed poor memory retention than the control group by demonstrating an escape latency of 63 s on day 5. A significant improvement ($p < 0.01$) in spatial memory was observed in the $AlCl_3$ + *R. officinalis*-treated group. However, better ($p < 0.05$) restoration of spatial memory was seen in the $AlCl_3$ + MPH- and $AlCl_3$ + Donepezil-treated groups. Escape latency for 5 days is represented graphically in **Figure 2A**.

A probe trial was conducted for the assessment of reference memory and exploratory behavior of mice for the previously placed invisible platform in the target quadrant. The $AlCl_3$-treated group reflected a significant decrease ($p < 0.0001$) in reference memory by making a smaller number of crossings over the platform position as compared with the control group. A number of platform crossings were significantly increased in the $AlCl_3$ + *R. officinalis*-treated group as compared with the $AlCl_3$-treated group; however, the $AlCl_3$ + Donepezil- and $AlCl_3$ + MPH-treated groups made a greater number of crossings than the $AlCl_3$ + *R. officinalis*-treated group (**Figure 2B**). A similar trend was seen in the number of entries where the $AlCl_3$-treated group showed the least number of entries in the target quadrant than the control and all the other experimental groups. Again, the $AlCl_3$ + Donepezil- and AlCl3 + MPH-treated groups showed a greater number of entries than the $AlCl_3$ + *R. officinalis*-treated group (**Figure 2C**). Likewise, a significant decrease ($p < 0.001$) in time spent in the target quadrant was observed in the $AlCl_3$-treated group compared with the control group and a significant improvement was observed after treatment with *R.* officinalis and Donepezil in the $AlCl_3$ + *R. officinalis*- and $AlCl_3$ + Donepezil-treated groups (**Figure 2D**). Overall, our results depicted in **Figures 2A–D** demonstrate that MPH and *R. officinalis* significantly improved cognition and alleviate the cognitive deficits induced by $AlCl_3$.

MPH and *R. officinalis* Induced Antidepressant Effects

The forced swim test was conducted to assess the antidepressant effect of MPH and *R. officinalis* on an $AlCl_3$-induced AD mice model having impaired cognitive functions. The $AlCl_3$-treated group exhibited depressive behavior by showing the least latency to immobility as compared with the control and other experimental groups. The $AlCl_3$ + MPH-treated group spent significantly more time struggling and hence delayed latency to immobility in comparison with the $AlCl_3$-treated group demonstrating that treatment aided in overcoming the effect of $AlCl_3$ on depression. However, $AlCl_3$ + Donepezil and $AlCl_3$ + *R. officinalis* comparatively delayed more ($p < 0.001$) latency to immobility than $AlCl_3$ + MPH (**Figure 3A**). Likewise, the $AlCl_3$-treated group displayed the highest number of immobile episodes as compared with all other experimental groups, and after treatment, a significant decrease in the number of immobile episodes was observed in the $AlCl_3$ + Donepezil- and $AlCl_3$ + *R. officinalis*-treated groups with $AlCl_3$ + MPH having the least number of immobile episodes as compared with the other treatment groups (**Figure 3B**). The $AlCl_3$-treated group also spent the greatest amount of time being immobile, which was significantly decreased via drug treatment in the $AlCl_3$ + Donepezil- and $AlCl_3$ + MPH-treated groups, respectively. A more significant decrease ($p < 0.05$) in time spent being immobile was observed after treatment with *R. officinalis* (**Figure 3C**). The overall findings depicted in **Figures 3A–C** show that MPH and *R. officinalis* significantly induce antidepressant effects and reduce depression-like behavior induced by $AlCl_3$.

R. officinalis Induced Anxiolytic Effect

An open field test was conducted to analyze the anxiety-like behavior in animal models. Animals that tend to spend more time in the center are considered less anxious. The $AlCl_3$-treated group showed more anxiety-like behavior by spending significantly ($p < 0.001$) lesser time in the center and more time in the periphery as compared with the control group (**Figures 4A, B**). The $AlCl_3$ + Donepezil- and $AlCl_3$ + *R. officinalis*-treated groups improved their performance by spending more time in the center as compared with the $AlCl_3$-treated group, demonstrating the antianxiety potential of Donepezil and *R. officinalis*, whereas the $AlCl_3$ + MPH-treated group spent the least amount of time in the center of the box (**Figure 4A**). Likewise, treatment with Donepezil and *R. officinalis* significantly decreased the time spent in the periphery in the $AlCl_3$ + Donepezil- and $AlCl_3$ + *R. officinalis*-treated groups, respectively, in comparison with the $AlCl_3$-treated group (**Figure 4B**). However, the $AlCl_3$ + *R. officinalis*-treated group showed more significant improvement than the $AlCl_3$ + Donepezil-treated group. It is of interest that the most anxious behavior was observed after treatment with MPH (**Figures 4A, B**). These results indicate the potential of *R. officinalis* to induce an anxiolytic effect.

Effects of MPH and *R. officinalis* on Amyloid Beta Plaque Burden

Congo red staining of the hippocampus showed the presence of Aβ plaques in the $AlCl_3$-treated groups compared with the control group. An almost similar number of plaques are seen in all $AlCl_3$ plus drug-treated groups and the $AlCl_3$-treated group. None of the tested groups showed a significant decrease in Aβ plaque burden (**Figure 5**). Therefore, it is likely that the selected doses of MPH and *R. officinalis* are not much effective to reduce the amyloid burden.

DISCUSSION

The current study investigated the effects of MPH and *R. officinalis* on various parameters including memory and learning, anxiety, and depression in the $AlCl_3$-induced AD mouse model.

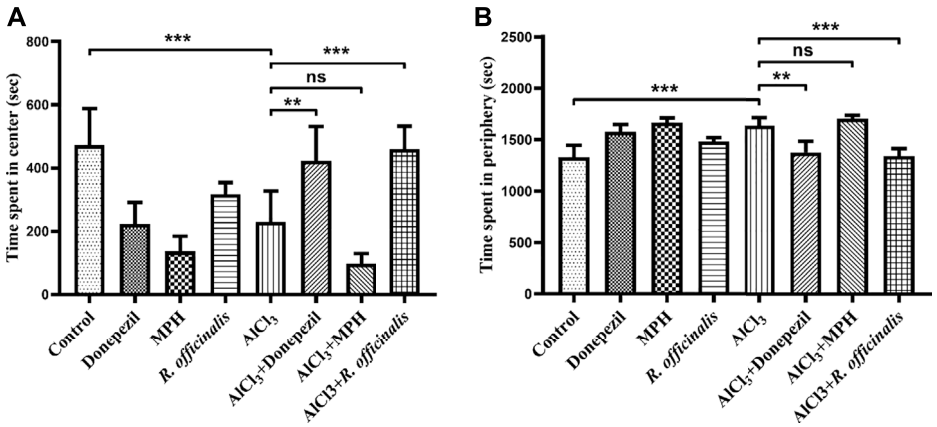

FIGURE 4 | Effects of Donepezil, MPH, and *R. officinalis* on anxiety and exploratory behavior in the open field test. **(A)** Graph depicting time spent in the center and **(B)** time spent in the periphery of the open field box. One-way ANOVA followed by the Bonferroni comparison test (mean ± SEM) was applied to analyze the data using GraphPad Prism. n = 8. ***$p < 0.001$, **$p < 0.01$.

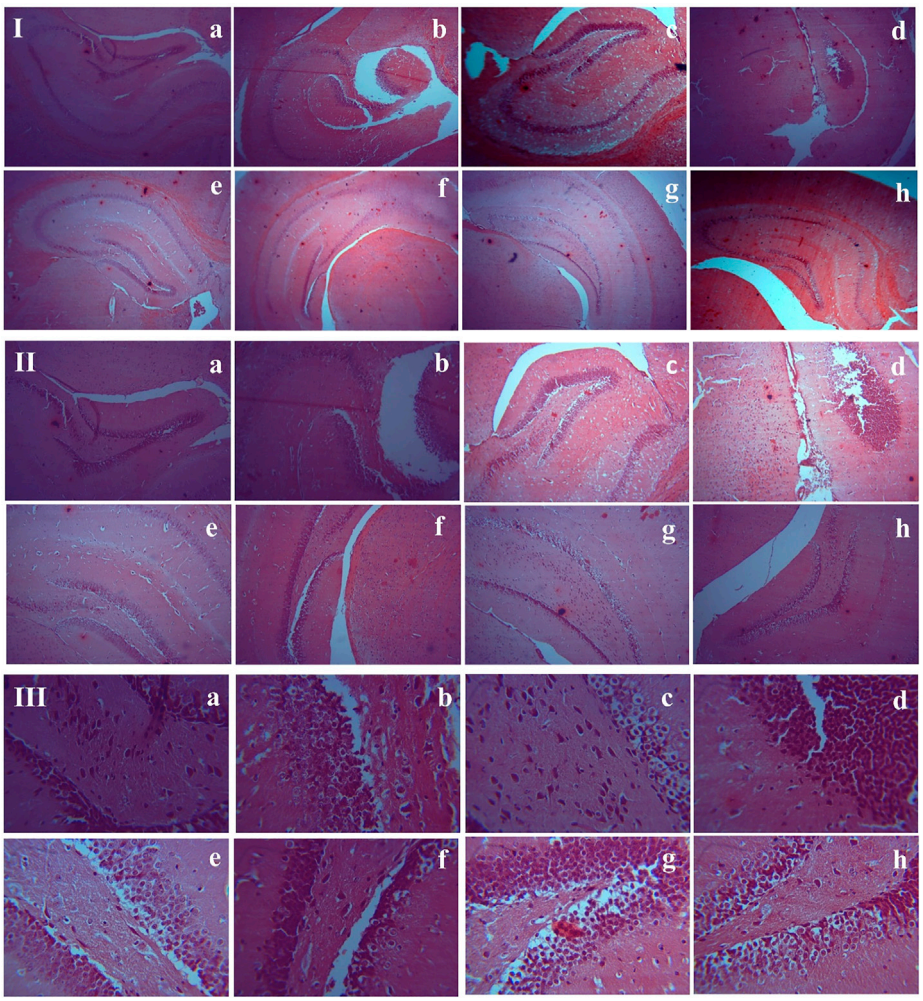

FIGURE 5 | Histological assessment of hippocampal tissues sections stained with Congo Red. **(I)** 4×, **(II)** 10×, **(III)** 40×. **(a)** Control, **(b)** Donepezil-treated, **(c)** MPH-treated, **(d)** *R. officinalis*-treated, **(e)** $AlCl_3$-treated, **(f)** $AlCl_3$ + Donepezil-treated, **(g)** $AlCl_3$ + MPH-treated **(h)** $AlCl_3$ + *R. officinalis*-treated.

TABLE 1 | Pharmacological properties of *R. officinalis*.

Subject	Route	Observed pharmacological effects	Diseased condition	References
Human	Oral	Anticholinesterase, antioxidant	Healthy volunteers	Dabaghzadeh et al. (2022)
Human	Oral	Improvement of mental energy and quality of sleep	Poor mental health	Araki et al. (2020)
Human	Oral	Reduced bacterial plaque and gingival bleeding	Gingival bleeding	Valones et al. (2019)
Humans	Oral	Memory enhancement, antianxiety, antidepressant, improved sleep quality	Healthy volunteers	Nematolahi et al. (2018)
Humans	Oral	Antianxiety, antidepressant	Healthy volunteers	Achour et al. (2022)
Humans	Inhalation	Decreased sleepiness and increased alertness	Sleepiness and alertness	Nasiri & Boroomand (2021)
Mice	Oral Gavage	Antidepressant, anti-inflammatory, rebalanced gut microbiota	Chronic restraint stress, hippocampus inflammation	Guo et al. (2018)
Mice	Oral	Memory improvement, synaptic regulation	$A\beta_{-1-42}$-induced AD	Mirza et al. (2021)
Mice	Oral Gavage	Anticancer	Colorectal cancer (xenograft tumor model)	Valdés et al. (2017)
Mice	Topical application	Antibacterial effect, wound healing	Wound with bacterial infection	Khezri et al. (2019)
Mice	Oral	Antidepressant, anxiolytic, modulation of oxytocinergic system in limbic system	Depression induced by tail suspension test (TST), anxiety model of LPS induced neuroinflammation	Sasaki et al. (2021)
Mice	I.P.	Memory enhancement, anti-inflammatory, neurogenic	$AlCl_3$-induced neurotoxicity	Khalid et al. (2020)
Mice	Inhalation	Reduction of stress and corticosterone in serum, increased dopamine level in the brain	Stress	Villareal et al. (2017)
Mice	Oral Gavage	Prevention of kidney function	CCl4-induced nephrotoxicity	Hamed et al. (2020)
Rats	I.P.	Pain relieving, anti-inflammatory	Neuropathic pain induced by chronic constriction injury of the sciatic nerve	Ghasemzadeh Rahbardar et al. (2017)
Rats	I.P.	Anti-inflammatory, antioxidant, reduced fibrosis	Postoperative peritoneal adhesion	Roohbakhsh et al. (2020)
Rats	Oral Gavage	Intestinal protection, antioxidant, anti-inflammatory	Ethanol-induced acute intestinal damage	Amaral et al. (2018)
Rats	Oral Gavage	Anti-inflammatory, antioxidant, reduced paw edema	Freund's adjuvant-induced arthritis	de Almeida Gonçalves et al. (2018)
Rats	Oral	Decreased bone loss, anti-inflammatory, antioxidant	Osteoporosis	Elbahnasawy et al. (2019)
Rats	Oral	Reduced edema, anti-inflammatory, antialgic	Carrageenan-induced paw edema	Borges et al. (2018)
Rats	Stomach tube	Enhancement of cognitive functions, anti-inflammatory, antioxidant	Ibotenic acid induced AD	Rezk et al. (2022)
Rats	Oral	Memory improvement	Lipopolysaccharide (LPS) induced memory deficits	Pusceddu et al. (2022)
Rats	Oral	Antioxidant	Renal toxicity and oxidative stress	El-Demerdash et al. (2021)
Rats	I.P.	Anxiolytic-like activity	Anxiety	Choukairi et al. (2019)
Rats	Oral Gavage	Neuroprotective, antinociceptive, antihyperalgesic	Diabetic neuropathy	Rasoulian et al. (2019)
Rats	Oral	Antidyslipidemic and antiatherogenic activity	Triton and saturated fat-induced (CSF) dyslipidemias	Santos Rodrigues et al. (2020)

Aluminum (Al) is the most abundant element in earth's crust and is in human use for centuries. Its widespread use and exposure originate from many sources including vaccine adjuvants, processed foods, cosmetics, medical treatments, cooking wares, and pharmaceuticals (Tietz et al., 2019). Chronic exposure to Al develops cellular toxicity and accumulation in various organs including the central nervous system. It binds to plasma transferrin and citrate molecules in the body and is transferred to the brain. The Al accumulation in the brain generates misfolded proteins and facilitates hyperphosphorylation and aggregation (Colomina and Peris-Samperdo, 2017; Niu 2018). $AlCl_3$ is commonly used to develop aluminum-induced animal models for AD, where it is suggested to cause shrinking of hippocampus pyramidal cells (Saeed et al., 2021), neural cell death, and cognitive deficits similar to AD (Zhang 2018).

MPH and other psychostimulants are often used as a therapy to enhance cognitive functions, reduce impulsivity, and induce wakefulness (Carlier et al., 2019). The majority of the studies emphasized that MPH improves cognitive efficiency and influences working memory, inhibitory control, and mental flexibility (Bolfer et al., 2017). Our results also showed a significant effect of MPH in improving spatial learning and memory. We compared the effects of MPH and *R. officinalis* with Donepezil on the $AlCl_3$-induced AD mouse model and found significant effects of MPH on spatial memory and reference memory in terms of the number of platform crossings and the number of entries in the target quadrant in the MWM test. *R. officinalis* also exhibit significant improvement in spatial memory as compared with the $AlCl_3$-treated group and demonstrated more significant effects in reference memory especially in comparison with Donepezil in terms of time spent in the target quadrant.

Depression is one of the most prevalent behavioral symptoms of AD (Yang et al., 2020). It is of interest that Donepezil showed antidepressant effects in the mouse forced swim test and chronic mild stress model in rats (Papp et al., 2016; Fitzgerald et al., 2020). However, MPH has also revealed significant improvement in depression symptoms in patients with Asperger syndrome (Golubchik et al., 2017). Our study compared the effects of *R. officinalis* with both Donepezil and MPH on depression-like behavior in the $AlCl_3$-induced AD model. Comparison of our forced swim results showed that all the tested compounds

significantly improved depression-like symptoms in the $AlCl_3$-induced AD model; however, *R. officinalis* exhibited significantly more antidepressant potential as compared with MPH and Donepezil by delaying more latency to immobility and spending more time being immobile. This can be credited to the antidepressant-like activity of one of the active compounds of *R. officinalis*, i.e., carnosic acid. Recent evidence reveals carnosic acid as a substantial modulator of the ADPN-FGF9 pathway via activation of PPAR-γ in adipocytes, a recently established factor in the development of depression (Wang X. Q. et al., 2021). Likewise, rosmarinic acid has shown antidepressant effects mediated through its increased antioxidant response (Wang J. et al., 2021) exhibiting neuroprotective effects via activation of $GABA_A$ receptors (Wang C. C. et al., 2021). In addition, Lataliza et al. suggested the substantial involvement of cannabinoid receptors/PPAR-γ signaling pathways in exerting the antidepressant-like effect of rosmarinic acid (Lataliza et al., 2021).

Moreover, it has been demonstrated that 60–90% of AD patients develop neuropsychiatric symptoms including anxiety disorder that could be treated with acetylcholinesterase inhibitors (AChEIs) (Cummings et al., 2016; Botto et al., 2022). AChEIs have varying effects on anxiety. Besides several disease-modifying effects of various new derivatives of AChEIs, they also possess promising effects on anxiety-like behavior that can be helpful for disease management (Giménez-Llort et al., 2017). Therefore, the current study also assessed the comparative anxiolytic potential of MPH, *R. officinalis*, and Donepezil in the $AlCl_3$-induced AD model. Our results showed that treatment with Donepezil and *R. officinalis* significantly improved anxiety-like behavior in the $AlCl_3$-treated AD model by increasing the time spent in the center arena of the open field box. A comparison of the results showed that *R. officinalis* displayed the strongest antianxiety potential. *R. officinalis* reduces the extent of anxiety; however, a substantial decrease in neural activity was also observed in a study by Choukairi et al., which can be attributed to the potential of its specific active compounds exerting their effects through modulation of certain neurotransmitter receptors (Choukairi et al., 2019). For instance, two major compounds of *R. officinalis*, i.e., rosmarinic acid and ursolic acid, exhibit anxiolytic and antidepressant-like effects observed in animal studies (Colla et al., 2015; Mirza et al., 2021).

Besides anxiolytic and antidepressant effects of *R. officinalis*, various other therapeutic properties were also documented by various research groups. Some of the recent significant findings are highlighted in **Table 1**, which represent the potential of *R. officinalis* as antinociceptive, anti-inflammatory, antiapoptotic, anticancer etc. (**Table 1**).

Of note, bioactive compounds of *R. officinalis* have limited ability to cross the blood–brain barrier; therefore, different delivery approaches were experimented with to enhance delivery through the blood–brain barrier. Kuo et al. demonstrated apolipoprotein E-modified liposomes conjugated with phosphatidic acid as a carrier for rosmarinic acid and quercetin to infiltrate the BBB to block $A\beta_{1-42}$-induced AD (Kuo et al., 2018). Likewise, attachment of specific ligand, targeted delivery by nanoparticles, and nanoemulsion through intranasal administration were used to rescue neurodegeneration (Fachel et al., 2020; Kuo et al., 2020; Long et al., 2020).

It is of interest that MPH exhibited increased anxiety-like behavior in the open field test in the current study. The effects of MPH on anxiety are still not clear. Some studies have shown that MPH either decreases (Jager et al., 2019) or increases anxiety (Zoratto et al., 2019). Different effects of MPH on anxiety have been debated, and such variations indicate the multidimensional and complex nature of emotional behaviors. There is a possibility that the procedures applied to assess anxiety only measure a specific idiosyncratic domain of the tested emotion. For instance, as reported by Boyette-Davis et al., the antianxiety effects of MPH are not affected based on the changes in locomotor behavior (Boyette-Davis et al., 2018). Therefore, various experimental approaches would be needed to completely assess the effects of MPH on anxiety. By contrast, acute administration of MPH has also been found to exhibit anxiolytic effects in the open field test, where MPH was administered 20 min prior to testing (Jager et al., 2019). Differences in dosage and treatment duration could be another possible reason for varying effects on anxiety (Zoratto et al., 2019). Although the physiological and behavioral effects of MPH are generally reversible after intermittent MPH chronic exposure, it is followed by prolonged abstinence (Kalinowski et al., 2020). However, withdrawal effects could also be the possible reason for high anxiety in the MPH-treated group in the present study.

A histopathological assessment revealed the formation of amyloid plaques in the $AlCl_3$-treated groups showing the development of AD pathology. Our results revealed that all the tested compounds failed to reduce the Aβ plaques burden. Although carnosic acid significantly improves cholinergic dysfunction and mitochondrial defects by reducing the Aβ-mediated toxicity (Chen et al., 2022), in our case, no substantial effect was observed by any of the tested drugs or *R. officinalis*. Perhaps the selected doses of our study are not much effective to reduce the Aβ plaques with a shorter treatment duration, which could be the possible reason for not being effective in reducing the amyloid burden.

These findings provide a preliminary data set on the therapeutic potential of *R. officinalis* possessing substantial anxiolytic and antidepressant activities. MPH and *R. officinalis* have significant effects on cognition with MPH being more effective than *R. officinalis* in restoring spatial memory. Nevertheless, MPH increases the anxiety-like behavior, which needs a further understanding of the complex molecular mechanisms involved in its mode of action.

CONCLUSION

Memory loss along with neuropsychiatric symptoms in AD leads to the requirement of a drug that can enhance memory and reduce behavioral despair. Donepezil has failed to stop the disease progression and is associated with various side effects, and MPH can aggravate psychological symptoms. Therefore, *R. officinalis*, a natural plant extract, could be more suitable to treat cognitive decline as well as psychological symptoms with minimal side effects. Further investigation of the mode of action of *R. officinalis* and the role of its various active compounds is warranted to indicate its therapeutic potential for AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Internal Review Board ASAB-NUST.

AUTHOR CONTRIBUTIONS

SZ, substantial contribution to conception and design of the study and finalization of the manuscript; SA, assistance in intraperitoneal injections, animal handling, behavioral analysis, and editing and formatting the manuscript; NM, all experimental work, data analysis, interpretation, and drafting the article.

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Therapeutic insights elaborating the potential of retinoids in Alzheimer's disease

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Alzheimer's disease (AD) is perceived with various pathophysiological characteristics such oxidative stress, senile plaques, neuroinflammation, altered neurotransmission immunological changes, neurodegenerative pathways, and age-linked alterations. A great deal of studies even now are carried out for comprehensive understanding of pathological processes of AD, though many agents are in clinical trials for the treatment of AD. Retinoids and retinoic acid receptors (RARs) are pertinent to such attributes of the disease. Retinoids support the proper functioning of the immunological pathways, and are very potent immunomodulators. The nervous system relies heavily on retinoic acid signaling. The disruption of retinoid signaling relates to several pathogenic mechanisms in the normal brain. Retinoids play critical functions in the neuronal organization, differentiation, and axonal growth in the normal functioning of the brain. Disturbed retinoic acid signaling causes inflammatory responses, mitochondrial impairment, oxidative stress, and neurodegeneration, leading to Alzheimer's disease (AD) progression. Retinoids interfere with the production and release of neuroinflammatory chemokines and cytokines which are located to be activated in the pathogenesis of AD. Also, stimulating nuclear retinoid receptors reduces amyloid aggregation, lowers neurodegeneration, and thus restricts Alzheimer's disease progression in preclinical studies. We outlined the physiology of retinoids in this review, focusing on their possible neuroprotective actions, which will aid in elucidating the critical function of such receptors in AD pathogenesis.

KEYWORDS

retinoids, neuroinflammation, RXRS, RARS, neurotransmission, neuroplasticity

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder marked with personality changes, memory and cognitive deficits due to loss of the neurons in the frontal cortex and hippocampus. The histological indicator of AD is amyloid plaques, comprising of insoluble amyloid-ß (Aß) peptides (Kabir et al., 2021). Overactive inflammatory astrocytes and microglia localized with the senile plaques are considered to be linked with the pathological lesions (Ayaz et al., 2019a). It is now commonly accepted that mostly affects older individuals over the age of 65 as a geing is a major trigger for Alzheimer's disease. AD is presently the most common neurological condition affecting over 15 million individuals globally (Andreeva et al., 2017). The world population of individuals with AD is constantly increasing. Clinical evidence links Alzheimer's disease to dementia and memory loss. AD is characterized by extra-neuronal Aß plaques deposition and intracellular neurofibrillary tangles in the temporal lobe. Aß plaques are made up of accumulated amyloid-beta peptides, whereas neurofibrillary tangles are made up of tau protein that has been hyperphosphorylated (Querfurth and LaFerla, 2010). Oxidative stress, neuroinflammation, and mitochondrial dysfunction are all triggered by the development of these aggregates, resulting in the loss of not only neurons but also white matter in the brain. New research reveals that the pathogenesis of Alzheimer's disease may be caused by a complicated interaction involving aberrant Aß and tau proteins. The amyloid hypothesis of AD claims that the buildup of Aß plaques in the temporal lobe of the brain is the basic pillar of neurodegeneration and memory deficits in AD patients (Musiek and Holtzman, 2015; Behl et al., 2020). The amyloid hypothesis's fundamental weakness is its failure to definitively establish the molecular mechanisms that link amyloidosis to NFTsfor neurodegeneration in Alzheimer's disease (Eriksen and Janus, 2007). There are numerous different possibilities concerning AD pathophysiology, and several natural compounds such as flavanoids, retinoids, lipoic acids have been developed to treat AD based on these beliefs (Ayaz et al., 2019b). The majority of Alzheimer's models are based on a single theory for the causation of AD, which is a serious flaw in the research. To create viable therapies that will treat the majority of instances, a full understanding of the condition is required. Researchers used different genetically comparable transgenic knock-in animals of AD and tau dysfunction associated with AD and dementia to investigate changes in retinoid signaling at the transcriptional levels in such models. Female rat hippocampal and frontal combined primary cultures were also used to undertake an early assessment of the therapeutic potential of a new family of synthetic retinoids (RAR-M1) that target both biological and non-biological receptors (Endres et al., 2014a; Khatib et al., 2020).

Vitamin-A analogs, both natural and synthetic, are known as retinoids. These chemicals are significant in memory because they are believed to play important functions in adult brain development (Das et al., 2014a). As a result, there is a surge of attention in discovering more about the biology and chemistry of known and new retinoids, as well as their therapeutic potential in the treatment of acute and chronic disorders like AD. Since retinoic acid (RA), a vitamin A derivative, can bind to nuclear receptors and regulate the expression of multiple genes in cells, it executes the majority of biological mechanisms (Lerner et al., 2012). Retinoids activate their target genes by interacting with nuclear receptors including retinoic acid receptors (RAR) and retinoid X receptors (RXR), which are transcriptional modifiers that are reported to be expressed in the prefrontal cortex, amygdala, and hippocampus regions of the brain (Goodman and Pardee, 2003a). These receptors bind to a specific DNA sequence and either suppress or promote target gene expression (Khorasanizadeh and Rastinejad, 2001). In animals, retinoid deprivation or mutations in the RAR and RXR genes have been linked to the suppression of spatial memory and learning, as well as the emergence of depression (Nomoto et al., 2012). In retinoid-deficit rats, suppressing RAR expression resulted in the accumulation of amyloid-beta (Aß) polypeptide in the vasculature, according to research (Shudo et al., 2009). Retinoids play a vital function in neuroprotection by preventing neuroinflammatory processes (Lee et al., 2009a). Microglia, have been shown to suppress the production of neuroinflammatory cytokines and chemokines (Goncalves et al., 2013a). To the improvement of cholinergic neurotransmission, retinoid receptor agonists were known to stimulate the expression of the choline acetyltransferase (ChAT) and acetylcholine transporter genes (Mufson et al., 2008). Retinoids are conventionally accepted as antioxidants, have significant role in the maintenance of brain activity during advanced age. AD patients have been observed with moderate levels of serum vitamin A. Clinically, it was reported that the cognitive abilities in the group of 442 patients of AD were improved with increased serum Vitamin-A levels (Mohammadzadeh Honarvar et al., 2017a). It has been observed that vitamin A and beta-carotene restrain the generation of amyloid-ß fibrils from amyloid precursor protein (APP) and induce variation in the fibrillar anatomy of amyloid beta proteins (Fahrenholz et al., 2010). Retinoic acid (RA), a dynamic metabolite of vitamin A, has been observed to regulate the gene expression relying on APP processing in nuclear receptors including RAR and RXR. Vitamin A deficiency potentiaiates the depoisition of Aß peptides and decreases the long term potentiation of the hippocampus in animals. Researches has revealed that RA potentaite the appearance of the MNSOD2 gene in neuroblast cells, thus decreasing oxidative stress, an essential pathological factor in AD. Studies revealed that memory and cognitive impairments, and reduced neuroplasticity, are due to RXR and RAR mutations

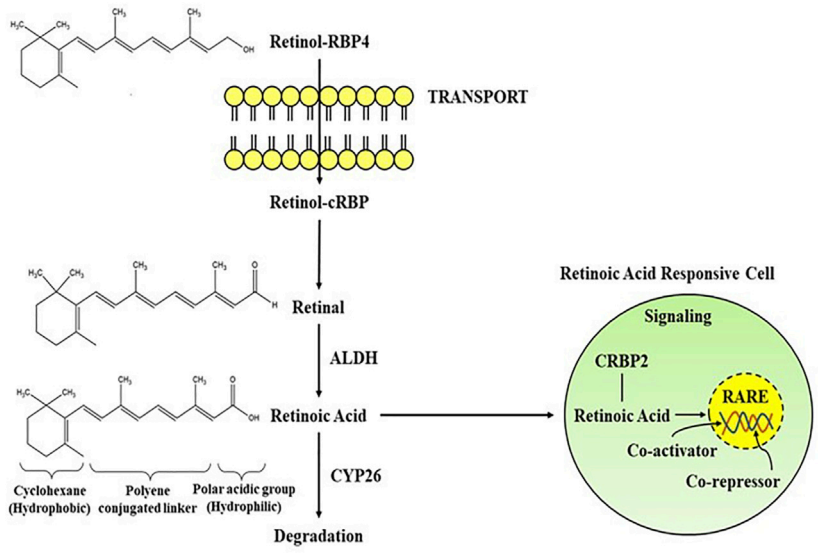

FIGURE 1
Schematic representation of the transport and signaling pathways of retinoic acid from retinol *via* the activity of alcohol dehydrogenase (ALDH) and degradation through CYP26. Retinoic acid mediates its activity by interacting with the RA receptors in RA responsive cell. RBP, Retinol binding protein; RARE, Retinoic acid response element.

(Connor and Sidell, 1997a; Etchamendy et al., 2003a; La Fata et al., 2014; Pierzchalski, 2015).In this review, we discussed the chemistry and biochemistry of various natural and synthetic retinoids, as well as their efficacy in preventing neurodegeneration in Alzheimer's disease.

Physiological portrayal of retinoids

The importance of retinoids in the growth and differentiation of the prenatal and postnatal brain has been recognised long ago (Jiang et al., 2012; Cunningham and Duester, 2015; Bonney et al., 2018). Yet, a substantial size of the data suggests that retinoid signalling is important in adult brain activity as well (Kour and Rath, 2016; Mishra et al., 2018). Vitamin A is the most widely occuring retinoid, regulating a number of physiolgical functions including embryogenesis, cellular proliferation, cell growth, and cell death, as well as proper brain functioning (Khillan, 2014). It is primarily synthesized from pro-vitamin A carotenoids, which may be found in a variety of colourful vegetables and fruits as well as in animal sources like egg yolks, and dairy products. Vitamin A carotenoids are synthesized by some microorganisms and photosynthetic plants which are metabolised to retinol in the small intestines of animals (Green and Fascetti, 2016). Some noteworthy scientific papers have already been published outlining the role of natural and synthetic retinoids in drug development and signaling cascades (Das et al., 2014b; Haffez et al., 2018; Chisholm et al., 2019). At present, there are number of major synthetic retinoids for their therapeutic use, even though some differences in haematological parameters occur following isotretinoin therapy, yet is the most excellent therapy for treating acne vulgaris in clinical studies (Gencoglan et al., 2018). Since, retinoids have conjugated double bonds, they are rapidly oxidised or isomerized by the action of oxidants, light, or intense heat. Increased ingestion of pro-vitamin A carotenoids is linked to a decreased risk of various brain disorders, including Alzheimer's disease, according to epidemiological research (Lakey-Beitia et al., 2017; Yang et al., 2017). Retinoids also improves visual acuity and protects against age-linked macular degeneration (Harrison, 2019). Therefore, natural and synthetic retinoids are now being explored intensively for effective neuroprotection in neuronal injuries and disorders (Chakrabarti et al., 2016a). The biosynthetic pathway of RA begins either with conversion of retinol to retinal or the manufacture of retinal (Figure 1) as provided by different food sources, often known as vitamin A (Sommer and Vyas, 2012). Two oxidation processes are required to synthesize retinoic acid from retinol. The activity of retinol dehydrogenase and the transition of NAD+ to NADH converts retinol to retinal (Hong et al., 2015). As in the following oxidation reaction, RA is synthesized from retinal with the activity of retinaldehyde dehydrogenases (RALHDs or ALDHs), which come in a variety of types (Kedishvili, 2016). Retinol attach to cellular retinol-binding proteins (CRBPs), whereas RA binds to cellular RA-binding proteins (CRABPs) in the cytoplasm (Napoli, 2017). CRBPs are classified into two classes (CRBP type I and type II); likewise, CRABPs are categorised into two classes (CRABP type I and type II), and they transport RA to

RARs and RXRs which are identical but differ in amino acid sequences. CRBPs are essential for the absorption and metabolism of retinol, whereas CRABPs are involved in the control of several RA signalling pathways and the availability of retinol to its receptors (Zhang et al., 2012). All RA isomers activate RARs, which function as heterodimers with RXR nuclear subtypes. Generally, retinoids occur naturally and are chemical conjugates of vitamin A. The RAR-RXR dimer regulates transcriptional activity by interacting with a retinoic acid response element (RARE) found in the gene promoter. Both *α* and *Γ* types of RARs are largely found in the adult brain, with higher expression in both the cortex and the hippocampus, while RARß, RXRΓ, RXRß, and RXR have limited distribution. Although levels might be significant in such confined locations; for example, RAR is expressed primarily in the hypothalamus and striatum. RAR protein's localized activity does not resemble one of their messenger RNA (mRNA) transcripts, suggesting that post-translational regulation of their representation is important. RA seems to have the ability to control the number of genes actively or passively through RARs (Hong et al., 2015; Kedishvili, 2016). The cytochrome P450 enzymes of the CYP26 family (Napoli, 2017), especially CYP26B1, are principally accountable for shutting down the network (Zhang et al., 2012).

Although the epigenetic roles of retinoids (regulation of transcription) are well-known, the non-genomic function is also important in its physiological activities. The non-genomic activity of retinoids is mostly assumed to be in intracellular pathways, such as stimulation of various kinase pathways, which permits faster indirect effects than the comparatively slow transcriptional processes. RAR is frequently involved in such cytosolic pathways, but retinoids can also interact with some other receptors like CRABP1 or protein kinase-Cα (Ochoa et al., 2003). The modulation of neuroplasticity through stimulation of mammalian target of rapamycin (mTOR) and ERK1/2 phosphorylation-induced mitogen-activated protein kinase (MAPK) is believed to include non-genomic processes (Zhong et al., 2018). RAR's interaction with the RNA-binding protein PurA is also being hypothesized to prevent axonal protein translation via encouraging mRNA transport to suppress ribonucleoprotein molecules. This inhibition is reversed by retinoids, enabling PurA-mediated axonal RNA transfer to recommence, resulting in neuronal growth stimulation. Aside from intracellular functions, Napoli and Chen found membrane-linked RAR-α in cholinergic neuronal cells, which increases neurogenesis through rapid ATRA-mediated neuronal translation (Chen and Napoli, 2008). Surprisingly, synapto genesis requires the integration of genomic and non-genomic mechanisms (Khatib et al., 2019). Furthermore, specific RAR agonists have different abilities to stimulate genomic and non-genomic systems, which can be engaged separately. RAR-α impacts the cholinergic as well as the GABAnergic and glutamatergic pathways. When RA acts on RAR, it increases the mRNA expression of Choline acetyltransferase (ChAT) and vesicular ACh transporter (VAChT), according to research done on mouse cell lines. As ChAT is necessary for memory encoding and the production of acetylcholine (ACh). Memory encoding and working memory are impaired when muscarinic ACh receptors are blocked, but additional information is enhanced when nicotinic ACh receptors are stimulated (Hasselmo, 2006) (Figure 1).

Retinoids and neuroinflammation

Dietary supplementation of carotenoids has been shown to play a crucial role in preventing several neu-rodegenerative diseases, including AD (Obulesu et al., 2011a). Retinoids are involved in neuronal patterning, differentiation, and axon outgrowth. Retinoid deprivation leads to impairment of normal brain development and function, resulting in the appearance of symptoms of different neurodegenerative diseases, including AD. Recent investigations indicate that retinoids can induce generation of specific neuronal cell types and also regenerate axons after damage (Maden, 2007). In addition, retinoids are involved in the maintenance of the differentiated state of adult neurons and neural stem cells as well as altered RA signaling levels. Many studies involving genetic analysis of AD have confirmed direct correlation between the genes that encode molecules involved in the RA signaling pathway and those that are considered to be involved in the pathogenesis of AD (Goodman, 2006a).

Retinoids are thought to perform through a variety of processes, according to a substantial amount of research. These chemicals have anti-inflammatory and antioxidant properties. Retinoids have also been discovered to play an important function as antioxidant enzymes as they contribute to the modulation of cytotoxic effects of ROS by eliminating free radicals from cells (Lushchak, 2011). ROS generation and deposition are enhanced in cells during oxidative stress environments like as metal exposure. Retinoids offer protection from this unstable state through a variety of methods, such as inhibition of ROS generation, free radicals clearance, subsequent activation of antioxidant enzymes, and modulation of defence signalling pathways including Nrf2 signalling (Alpsoy et al., 2009). In this view, clinical data suggests that retinoid levels in the liver have been reported as a defence mechanism towards ROS-mediated metal exposure (Defo et al., 2012). Retinoic acid (RA) has been shown to protect neurons from oxidative stress and apoptotic death by lowering glutathione levels (Lee et al., 2009b). In hippocampus cells, it also improves superoxide dimustase (SOD1 and SOD-2) activity (Obulesu et al., 2011b). Furthermore, retinoids are involved in the development and proper functioning of several cells and organs of the immune system, indicating that they play an important regulatory role on various inflammatory responses.

The impaired generation of RA-dependent tolerogenic macrophages and dendritic cells in Vit A-deficient cells has been shown to exacerbate inflammatory response (Saurer et al., 2007). Study found that appropriate retinoid levels are required for proper functioning of epithelial barrier stability (Filteau et al., 2001). Retinoids, when consumed in sufficient amounts, have been shown to reduce inflammation in animals (Kang et al., 2007). Also, the anti-inflammatory actions of retinoids involves the inhibition of the synthesis of inflammatory cytokines by reducing the translocation of the NF-kappaB transcription factor (Horton et al., 2001). Additionally, RA has a profound suppressive effect on T-cells (Th cells) which are involved in neuroinflammation whereas RA stimulates Th2, a subtype of T-cell that has anti-inflammatory properties (Nozaki et al., 2006). Furthermore, RA has been demonstrated to increase FoxP3 T-cells, which decrease the inflammation (Kim, 2011). These findings suggest that retinoids are anti-inflammatory agents which may have clinical efficacy in neuroinflammatory disorders including AD (Mohammadzadeh Honarvar et al., 2017b).

Microglia maintain brain homeostasis in normal circumstances. When pathogenic triggers, such as proinflammatory cytokines, pH changes, or hypoxia, are detected, the cells undergo a metamorphosis known as "activation," which can lead to a chronic prolonged, vicious cycle of "sub-threshold" neuroinflammation. This moderate, yet continually sustained pro-inflammatory condition is thought to represent the neuronal component supporting neurodegenerative pathophysiology (Dantzer et al., 2008). Additionally, cytokines activate indoleamine-2,3-dioxygenase (IDO), a major enzyme in the kynurenine pathway (KP), which destroys the serotonin precursor tryptophan (Hochstrasser et al., 2011) culminating in a microglial-mediated rise in the neurotoxic NMDA-receptor agonist quinolinic acid (Schwarcz et al., 2012). The kynurenine pathway not only serves as a marker for inflammatory activation but also serves as a link between neuro-inflammation and transmitter imbalances, which are linked to a variety of neurodegenerative diseases. As a result, microglial stimulation is a viable therapeutic target for a variety of neurological diseases. Also, Aß causes activation of microglia and austrocytes to produce proinflammatory mediators, while retinoic acid can prevent the development of these proinflammatory cytokines by interacting with RARs, which are found in astrocytic and microglial cells. Retinoids have been shown to stimulate RAR and RXR, allowing these cells to adjust actions and limit production. Among the most fundamental tasks in the therapy of AD is to inhibit neuroinflammatory reactions. Previous research has found that retinoic acid can help in reducing neuroinflammation in neurodegenerative disorders. By reducing the transcriptional activity of the NFK-B, RA was believed to decrease the Aß-mediated generation of TNF-α and suppress the activity of inducible nitric oxide synthase (iNOS) in microglial cells (Kaur et al., 2006). Retinoids also reduce Aß-mediated neuroinflammation, amyloidogenesis, and cognitive impairments in animal models according to the latest research (Behairi et al., 2016). In transgenic mice, RA stimulates neural stem cell growth while suppressing microglial reactivity, resulting in hippocampal neurogenesis (Takamura et al., 2017). In an animal model with Aß-induced neuroinflammation, anti-inflammatory functions of a RAR ligand Am80 (Tamibarotene) (Table 1) were examined, and the analysis indicated that Am80 might endorse the generation of brain-derived neurotrophic factor (BDNF), thus providing cytoprotective outcomes in disease states (Katsuki et al., 2009). Upon administration of Am580 suppressed inflammation-mediated neuronal loss in cultured neurons (Jarvis et al., 2010). In numerous neurological diseases, namely Alzheimer's disease, RA implies a vital function in suppressing neuroinflammatory reactions and encouraging clearance of Aß additional study is presently being taken for a better comprehension of the microscopic basis for the mechanism of actions of retinoids and carotenoids in the reduction of neuroinflammation in AD. Both retinoic acid and carotenoids provide effective anti-oxidative and anti-inflammatory actions and thus can be used in neuroprotection. They can slow the production and buildup of amyloid plaques, reduce the peroxidation of lipids, and inhibit the release of pro-inflammatory factors, all of which enhance mental abilities (Mohammadzadeh Honarvar et al., 2017c). All such studies have suggested that retinoic acid is performed *via* different routes to provide highly effective neuroprotection in AD. A recent study showed that RA reduced neurotoxicity in the rat brain via regulating the activity of Sirtuin 1, a class 3 histone deacetylase belonging to the Sirtuin protein family, as well as NFκ-B (Priyanka et al., 2018).

RA is a CNS morphogen that is recognized to prevent neuroinflammation (Hellmann-Regen et al., 2013) and microglial activation and also, it is important for neuronal growth. RA is a powerful neuroprotective drug that has been linked to neuroplasticity (Maghsoodi et al., 2008). In neurological diseases, each of these pathways is reported to be disturbed. Furthermore, there is a considerable amount of direct evidence supporting retinoid signaling's role in the pathophysiology of AD (Bremner and McCaffery, 2008) and other neurological diseases (van Neerven et al., 2008), implying that local brain RA might act as an "endogenous antidepressant." Moreover, the commonly prescribed and well known antidepressant fluoxetine inhibits RA degradation (Wietrzych-Schindler et al., 2011), implying that fluoxetine's neuroprotective, anti-inflammatory, and perhaps anti-depressant effects are all achieved *via* RA signaling (Hellmann-Regen et al., 2015).

TABLE 1 The schematic data revealing the potential role of synthetic and natural retinoids as well as vitamin A derivatives in ameliorating the pathogenesis of Alzheimer's disease in various experimental models through distinct mechanisms/pathways.

S No.	Compound (natural and synthetic)	Biological action through receptor subtype	Potential effects/actions mediated by the corresponding agents	References
1.	Bexarotene	Synthetic RXR Agonist-selectively activates RXRα, RXRß, and RXRΓ subtypes.	• Reverse AB25-35 insulin reduction • Increase ApoE secretion by RXR activation • Reduce amyloid beta agglomeration in neurons and promotes its clearance from the brain • Improves spatial memory	Duester (2022)
2.	Am 80 or Tamibarotene	RXR/RAR Agonist	• Reduces insoluble Aβ40-Aβ42 levels • Maintains cortical cholinergic neurotransmission • Reduces anxiety and personality changes • Reduces BACE-1 expression through Nf-kB signaling	Maggio and Vlachos (2014)
3.	HX 630	RXR Agonist- selectively acts through nuclear receptor subtype RXR and little effect on other subtype	• Improving learning ability • Reduced microglial activation	Fukasawa et al. (2012a), Tousi (2015)
4.	Am 580	RAR Agonist- a stable benzoic acid derivative of retinoic acid which selectively activates RARα isomer	• Reduces Aβ aggregation both intracellularly and extracellularly	Fukasawa et al. (2012b)
5.	All-trans retinoic acid	Retinoic acid isomer	• Downregulation of BACE-1 expression • Modulates NF-kB signaling pathway • Increase IL-10 release • Imrove cognitive and memory abilities • Downregulates NOS production	Kawahara et al. (2014)
6.	Acitretin	Synthetic retinoid	• Increase APPs-a levels in CSF of mild to moderate AD patients • Decrease production of inflammatory cytokines • Increases expression of ADAM10 • Increases IL-6 levels in CSF	Sodhi and Singh (2014)
7.	Am 80 + HX 630	RXR/RAR Agonist	• Decrease neuroinflammation and microglial activation • Production of BDNF • Improve spatial learning	Liu et al. (2015)

Retinoids in ameliorating amyloid-ß

Lower intrinsic levels of retinoids have been linked to cognitive loss in the elderly (Huang et al., 2018), and have been found to decrease in the ageing mouse brain (Touyarot et al., 2013). This reduction affects cognition since RA promotes neurogenesis and neuroplasticity, both of which are necessary for memory and learning (Shearer et al., 2012). If low RA levels induce AD, it is reasonable to expect that enhancing them would be beneficial, and multiple *in-vitro* investigations have shown that RA lowers amyloid-beta toxicity (Sahin et al., 2005). Moreover, a vitamin A-deficient diet in mice causes disturbance in RA signalling pathway and Aß accumulation in the blood vessels of frontal brain neurons, which can be restored by RA treatment (Husson et al., 2006a). Increasing the RA signalling through its receptor agonists promotes cognition in transgenic mice models of AD, clears Aß in microglia and neurons, and has a powerful anti-inflammatory effect (Goncalves et al., 2013b). As a result, synthetic retinoids might be used to treat Alzheimer's disease and other neurodegenerative diseases. Because of its multiple therapeutic benefits, synthetic retinoid tamibarotene (Am80) is being explored intensively as a possible medication for Alzheimer's disease which tends to reduce the level of insoluble Aß42 in APP23 AD model mice, according (Kawahara et al., 2009) As such acitretin is under investigation, that has been shown to raise the levels of the α-secretase of amyloid-protein precursor (APP), promoting the non-amyloidogenic process in neuroblastoma cells and lowering Aß levels in APP/PS-1 AD model (Tippmann et al., 2009). Acitretin has also been shown to pass the blood-brain barrier in rodents (Holthoewer et al., 2012). Endres et al. (2014b) studied the effects of oral acitretin treatment on α-secretase-acquired APP levels in the cerebrospinal fluid (CSF) of patients with AD. Additionally, acitretin raised APPs- levels and increased non-amyloidogenic processing of APP in humans, according to the studies (Endres

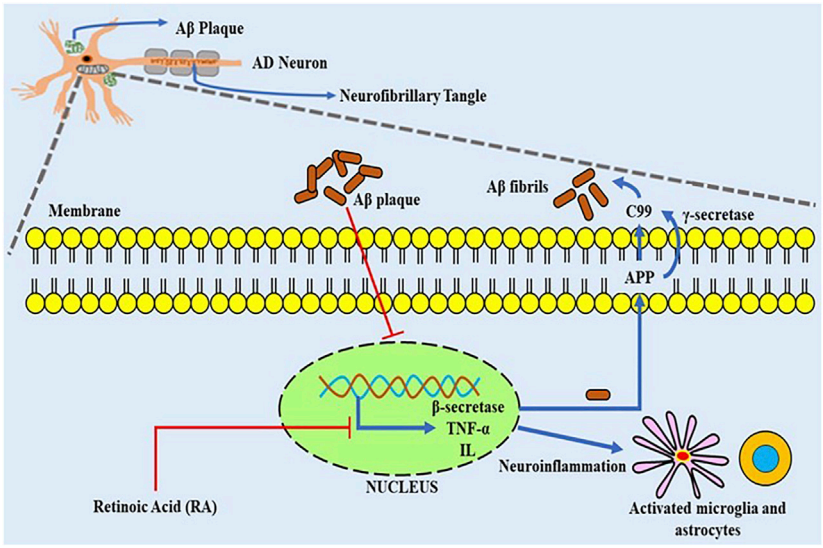

FIGURE 2
The overactivation of the ß- and Γ-secretases complexes may lead to the formation of Aß fibrils which successively leads to the generation of amyloid-ß plaques and neuroinflammation by activating the pro-inflammatory cytokines like TNF and IL. Such type of overactivation can be inhibited by the activity of retinoids like retinoic acid (RA). TNF-α, Tumour necrosis factor-alpha; IL, interleukins; Aß, amyloid beta; APP, Amyloid precursor protein; C99, carboxyl fragment 99.

et al., 2014b). Although some experimental data on the retinoids utilization in clinical trials is available, research on their possible therapeutic efficacy in AD is still in its initial stages. The data from a clinical trial of bexarotene (RXR ligand) in patients of AD showed that it may decrease cerebral amyloid and enhance plasma Aß1–42 in ApoE4 noncarriers (Cummings et al., 2016). Furthermore, acitretin entered Phase 2 trials in 2010, with preliminary findings showing a 25% rise in APPs-α levels in the CSF in the treatment group of AD patients (Endres et al., 2014c). Additionally, acitretin modulates certain genes connected to the pathogenesis of Alzheimer's disease, including choline acetyltransferase (chAT). Gonçalves and others tested the effectiveness of numerous retinoid receptor agonists, namely AM 580, CD 2019, and CD437, that are specific RARα, RAR, and RAR *Γ* agonists, accordingly, on the AD experimental model. They showed that activating the RAR receptor signaling pathway promotes Aß removal by increasing the expression of NEP and IDE enzymes and modifying glial cell production of the pro-inflammatory cytokine TNF-α (Kobayashi et al., 1994) (Figure 2).

As a result, a clear link between both reduced IDE mRNA levels and increased Aß deposition was already reported in the AD brain. Furthermore, reducing IDE expression may increase the likelihood of developing AD. Additionally, NEP-associated proteolytic processing of Aß has been shown to activate the signaling of RAR in brain cells (Goncalves et al., 2013a). Therefore, preliminary studies clearly show that natural and artificial retinoids can control Aß production and accumulation, suggesting that retinoids could be employed as a promising therapy for AD pathology as shown in Figure 2. Acitretin, a biosynthetic retinoid, is now being tested in humans under Phase 2 clinical trials. Although, there seems to be no experimental evidence supporting this treatment in Alzheimer's patients. AD is related to impaired removal of Aß from the brain which is modulated by APOE. IDE is a protein that dissolves Aß deposits and is found in the brain (Manzine et al., 2019). RA regulates IDE transcription, and the gene's promoter contains a RAR-α response element (Vekrellis et al., 2000). APP is a protein that aids in the production of Aß but is not cytotoxic and stimulates nerve growth (Melino et al., 1996). Also, the MAPT promoter has been demonstrated to be regulated by RA. The examination of MAPT's (König et al., 1990) nucleotide coding region discovered that it includes a RAR-α response element (Goedert et al., 1989), starting at 5′ -16. The genotype has four distinct TGACC domains, each of which potentially provides retinoid responsiveness (Duester et al., 1991). Variants of one or more RARs may alter the transcriptional activity of such components, according to the latest findings. In contrast to RA, transforming growth factor- ß (TGF-ß), a mediator crucially engaged in AD, Aß plaque generation (Hall et al., 1992), neurological damage, and neuroinflammation, favorably regulates APP translation (Burton et al., 2002). The treatment of TGF-2ß, which decreases amyloid load in mouse models, thus appears to shield terminally differentiated cells encoding TGF-B2 targets against Aß toxicity (Grammas and Ovase, 2002). TGF-2 processes are increased by RA and decreased by their

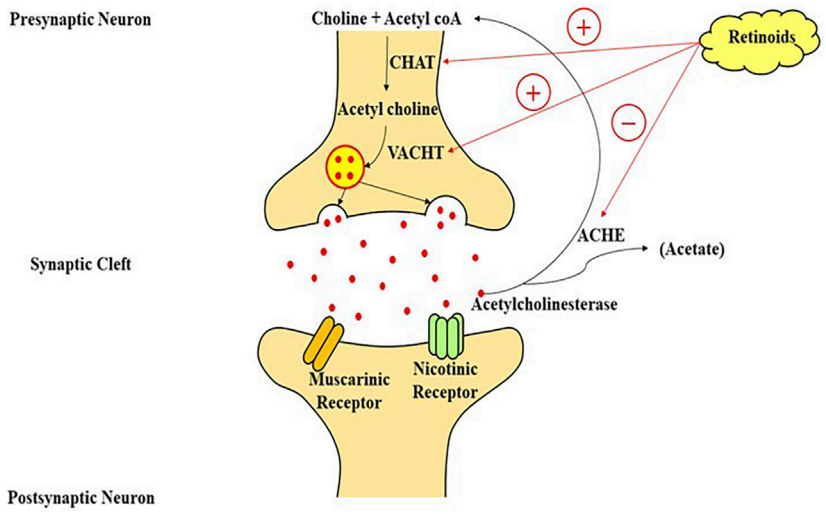

FIGURE 3
A model of retinoic acid signaling in the central nervous system, which plays an important role in the stimulation of transcriptional machinery to modulate the cholinergic neurotransmission through the production of choline-acetyl transferase mRNA and acetylcholine on the interaction of retinoic acid with its nuclear receptors. CHAT, choline acetyltransferase; VACHT, vesicular acetylcholine transporter; acetyl coA, acetyl coenzyme A; ACHE, acetylcholinesterase enzyme.

insufficiency (Wyss-Coray et al., 2001). In retinoid-deficit rats, RA restores TGF-ß2 in a tissue-specific manner. As a result, RA may enhance APP synthesis in healthy neurons through the TGF-ß signaling, including SMAD4, which is highly labeled in the AD brain. Transthyretin (TTR) may eliminate or inhibit the production of Aß, according to studies, and greater degrees of this seems to reduce amyloid-induced toxicity (Freemantle et al., 2002). TTR contents were found to be decreased in AD (White and Kelly, 2001), but no TTR alterations were found in an AD sample. Enhanced TTR does have other effects, including stabilizing RARG2 and decreasing the receptor's responsiveness to RA. The MAPK pathway is inhibited to accomplish this goal. All of the foregoing empirical evidence reinforces the notion that retinoid dysfunction is a critical component tin the emergence of Aß toxicity. This is especially important given the age-related reduction in retinoid availability seen in both healthy and AD humans (Serot et al., 1997). It will be important to analyze these individuals in the coding, 5′, 3′, and promoters to see whether these genetic changes in the retinoid receptors at regions are associated to increases AD susceptibility (Figure 2).

Retinoids mediated neurotransmission in Alzheimer's disease

Multiple neurotransmitter channels, particularly the catecholaminergic and cholinergic pathways, are disrupted in AD (Mecocci et al., 2002). Cholinergic neurons in the frontal cortex which extends to the neocortex, amygdala, and hippocampus degenerate, which is a characteristic of AD. The depletion of cholinergic neurons causes cognitive issues in experimental animals (Veng et al., 2003; Trillo et al., 2013), and impaired cholinergic signaling is associated with the initial stages of dementia. Treatment of cholinesterase inhibitors (drugs that stop the Ach metabolism and extend its activity in the cortex) was shown to enhance cognition in AD models, and these medications are now being used to address the symptoms of cognitive impairment in patients of neurological diseases (Hunter et al., 2004). Retinoids, which have neurotrophic actions on cholinergic neuronal cells and are markedly reduced in AD patients (Santucci et al., 1989), may serve as a complementary therapy for AD characteristics. RAR stimulation can increase the production of choline acetyltransferase (ChAT) and the VAChT protein, both of these aid in the transportation of Ach into presynaptic vesicles for their release (Goodman, 2006b). Retinoids have been shown to boost Ach and ChAT mRNA levels (Figure 3) (Berse and Blusztajn, 1995a). Dysfunction of monoaminergic pathways, such as the dopaminergic and noradrenergic systems, has also been linked to AD. As a result, the locus coeruleus, a brain's primary noradrenergic center with considerable distribution in the cortical regions, shows severe deterioration in AD. In AD, tyrosine hydroxylase (a flow-restricting protein for noradrenaline and dopamine production) and dopamine-beta-hydroxylase (DBH, the enzyme essential for noradrenaline generation) levels are also diminished (Ayaz et al., 2021). Furthermore, AD is linked to a

lower extent of norepinephrine in the cortex (Mann et al., 1980), and dopamine in the cortex, striatum, and amygdala (Iversen et al., 1983). The trophic effects of RA on the monoaminergic system may help in the mitigation of AD signs because they govern the activity of tyrosine and dopamine hydroxylase (Reinikainen et al., 1988) and could also explicitly regulate the articulation of dopamine D2 receptors by interacting with the RARE promoter site (Pinessi et al., 1987). Some researchers used a delayed recall training test to illustrate the potential usefulness of RA in improving learning and memory. As previously described in regards to RA and AD, this highly utilized scenario for memory is believed to necessitate stimulation of receptors in the 3 neuromodulatory systems: 1) muscarinic cholinergic receptors, 2) dopamine receptors, 3) and adrenergic receptors. The cholinergic receptor antagonist scopolamine was shown to impair memory as predicted, while RAR and RXR agonists were discovered to restore cognitive performance. While the specific process for this impact has yet to be discovered, Shudo and others hypothesized that a retinoid-mediated increase of D2 receptor activation might be one possibility (Kim et al., 2001). Retinoids and forskolin, an adenylate cyclase agonist, had already been demonstrated to enhance the quantity of ChAT mRNA in murine cells. Others have previously found that drugs that elevate cytoplasmic cAMP amount influence ChAT activity transcriptionally in a range of experimental conditions (Samad et al., 1997a; Ledesma and Dotti, 2012; Chakrabarti et al., 2016b). By analyzing the identical blots in sequence with ChAT and VAChT cDNA after a 48-h exposure of the cells with optimum amounts of RA, studies were able to effectively examine the effects of retinoids on ChAT and VAChT mRNA (Walsh and Selkoe, 2007).

Alteration in cholinergic system, which result in a reduction in Ach, are one of the characteristics of AD. The abnormal scopolamine-induced release of Ach due to blocked presynaptic autoreceptor in vitamin A deficient rats, results in cognition loss. Lower synthesis of ChAT, as well as brain cell death induced by RA and the gene promoter, are likely to be the causes of ACh release impairment (Gessel et al., 2012; Saleem et al., 2021). Increased ChAT appears to be favourable, and it has been proposed that RA regulates the vascular ACh transporter as well (Pedersen et al., 1995). In a passive avoidance experiment, retinoid restored scopolamine-derived cognitive loss (Berse and Blusztajn, 1995b). Retinoids also have a role in the expression of tyrosine hydroxylase, dopamine hydroxylase, and the dopamine D2 receptor. Dopamine receptors activity is regulated primarily by RA via interaction with a RA response element (RARE) at the promoter (Shudo et al., 2004).

Hence, retinoid signalling appears to have a function in regulating both acetylcholine production (through ChAT) and cholinergic availability at synapses (*via* AChE) which might serve a function in the pathogenesis and treatment of Alzheimer's disease. Yet, further research is required (Samad et al., 1997b) (Figure 3).

Retinoids in potentiating neuroplasticity

Retinol/Vitamin A is a fat-soluble vitamin whose major metabolite, retinoic acid, performs several biological functions comparable to hormones. The hippocampus and adjacent regions produce and metabolise RA, as well as express retinol-binding protein (Lane and Bailey, 2005). The majority of studies on the involvement of retinoids in memory and learning have used Vitamin A deficient (VAD) rats, RA receptor (RAR) transgenic mice, and ageing mouse models, etc. Etchamendy showed that VAD diet in mice model had dramatically decreased spatial memory and learning after 31 weeks, but that after a few days of RA therapy, the behaviour reverted to normal (Misner et al, 2001). VAD-mediated learning and spatial cognition deficits in rats may be facilitated by RA and RARs, according to some investigations (Etchamendy et al., 2003b). RA activates the RAR, a nuclear receptor that controls genes regulating the neuronal proliferation and differentiation, neurite outgrowth, synaptic plasticity, and other activities (Hernández-Pinto et al., 2006; McCaffery et al., 2006). The downstream pathways in RA activation, however, are unknown. Long-term potentiation (LTP) is the most common form of neuroplasticity, which is thought to be the cornerstone of memory and learning. RA levels and RARs, as well as neurogranin (RC3) and neuromodulin (GAP43), which together play a key role in modulating the uptake of Ca^{2+} and Ca/CaMK in neuroplasticity, have all been investigated in analyses of LTP and long-term depression (LTD) and the influence of VAD (Mark et al., 2006). Various *in-vitro* investigations have revealed that NMDA-NR1 may be a promising target for RA in regards to glutamate receptors. In neural development, RA might stimulate NMDA-NR1 subunit expression in human pluripotent adipose tissue stromal cells (Krucker et al., 2002). During growth, the gene expression of NMDA-NR1 can alter dynamically, such that certain quantity of NR1 subunits can be observed throughout all regions of the newborn rats brain, but 3 weeks after birth, this expression appears to reach its peak, before progressively declining to adult human levels (Kulikov et al., 2007). Such arrangement was observed to be comparable with RAR expression in neural tissue in some previous studies (Luo et al., 1996). The link between Ca^{2+}, NMDA receptors, and RA signalling *in vivo*, on the other hand, is yet unknown. LTP and LTD, which are the most fundamental processes of cognitive performance, are initiated by Ca^{2+} influx (Jiang et al., 2011). The LTP and LTD were observed to be impaired in RAR or RAR-RXR knockout mice (Bliss et al., 2006). (Figure 4) Furthermore, in cultured hippocampus, acute RA treatment enhances small excitatory post-synaptic activity magnitude (Chiang et al., 1998). The NMDA receptor, as well as other glutamate and non-glutamate receptors (such as TrkB), can control Ca^{2+} influx in neurons (Aoto et al., 2008). Many types of LTP and LTD have been linked to NMDA receptors (Lynch, 2004). The major

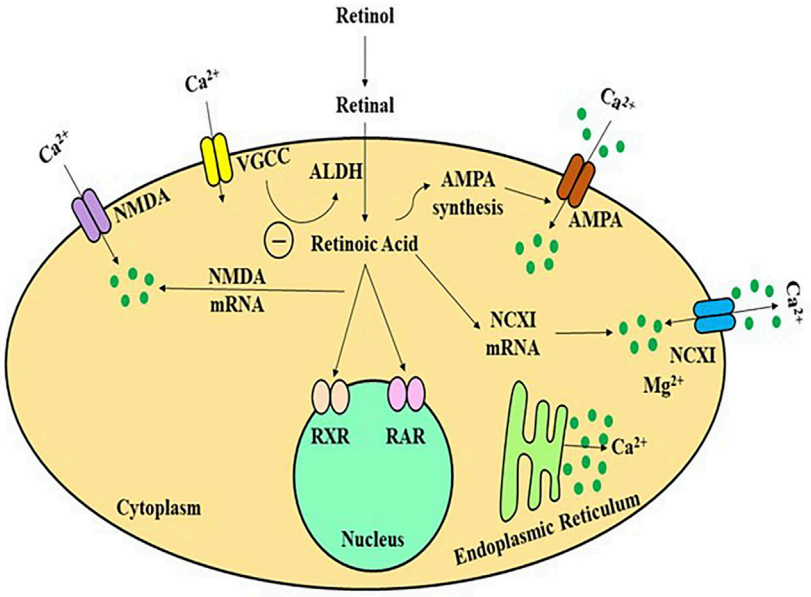

FIGURE 4
A schematic framework of activity-dependent retinoid generation in neurons. Appropriate synaptic transmission inhibits RA production in a Ca^{2+}-dependent manner by activating glutamate receptors and L-type Ca^{2+}channels. Limiting glutamate receptors or L-type Ca^{2+} channels reduces dendritic L-type Ca^{2+} intake and de-represses retinoid production, allowing AMPARs to be translated and inserted synaptically neural cells to increase the calcium influx. ALDH, alcohol dehydrogenase; AMPA, amino-hydroxy-methyl-isoxazolepropionic acid receptor; NMDA, N-methyl-D-aspartate receptor; VGCC, volatge gated calcium channel; RAR/RXR, retinoic acid receptors.

mechanism for Ca^{2+} influx into neurons through the post-synaptic membrane is thought to be interaction with NMDA receptors (Figure 4) (Isaac et al., 2009). Complex cognitive activities, including memory generation, necessitate RAR activation *via* all-trans-RA (ATRA) to promote neuroplasticity, according to some studies (Fukumori et al., 2010).

Calcineurin is a calcium-binding enzyme that is important for the link between calcium and RA in neuronal cells. Calcineurin suppression promotes the generation of RA. These alterations occur by stimulating the RA generation and RAR stimulation (Etchamendy et al., 2001). RAR is a protein that stimulates the production of calcium-permeable channels in this network. It is nongenomic because it does not involve its traditional job of controlling transcriptional activity, but rather the fast activity of RAR in the cytosol to control protein expression. RAR is continually carried to the nerve cells, where this functions as an mRNA-binding protein, preventing the production of mRNA like glutamate receptor 1 (GluR1), a critical component of the AMPA receptor calcium receptor. When RA interacts with RAR, it causes the receptor to alter the shape and the detachment of GluR1 mRNA, allowing it to be translated (Arendt et al., 2015). The postsynaptic surface is injected with newly translated GluR1 subunit-containing AMPA channels, permitting calcium uptake into the neuron, thus reinforcing the excitatory contact and turning off RA generation, disrupting the neuroplasticity. Thus the loss of neuroplasticity can be cured by shrinking inhibitory synapses as well as the stimulation of excitatory nerve terminals.

The results of this investigation revealed that RA-mediated morphological and physiological neuroplasticity in adult human cortical lines requires mRNA transcription, as evidenced by recent studies on the importance of protein synthesis in neuroplasticity (Poon and Chen, 2008). Synaptopodin was found as a target and regulator of RA-mediated neuroplasticity at the molecular level. Synaptopodin is an actin-regulating protein related to the spinal machinery (Biever et al., 1979), a membrane expansion of the endoplasmic reticulum observed in a subset of the telencephalic dendritic spine. The findings of such studies showed that 1) synaptopodin complexes are noticed in roughly 70% of nerve cells in the cortical region 2) synaptopodin is an indicator of the human spine system; 3) synaptopodin complexes and spine systems are observed in large nerve cells; and 4) a plasticity-mediating signal, such as RA treatment, stimulates the transformation of synaptopodin complexes, spine system. The significance of synaptopodin and the spine system in neuroplasticity is uncertain; although, it has been suggested that they play a role in the local synthesis of protein and regulation of cytosolic calcium dynamics (Deller et al., 2003). Synaptopodin interacts with actin and -actinin and has been proposed to control spinal mobility and long-term

spinal integrity *via* Rho-A signaling (Lenz et al., 2021). Synaptopodin and myosin V was discovered recently to be associated with this mechanism (Yap et al., 2020). Synaptopodin might thus be involved in the structural and functional alterations seen at synaptic vesicles undergoing NMDA receptor-induced neuroplasticity (Jedlicka and Deller, 2017). Both retinoids and synaptopodin have already been related to the homeostatic neuroplasticity in mouse neural tissue, as well as the production and transport of Ca^{2+}-dependent AMPA receptors. In diseased states of the brain, changes in retinoid signaling and synaptopodin activity have been linked to neuroplasticity abnormalities (Soden and Chen, 2010). As a result, in the neural tissue of patients with AD and cognitive impairment, changes in synaptopodin activity and retinoid signaling have now been found. Considering that retinoids have been suggested as a promising medicinal route for AD-linked cognitive impairment (Endres et al., 2014a). RA may work by altering the expression of synaptopodin and hence enhancing the capability of adult neural cells to enhance neuroplasticity. Eventually, VAD can harm cognitive function throughout postnatal life. One mechanism behind this phenomenon might be VAD-mediated downregulation of neural RAR, which results in lower production of NMDA-NR1 through no direct transcription, impairing neuronal Ca^{2+} excitability and decreasing LTP (Figure 4; Table 1).

Antioxidative actions of retinoids

Retinoids, vitamin A including carotenoids and beta-carotene, are lipophilic molecules produced by plants and animals which support various processes for cell growth, development, and differentiation (Duester, 2008; Shannon et al., 2017). Vitamin A is ingested in the form of pro-vitamin A carotenoids from plants and preformed vitamin A from animal-derived food which are modified to all-trans-retinol through a reactive pathway in the intestine. Through diet, carotenoids directly acts as ROS scavengers *via* energy transfer (Muller and Bohm, 2011). The deficiency of vitamin A and its metabolites were studied for their coalition with cognitive impairment in adult animals, thus highlighting the importance of sufficient vitamin A levels. Carotenoids are categorized as- 1) Non-pro-vitamin A carotenoids like lutein and 2) pro-vitamin A carotenoids including β-carotene and retinal, and non-pro-vitamin A carotenoids such as lycopene and lutein (Mohammadzadeh Honarvar et al., 2017d). Retinoids perform by various mechanisms and reach out the free radicals in mitochondria, plasma and cell membranes through electron transfer, physical scavenging, and hydrogen abstraction (Woodall et al., 1997). Also, they can react indirectly with several signaling pathways, such as the nuclear factor elytroid-2-related factor 2 (Nrf2), mitogen-activated protein kinase (MAPK), and NF-κB (Palozza et al., 2003; Ben-Dor et al., 2005). The antioxidative effects of retinoids includes quenching of singlet oxygen molecules and scavenging peroxyl radicals. Centrally located retinol-binding proteins (RBPs) are known to control the transport of retinol through the BBB into the brain (MacDonald et al., 1990). Higher levels of retinoids were implicated in the frontal lobe cortex of the postmortem human brain (Craft et al., 2004). ß-carotene, a precursor of retinoic acid, has also been observed in the singlet oxygen scavenging, free radical quenching, and lipid anti-oxidation in plasma. Thus, ß-carotene is considered to be localized in the lipid core of the membranes and implied as one of the favourable antioxidant to scavenge hydrophobic radicals in the membrane for clinical utilization (de Oliveira et al., 2012). Retinoids are structural derivatives of vitamin A which are reported to be involved in normal brain functioning including neuronal growth, development, and differentiation. Retinoids are known to regulate the glucocorticosteroids abundance in the brain which is an essential physiological process that can be observed in various stress-linked conditions to preserve the neuroplasticity within the hippocampus (Bonhomme et al., 2014). Under the oxidative stress conditions including metal injury and ROS accumulation, retinoids preserve the cells against this imbalance through several pathways, such as Nrf-2 and other defensive mechanisms like inhibition of ROS generation, free radical scavenging, induction of antioxidant enzymes. It has also been reported that RA has a neuroprotective role against apoptosis and oxidative stress *via* decreasing glutathione and restoring SOD-1 and SOD-2 in the hippocampus (Ahlemeyer et al., 2001). The role of retinoid signal transduction in the control of dopaminergic neurotransmission was noticed during the high levels of RA-synthesizing enzymes and RAR, which potentially has a significant action on regulation of cell survival, adaptation, and homeostatic regulation of the dopaminergic system (Lévesque and Rouillard, 2007). Retinoid expression plays a significant function in memory and cognitive performance, and synaptic plasticity as well (Crandall et al., 2004). RA supplementation upregulated μ-type opioid receptor 1 (MOR1) and its cascade and alleviated dyskinetic movements, which is a known result of long-term therapy of L-DOPA, in animal model (Pan et al., 2019). Additionally, RA induced the neuroprotective action on dopamine neurons in MPTP-treated mice model of AD. Administration of RA-loaded polymeric nanoparticle largely curbs the dopamine neuron loss in the substantia nigra and axonal innervations in the striatum (Esteves et al., 2015). Moreover, a research on synthetic retinoid revealed the reduction in neuroinflammation, Aß load, and oxidative stress with age-linked cognitive improved in AD patients (Bitarafan et al., 2016).

Future perspectives

Growing data suggests that retinoid insufficiency may have a role in the pathology of AD, including enhanced Aß accumulation and memory loss (dos Santos Guilherme et al., 2019). Retinoid serum levels were found to be lower in AD patients in a meta-analysis (Das et al., 2019). RA transmission, on the other hand, have been shown to reduce the development of AD in rats (Bonnet et al., 2008; da Silva et al., 2014) and might be used as treatment strategy in patients with AD (Reinhardt et al., 2016). In developing countries, VAD is significantly more common among pregnant women and school—age children (Kitaoka et al., 2013). Vitamin A deficiency may contribute to AD development by disrupting APP processing and resulting in Aß buildup. In AD mouse models, it was discovered that VAD raised Aß levels. Retinoids were implicated in the transcriptional control of the APP and BACE1 genes *via* NF-B and RARs in earlier studies (West, 2002; Endres et al., 2014d). In the mouse model of AD, retinoid deficiency promoted BACE1-induced APP cleavage and Aß production, facilitating senile plaque accumulation as well as cognitive impairments. Additionally, the VAD-induced Aß rise may impede RA production, aggravating AD pathogenesis (Koryakina et al., 2009). A production and clearance are both important factors in controlling Aß levels in the brain. Microglia, a phagocyte and a innate immune cell in the brain, play critical role in removal of Aβ peptides (Husson et al., 2006b). It was found that inhibiting RAR reduced Aß clearance by microglia in some investigation, implying that RAR-dependent Aß breakdown is assisted by microglia (Goncalves et al., 2013c).

In vitro data imply that all-trans-retinoic acid may also affect tau protein production, particularly the amount of phosphorylated forms of tau (Lee and Landreth, 2010). The utilization of retinoids with the goal of rectifying or decreasing neurodegenerative effects may also include the regulation of neuroinflammation, another pathological mechanism that causes neuronal and synaptic loss in diseases like AD. *In vitro*, amyloid-ß increases the production and release of the inflammatory cytokines such as tumour necrosis factor- and inducible NOS in microglial cells of AD model. Studies on the clinical benefits of omega-3 fatty acids may actively support the neuroprotective effects of retinoids in dementia. A nutritional investigation of patients with various kinds of dementia revealed that a diet high in antioxidants and polyunsaturated fats, especially vitamin A, may have a preventive role (Zeng et al., 2017). Retinoid signalling has already been proposed as a possible target for developing new Alzheimer's therapy (Charpentier et al., 1995). Because there is contradictory data in the field, it is difficult to say if AD patients have a dysfunction of RA expression. Rinaldi et al. (2003), Larrieu and Layé (2018) found that serum levels of retinols are lower in AD patients, while Connor and Sidell (Goodman and Pardee, 2003b) found that hippocampus retinoid content is equivalent in AD and control groups. Corcoran et al. (2004), Rinaldi et al. (2003) found lower levels of RAR and RALDH2 in AD brains, indicating that RA signalling is likely to be impaired in patients. Studies in aged mice indicate that RAR levels are reduced which may eventually causes impaired RA-mediated actions. Likewise, biological stimulation of RA signalling can restore the memory and learning as reported in aged animal models (Connor and Sidell, 1997b). However, the pathogenesis of Alzheimer's disease is complicated, involving many biological activities, and retinoids can influence these systems by influencing expression of genes and serving as an antioxidant. Particular data like "the retinoid activity in AD" should be interpreted with caution. To investigate retinoid treatment for Alzheimer's disease, researchers must first determine the specific involvement of retinoid and its receptors in the many mechanisms that regulate plaque generation, neurofibrillary tangles, cholinergic signaling, and ApoE activity in the adult brain *in vivo* (Corcoran et al., 2004). It has been proposed that retinoid signalling is a strong, plausible avenue for future Alzheimer's treatments. It is uncertain if Alzheimer's patients have retinoid signalling given that RA has been utilised in the treatment of AD and there are contradicting results. For example, GL Fata and others observed the serum level of vitamin A in Alzheimer's decreased While Connor and Seidel suggested that hippocampal levels of retinoids in Alzheimer's is similar to that of controls, A decline in RARα concentration and retinal dehydrogenase 2 in Alzheimer's brain, indicating that retinoid signaling may be impaired in patients, In older mice showed that the frequency of RAR decreased, which may eventually lead to a reduction in retinoic acid-dependent effects. In fact, perceptive deficiencies seen in older mice can be inverted by activating retinoic acid. However, the pathogenesis of Alzheimer's disease includes numerous and intricate signalling pathways, and retinoids may alter these processes by controlling gene expression in addition to having antioxidant characteristic consideration of vitamin A and its receptors' effects on numerous pathways, including plaque formation, neuroinflammation, and neurotransmission, is important in order to treat AD with retinoids (Eftekhari et al., 2021; Ayaz et al., 2022).

Conclusion

Retinoid signaling is clearly involved in neurodegenerative diseases like AD, as well fdas memory and learning. The relevance of sufficient Vitamin A status for such cognitive activities has been highlighted in adult mice and rats, but the exact targeting of retinoid signalling pathways behind these pathology have yet to be identified. Remarkably little is focused on the impact of ATRA signalling on other brain

functions, given the enormous number of neuronal functions that could potentially be controlled by retinoids in the adult brain. Transgenic mouse models have revealed a role for retinoids in cholinnergic signaling, gene transcription, neuroplasticity, amyloid plaque formation. Nevertheless, such data may not clearly distinguish between the well-described protective effects of retinoids and neurodegeneration. Future study in this new subject is needed to enhance our knowledge of the role of retinoids in the brain. Retinoids, retinoid ligands, and inhibitors have a lot of promise as treatments for Alzheimer's, and probably other neurodegenerative disorders. However, we need to learn more about the neuronal genes and molecular pathways that are precisely regulated by retinoids, as well as the physiological consequences in the brain, before such interventions may be explored.

Author contributions

Conceptualization, TB and DK; methodology, TB and AS; investigation, TB, DK, and RKS; resources, TB and SB.; data curation, TB, HM, and MA; writing-original draft preparation, TB and DK; writing-review and editing, HA and AM; visualization, AS and TB; supervision, TB and SB.

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Conflict of interest

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Publisher's note

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Investigation of Natural Compounds for Therapeutic Potential in Streptozotocin-induced Diabetic Neuroinflammation and Neuropathic Pain

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Diabetic neuropathy (DN) is a serious microvascular complication of diabetes mellitus (DM) that impacts the nervous system. Several risk factors are involved in the progression and maintenance of DN-associated pain, such as higher expression of various inflammatory mediators, e.g., tumor necrotic factor-alpha (TNF-α), nuclear factor-kappa B (NF-κB), and cyclo-oxygenase-2 (COX-2). The present research explores the neuroprotective potential of natural isolates, including berbamine, bergapten, and carveol, on the DM-induced neuroinflammation and neurodegeneration that cause neuropathic pain. The study utilized computerized techniques, including computational analysis (a docking assay and a molecular dynamic simulation) before moving to *in vivo* protocols. Diabetic neuropathy was induced by intraperitonial injection (IP) of streptozotocin (65 mg/kg), and the animal subjects (rats) were kept for 4 weeks for the development of DN. Once diabetic neuropathy was confirmed, the subjects were treated with berbamine, bergapten, and carveol until the sixth week (i.e., 2 weeks of treatment). At the sixth week, the rats were sacrificed, and the sciatic nerve and spinal cord of each was collected for further molecular investigation. Docking and a molecular dynamic simulation (MDS) delivered the information that the natural compounds (berbamine, bergapten, and carveol) were interacting with the selected target protein (i.e., mitogen-activated protein kinase). After IP, it was found that berbamine, bergapten, and carveol had

Abbreviations: BBM, berbamine; BRG, bergapten; CAR, carveol; STZ, streptozotocin; GSH, glutathione; GST, glutathione s-transferase; iNOS, inducible nitric oxide synthase; LPO, lipid peroxidase; H&E, hematoxylin and eosin; IHC, immuno-histochemistry; COX-2, cyclooxygenase; TNF-α, tumor necrosis factor-alpha; NF-κb, nuclear factor kappa B; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffer saline; H_2O_2, hydrogen peroxide; DTNB, 5, 5′-dithio-bis-2-nitrobenzoic acid; CDNB, 2,4-dinitrochlorobenzene; DAB, diaminobenzidine peroxidase; TCA, trichloroacetic acid; REC, research and ethical committee; IP, intraperitoneally; SN, sciatic nerve; SC, spinal cord; NO, nitric oxide; HRP, horseradish peroxidase; PCR, polymerase chain reaction.

ameliorated mechanical allodynia and thermal hyperalgesia by the 28th day of the study (2 weeks after treatment) without affecting blood glucose levels. Berbamine, bergapten, and carveol markedly elevated the levels of glutathione (GSH) and glutathione s-transferase (GST), in both the sciatic nerve and spinal cord, and also reduced lipid peroxidase (LPO) and nitric oxide (NO). The abovementioned natural isolates reduced pathologic alterations provoked through DN, a finding confirmed through histopathological assays (hematoxylin and eosin staining and immuno-histochemical analysis). Treatment down regulated higher expressions of the inflammatory mediatorcyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), and nuclear factor kappa B (NF-κB), as confirmed by ELISA and polymerase chain reaction (PCR). The outcomes of berbamine, bergapten, and carveol are compared with those of pregabalin as a positive control group. Compared to pregabalin, treatment with the aforementioned three natural compounds improved nociception and reduced hyperalgesic effects, and consequently reduced pain perception and inflammation. Our results suggest the mechanism for the neuro-protective impact of berbamine, bergapten, and carveol might possibly be arbitrated via COX-2, TNF-α, and NF-κB, and regulated by mitogen-activated protein kinase, ultimately ameliorating STZ-provoked, DM-induced neuroinflammation and neurodegeneration, and associated neuropathic pain.

KEYWORDS

natural compounds, molecular dynamic simulation, diabetic neuroinflammation, neuropathic pain, ELISA, PCR

Introduction

Diabetes mellitus (DM) is a most serious metabolic syndrome, and the fifth leading cause of death worldwide, being characterized by persistent hyperglycemia (Go et al., 2015). According to the International Diabetes Federation (IDF), in 2021 Pakistan had a population of 33 million diabetics, which was 70% higher than in 2019. Furthermore, 26.9% of those affected with diabetes remain undiagnosed (IDF, 2021). DM can result in serious complications, influencing various organs, such as the nerves, kidneys, heart, blood vessels, and eyes (Wild et al., 2004). Diabetic neuropathy (DN) is a microvascular complication of persistent DM that affects the nerves and results in significant disease and death rates (Kallinikou et al., 2019). Estimates of the high prevalence of DN among those diagnosed with diabetes in Pakistan range from 36% to 68% (Ali et al., 2013). Distal symmetrical sensory neuropathy is the most widely known type of DN, or recognized polyneuropathy. Loss of motor and sensory nerves are attributes of DN. Ongoing hyperglycemia contributes to neurotic changes, such as tightening of the neuronal capillary, axonal condensing, demyelination of nerves, injury to nerve fibers, and neuronal damage (Sasaki et al., 2020). Studies of the etiology of DN have distinguished numerous bio-chemical mechanisms of nerve and neurovascular impairment, amongst which neuronal injury may be ascribed to elevated levels of oxidative stress (Suryavanshi et al., 2020). Inside the peripheral nervous system, both chronic and acute DM are sources of oxidative stress, and trigger the progression of DN (Heydari et al., 2014). Modifications in neuronal activity within the peripheral and central nervous systems, and the further stimulation of glial and immune cells, can lead to the pathogenesis of neuropathic pain. Peripheral nerve injury is accountable for the onset of numerous inflammatory arbitrators, as well as chemokines and cytokines significant for the development and preservation of neuropathic pain (Woolf and Ma, 2007). Hyperglycemia also stimulates the transcription nuclear factor (NF-κB), which in turn causes overexpression of specific gene sequences that control the regulation of numerous inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2) (Ahmed et al., 2016). The medical demonstrations of DN produce distinct neuropathic manifestations: impulsive pain like burning, electric shock or a penetrating sensation, thermal and mechanical hypersensitivity, or alternatively, lack of pain, but an accompanying lack of sensitivity (Boulton, 2012). The current research work was designed to assess the protective effect of berbamine, bergapten, and carveol on streptozotocin-induced DN in rats, targeting various proteins, such as mitogen-activated protein kinase (Figure 1) for *in silico* analysis, and nuclear factor kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2) for *in vivo* investigations.

Hence, these compounds, i.e., berbamine (BBM), bergapten (BRG), and carveol (CAR), as shown in Figure 1, are explored for their potential therapeutic use in the neuropathic pain from STZ-induced diabetes. Natural compounds exhibit promising

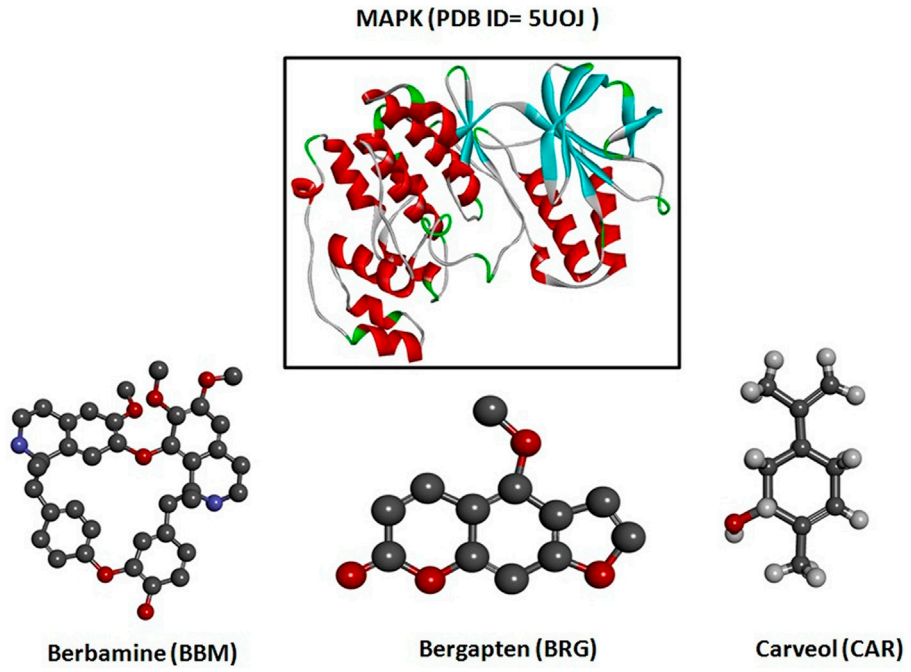

FIGURE 1
3D structures of berbamine, bergapten, and carveol and mitogen-activated protein kinase (MAPK, PDB I.D: 5UOJ) drawn through Chem Draw and Discovery Studio Visualizer client 2016.

neuroprotective potential, as per recently published literature (Faheem et al., 2022a). Berbamine (BBM) is a bisbenzylisoquinoline alkaloid from *Berberis amurensis* Rupr. It possesses anti-cancerous and anti-inflammatory properties, multidrug resistance, and synergistic activities. Psoralen is a furocoumarin-derived natural chemical from the *Ammi majus* plant. Methoxsalen-based furocoumarin, including bergapten (5-methoxsalenpsoralen), has been researched in relation to cancer, vitiligo, and psoriasis. The caraway plant yields carveol (CAR), an essential oil ingredient cultivated globally. Caraway includes pinene, thujene, phellandrene, camphene, limonene, and carvone (Faheem et al., 2022b). The selection of these compounds is based on their anti-inflammatory potential. The compounds were administered and, according to the findings, therapy with these natural chemicals (BBM, BRG, and CAR) down regulated NF-κB, which resulted in an attenuation of the diabetic-induced neuropathic pain caused by STZ. The aforementioned natural substances are thought to work by inhibiting the activity of the transcription factor NF-κB, which in turn reduces the production of pro-inflammatory cytokines, such as COX-2 and TNF-α, which may be suggested as a mechanistic route. In addition, the treatment improved antioxidant enzymes (GSH and GST) and decreased LPO and iNOS, thus preventing oxidative stress and free radical generation, halting the progression of STZ-mediated DN, and hence attenuating neurodegeneration and neuroinflammation.

Material and methods

Chemicals

Chemicals used were streptozotocin (STZ) formaldehyde, ketamine, 5% dimethyl sulphoxide (DMSO), hydrogen peroxide (H2O2), di-hydro-dithiobis-nitrobenzoic acid (DTNB), lipid peroxidase (LPO), PBS tablets, normal saline,1-chlor-2,4-dinitrobenzene (CDNB), xylaxine, GST, proteinase K, reduced glutathione (GSH), streptozotocin (STZ), ethanol, trichloroacetic acid (TCA), liquid nitrogen, trizol, chloroform, isopropanol, 70% ethanol, RNase-free water, ELISA kits including NF-κb by E-lab sciences (Catalogue number: E-EL-R0674), TNF-α by E-lab sciences (Catalogue number: E-EL-R0019) and COX-2 by Nanjing pars Biochem (Catalogue number: PRS-30205Ra) and pregabalin. The chemicals were all purchased from Sigma Aldrich.

Animals

Male Sprague-Dawley rats weighing 200–250 gm were utilized in the study. These rats were kept under a 12 h light–dark cycle in animal housing at the Riphah Institute of Pharmaceutical Sciences, Islamabad, with a precise environment (temperature: 25 ± 2°C and humidity: 55% ± 5%). The animals

were allowed standard food and water *ad libitum*, and were divided into different groups for the experiments. All experimental work was executed in accordance with the guiding principles of the Institute of Laboratory Animal Resources, Commission on Life Sciences University, National Research Council (1996), and was accepted by the Ethical Committee of the Riphah Institute of Pharmaceutical Sciences (Ref. No. REC/RIPS/2019/28).

Animal grouping and dosing

The rats were divided into 6 groups (n = 6 rats per group).

Group I (Non-Diabetic: ND) served as the saline group (NaCl 0.9%; 10 ml/kg) without STZ.

Group II (Diabetes Neuropathy: DN) served as a disease group (STZ; 65 mg/kg body weight).

Group III (DN + BBM) served as a treatment group (STZ; 65 mg/kg + BBM [1, 5, and 15 mg/kg]).

Group IV (DN + BRG) served as a treatment group (STZ; 65 mg/kg + BRG [25, 50, and 100 mg/kg]).

Group IV (DN + CAR) served as a treatment group (STZ; 65 mg/kg + CAR [5, 10, and 20 ml/kg]).

Group VI (DN + PBN) served as standard group (STZ; 65 mg/kg + pregabalin [PBN: 30 mg/kg]).

The dosing started 4 weeks after the induction of diabetes and continued until the sixth week. All behavioral investigations were performed after confirmation of STZ-induced diabetes mellitus associated DN on the 28th, 31st, 35th, 38th and 42nd days after induction. After six weeks the rats were sacrificed, and tissue samples of their sciatic nerves and spinal cords were collected for histopathological and molecular investigation.

Docking studies

Molecular docking or computational analysis was carried out to study the different therapeutic effects of the test compound by focusing on good binding affinity at the target site. The docking studies of berbamine (BBM), bergapten (BRG), and carveol (CAR) were performed using Auto Dock Vina 4.0 version (Scripps Research, San Diego California) and PyRx (Scripps Research, San Diego California) software against designated targets involved in DN and inflammation, such as mitogen-activated protein kinase (MAPK PDB ID: 5UOJ). ChemSketch (http://www.acdlabs.com) was used to prepare the ligand, and afterwards, Discovery Studio Visualizer -2016 (DSV- 2016; Dasault System Biovia Corp) converted it into a three-dimensional structure saved in a protein data band (PDB) file. Target proteins were retrieved from the protein data bank in PDB format, and refined by the DSV 16. The docking was performed through selection of amino acid residue from the active site and not against entire proteins; and binding affinity value was articulated in kcal/mol. PyRx software was used to obtained the best pose for the ligand receptor complex. The schematically depicted hydrogen bonding, hydrophobic bonds, and amino-acid residues participating in H-bonding of the best docked pose for the ligand protein-complex was achieved through post docking analysis (Malghani et al., 2020).

Molecular dynamic simulation

Molecular dynamics simulations of three complexes, i.e., BBM-MAPK, BRG- MAPK, and CAR-MAPK, were performed using the Desmond software package (Ivanova et al., 2018). Complexes were first exposed to an orthorhombic box with their respective number of water molecules for complexes by a simple point charge water model, with optimized potential for liquid simulations. After exposure to an isotonic environment through the addition of NaCl, the system was made electrically stable by adding counter ions, a Nose–Hoover thermostat maintaining a 300-k temperature, and pressure of 1.01325 bars by Martyna–Tobias–Klien barostate with 100 ns as the time of the simulations. Determination of electrostatic interactions was performed using the Mesh Ewald method (Faheem et al., 2021).

Induction and evaluation of diabetes and diabetic neuropathy in rats

After overnight fasting, the rats were subjected to a single intraperitoneal (IP) injection of streptozotocin (STZ; 65 mg/kg) solution for induction of DM. Blood samples were collected from the rats' tail veins 48 h after STZ administration and assessed for plasma glucose levels (PGLs) with an Accu-Check Performa gluco-meter. The rats with PGLs above 400 mg/dl were taken into consideration (Ostovar et al., 2020). After 28 days, treatment was initiated and was continued till 14th day. The animals were subjected for behavioral assays (mechanical and thermal hyperalgesia) and then molecular investigation (Yadav et al., 2014).

Plasma glucose levels assessment

In the present study, the PGLs of the rats were taken into consideration as metabolic parameters. The PGLs of the rats in each group were estimated after 48 h, and on the 14th day and the 28th day after STZ administration (Selagzi et al., 2008).

Mechanical allodynia

Von Frey filaments were used to assess the mechanical allodynia on the 14th day of treatment (Iqbal et al., 2020).

After habituation, the rats were placed in a cage with a mesh floor, and the Von Frey filaments were applied from below the mesh floor on the mid planter surface of each hind paw. Force was applied with lower pressure increasing to higher pressure perpendicularly on hind paws following filament bends of 2–3 s. Abrupt withdrawal responses of paws were observed.

Thermal hyperalgesia

After habituation, thermal hyperalgesia was evaluated by placing the hind paws of the rats on a hot-plate, adjusted to a constant temp (55 ± 0.5°C) on the 28th day for confirmation of DN (Suryavanshi et al., 2021). The cutoff time was 20 s to avoid paw tissue damage (Khairnar et al., 2020). Increased sensitivity to thermal stimulus was estimated through this hot-plate test.

Antioxidant assays

Investigation of oxidative stress markers in the sciatic nerves and spinal cords of the rats was significant in discerning the damage caused by STZ-induced DN. A tissue sample from both the spinal cord and sciatic nerve of each rat was homogenized in a phosphate buffer containing phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor at 4°C. Centrifugation of the sample homogenate of both the sciatic nerve and spinal cord was performed at 3,000× *g* for 10 min and the supernatant separated and used for further investigation (Imran et al., 2020).

Glutathione-S-transferase (GST) activity

1-Chloro-2,4-dinitrobenzol (CDNB) was used as a substrate to determine GST activity in homogenized tissue samples, as demonstrated previously, with a slight amendment (Shah et al., 2018). Each well was filled with 20 µl of the collected supernatant, a freshly prepared solution of 20 µl of 1 mM CDNB, 100 µl of buffer solution, and 15 µl of 5 mM reduced glutathione. An ELISA micro plate reader with a 412-nm wavelength was used to measure the absorbance of GST at room temperature.

Reduced glutathione (GSH) activity

The decreased levels of GSH were determined by a method employed previously, but with a slight modification (Shah et al., 2018). Each well was filled with 10 µl supernatant mixed with 75 µl of 0.6 mM 5,5-dithio-bis (2- nitrobenzoic acid) (DTNB) in 0.2 M sodium-phosphate buffer with pH of 8, and after that 100 µl of phosphate buffer was added to each well. The 0.2 M phosphate buffer and 0.6 M DTNB solution was used as control. The absorbance of all samples was measured using the ELISA micro plate reader at a wavelength of 340 nm at room temperature. The purpose was to determine the real absorbance from the tissue sample, subtracting the absorbance of the control, which then implies the GSH level.

LPO assay

Lipid peroxidation was performed employing the previously used protocol with a slight modification to estimate the thiobarbituric acid (TBA) reactive substances from tissue samples of the sciatic nerves and spinal cords of rats by colorimetric method (Shah et al., 2018). The tissue samples from each rat were homogenized separately. Centrifugation of sample homogenates was carried out at 3,000× *g* for 10 min and the LPO assay supernatant was collected. In this assay, 30 µl of the collected supernatant was mixed with 10 µl ferric chloride, 20 µl ascorbic acid and 36 µlk + buffer. The above mixture was then incubated in a water bath at 37°C for 1 h. To halt the reaction, 65 µl 0.8% TBA and 65 µl of trichloroacetic acid (TCA) were added, and this mixture was incubated at 100°C in a water bath for 20 min. The tubes containing samples were placed into ice-cold water and then centrifuged for 10 min at 2,500× *g*. The supernatant was then measured at a wavelength of 540 nm using a plate reader to estimate the absorbance of TBARS.

Nitric oxide assay

After homogenization in a phosphate buffer, containing phenyl methyl sulfonyl fluoride as a protease inhibitor, the sample (of sciatic nerve and spinal cord) was centrifuged at 4,000×g for 10 min at 4°C, and the supernatant was collected and processed for determination of nitric oxide (NO) (Kumar et al., 2010).

Histological examination

For histopathological examination and extraction of the sciatic nerve, the rats were sacrificed the sixth week after DM induction. The entire sciatic nerve of each rat was kept in a solution of 4% formaldehyde for fixing. Subsequently, the tissues were sectioned at 4 um coronal sections, cut using a rotary microtome, and fixed in a 4% formaldehyde solution. Later the fixed sciatic nerve sections were paraffinized for the following morphological analysis.

Immuno-histochemical investigation

An immuno-histochemical (IHC) investigation was performed according to a previously well-defined procedure

Primer	Tm	Sequence (5′-3′)
Rat_GAPDH_F	62.5°C	TCTTCCAGGAGCGAGATCCC
Rat_GAPDH_R	62.5°C	TTCAGGTGAGCCCCAGCCTT
Rat_TNF_α_F	56.1°C	CTTCAAGGGACAAGGCTG
Rat_TNF_α_R	56.1°C	GAGGCTGACTTTCTCCTG

with slight modifications (Faheem et al., 2020). The tissue (spinal cord) was fixed in paraffin on slides, deparaffinized with xylene 100%, and rehydrated by washing with graded dilutions of ethanol (100%, 90%, 80%, and 70%). Slides were processed via enzymatic procedure for antigen retrieval with the addition of proteinase K. PBS was used to wash the tissue sample slides for 5 min and this was repeated three times. To extinguish endogenous peroxidase action, the slides were submerged in 3% hydrogen-peroxide for 10 min at room temperature. PBS was used to wash the slides again, and then blocking serum, such as 5% normal goat serum (NGS), was applied to every slide containing test tissues at room temperature and they were kept for incubation for 2 h. Subsequent slides were incubated with the primary antibodies, which included anti-NF-κb, anti-TNF-α, and anti-COX-2, and kept for overnight incubation at 4°C. The next day, 0.1 M PBS was utilized to wash the slides and repeated 2 times, before they were again incubated with biotinylated secondary antibodies for 1.5 hours at room temperature. Again, PBS was used to wash the slides, and a

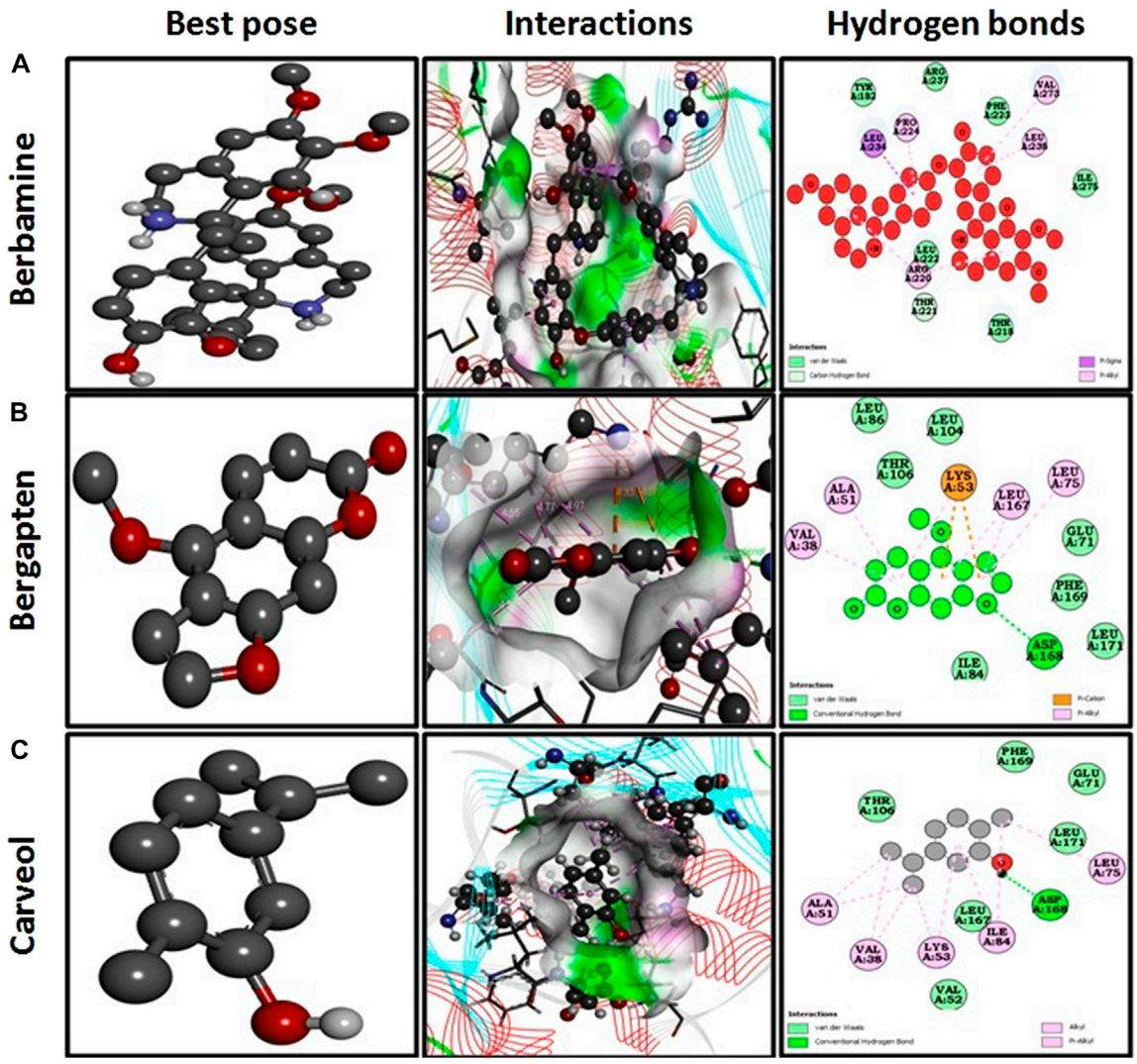

FIGURE 2
Represents best pose, interactions, and hydrogen bonds of berbamine **(A)**, bergapten **(B)** and carveol **(C)** and pregabalin against mitogenactivated protein kinase (MAPK, PDB I.D: 5UOJ) obtained through DSV 2016.

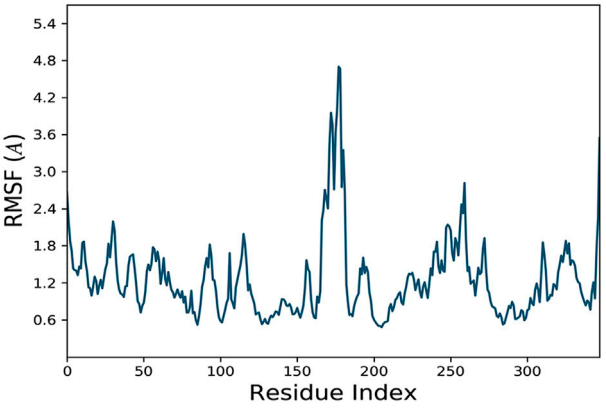

FIGURE 3
Represents root mean square fluctuation (RMSF) of mitogen-activated protein kinase (MAPK, PDB I.D: 5UOJ) obtained through Desmond software package.

humidifier box was then used for incubation, after application of avidin–biotin complex (ABC) reagents to the slides for 60 min. The solution of 0.1% diaminobenzidine peroxidase (DAB) was used for staining the slides after washing with 0.1 M PBS. The sample slides were washed with deionized water then dehydration was carried out with graded conc. (70%, 80%, 90%, and 100%) of ethanol. Slides were dried in the open air, cleared with xylene, and mounting media was used for fixing the cover slips. IHC images of the slides were taken with a light-microscope in TIF format and then ImageJ software was used for further quantification.

ELISA analysis

Commercially available ELISA kits were utilized to estimate expression of inflammatory markers, including NF-κb, COX-2, and TNF-α, as per the manufacturer's instructions. Silent Crusher-M (Heidolph-Germany) was used to homogenize approximately 50 mg tissue samples of the sciatic nerve stored at-80°C, using 0.1 M PBS containing protease inhibitor as PMSF (phenylmethylsulfonyl-fluoride). The centrifugation for the subsequent homogenate was performed for 20 min at 3000 RPM at 4°C and then the supernatant carefully separated from the top-evading pallet at the bottom. Using a bi-cinchoninic acid kit (BCA), the protein conc. was measured for every group. The 96-well plates containing the supernatant of sciatic nerve samples were processed with targeted antibodies. The ELISA micro plate reader was used to estimate expression of inflammatory markers, which are TNF-α and COX-2. The readings were taken 3 times by repeating the procedure. The resultant values of the inflammatory markers were expressed in picograms per milliliter to the overall protein content (Faheem et al., 2022a).

Real-time polymerase chain reaction

Following the instructions of the manufacturer, 200 mg of frozen tissue from the spinal cord was weighed and finely crushed in a prechilled pestle and mortar with liquid nitrogen. Homogenized tissue was kept at room temperature. Then, 1 ml of Trizol was added to the homogenate and transferred to 1.5 ml microfuge tubes. Tubes were gently inverted 4–5 times. The homogenate was incubated at room temperature for 5 min, 400 µl of chloroform was added and incubated at RT for 3 min, and then centrifuged at 12,000 rpm for 10 min at 4°C for phase separation. The aqueous upper layer was transferred to a new 1.5-ml tube placed on ice, and isopropanol was added in equal ratio. Tubes were incubated on ice (−20°C) for 10 min in a horizontal position to precipitate down RNA. Samples were centrifuged at 4°C and 12,000 rpm for 10 min. The pellets were washed twice with 1 ml of 70% ethanol at 7,500 rpm for 5 min at 4°C, and air dried completely. Then, 40 µl of RNase-free water or DEPC was added. RNA can be stored at −80°C until downstream application. cDNA was amplified by real-time PCR, using the same amount of cDNA, and employing a thermocycler as the method of execution. The expression of the mRNA was standardized according to the levels of expression of GADPH. The $2^{\Delta\Delta\text{-CT}}$ technique was used for real-time quantitative PCR, which allows for the determination of the relative gene expression (Imran et al., 2021). The forward and reverse primer sequences of rat GAPDH and TNF-α are shown as follows.

Statistical analysis

The outcomes from the current study have been stated as mean ± standard error of the mean (SEM). The results of the behavioral studies were investigated through one-way ANOVA accompanied by *post hoc* Tukey test. GraphPad Prism 8.0 software (United States) was used to evaluate graphical data. The statistically significant difference was considered for values of $p < 0.05$ (symbols; *or#). Morphological statistics of the sciatic nerve were examined by ImageJ software (United States, version 1.46). The significant difference of the DN group compared to the saline group is denoted by symbol "#," whereas the significant difference of the treatment groups compared to the DN group is denoted by symbol "*."

Results

Molecular docking

Docking investigations of the ligands berbamine, bergapten, and carveol were performed against proteins targets MAPK. Virtual results showed that berbamine, bergapten, and carveol

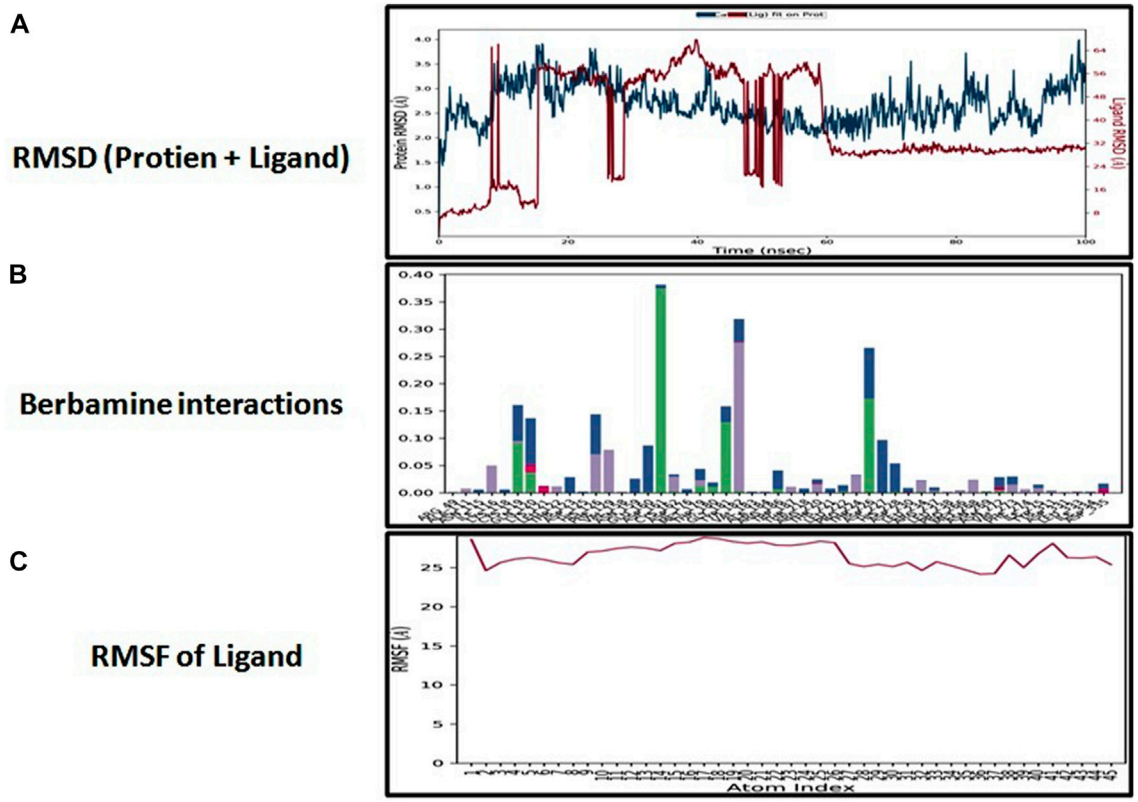

FIGURE 4
Represents root mean square deviation (RMSD) of mitogen-activated protein kinase (MAPK, PDB I.D: 5UOJ) and berbamine **(A)**, interactions (hydrogen bonds, hydrophobic interaction ionic and water bridges) of berbamine **(B)**, and root mean square fluctuation (RMSF) of berbamine **(C)** obtained through Desmond software package.

possess binding affinity of 6.9, 6.5, and 5.8 kcal/mol against MAPK. Bergapten and carveol also formed one hydrogen bond against MAPK at ASP 168, as shown in Figures 2A–C.

Molecular dynamic simulation

The docking analysis was further elaborated through molecular dynamic (MD) simulation, utilizing a Desmond software package. MD simulations of complexes of BBM-MAPK, BRG-MAPK, and CAR-MAPK were performed for 100 ns under a physiological environment. The results of the Desmond software package were received in the form of root mean square fluctuations (RMSF) of protein, as shown in Figure 3. The result for BBM in the form of the RMSD, hydrogen bonds, and the RMSF of the ligand are displayed in Figure 4. The BBM is interacting with the MAPK along the trajectory time of the simulation (100 ns) multiple times, as shown by Figure 4A. The BBM formed a hydrogen bond with multiple amino acids, including CYS119, GLY120, ASP161, MET179, THR 180, GLY181, THR226, and ASN276, as shown in Figure 4B. The result show a high RMSF, indicating reversible binding and less stability of BBM with the MAPK on the aforementioned environmental parameter, as shown in Figure 4C. The result for BRG in the form of the RMSD, hydrogen bonds and the RMSF of the ligand are displayed in Figure 5. The BRG is engaged with the target protein for more than 75% of the entire simulation duration, as shown in Figure 5A, providing stable hydrogen bonds at ASP168 and PHE169, as shown in Figure 5B. The result show reversible binding of BRG against the MAPK, but unlike BBM, BRG provides stable interactions, as reflected in the range of the RMSF values, as shown in Figure 5C, and in its hydrogen bonding. The result for CAR in the form of the RMSD, hydrogen bonds, and the RMSF of the ligand are displayed in Figure 6. CAR shows 35% engagement with the target protein, as shown in Figure 6A, proving hydrogen ASN26, SER28, VAL51, ALA107, GLU109, MET110, ASP168, and PHE189, as shown in Figure 6B. The result shows reversible binding of CAR against the MAPK, indicating unstable interactions, as reflected by the high RMSF value shown in Figure 6C.

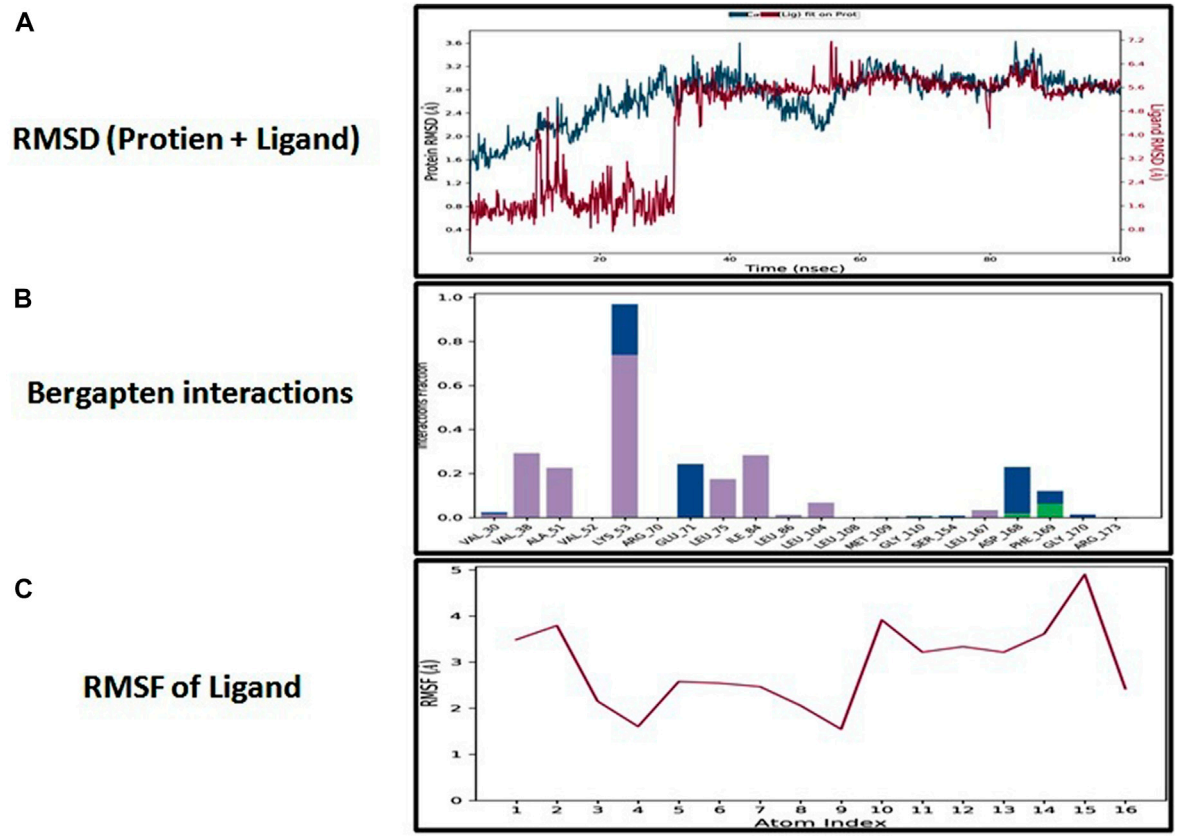

FIGURE 5
Represents root mean square deviation (RMSD) of mitogen-activated protein kinase (MAPK, PDB I.D: 5UOJ) and bergapten **(A)**, interactions (hydrogen bonds, hydrophobic interaction ionic and water bridges) of bergapten **(B)**, and root mean square fluctuation (RMSF) of bergapten **(C),** obtained through Desmond software package.

Plasma glucose level

The PGL was obtained at 48 h, and on the 14th day and the 28th day after STZ administration, and it was observed that the PGL was significantly high compared to the non-diabetic group, as shown in Table 1.

Effect of paw withdrawal threshold

The effects of berbamine (BBM), bergapten (BRG), carveol (CAR), and pregabalin (PBN) on mechanical allodynia on the 28th day of streptozotocin (STZ)-induced diabetic neuropathic pain are shown in Table 2. The treatment with natural substances led to a considerable improvement in mechanical performance. In a nutshell, BBM enhanced the paw withdrawal threshold (PWT) compared to the STZ-induced neuropathic pain group, at doses of 5 and 15 mg/kg on day 28. When compared to STZ-induced neuropathic pain, BRG substantially increased the PWT at doses of 50 and 100 mg/kg on day 28. In comparison to STZ-induced neuropathic pain, the PWT was considerably improved by CAR on day 28 at both 10 and 20 ml/kg.

Effect on thermal hyperalgesia

Table 2 displays the effects that berbamine (BBM), bergapten (BRG), carveol (CAR), and pregabalin (PBN) had on thermal hyperalgesia (TH) on the 28th day of streptozotocin (STZ)-induced diabetic neuropathic pain. The therapy with natural chemicals resulted in a substantial rise in TH. When compared with STZ-induced neuropathic pain, BBM increased paw TH at 5 and 15 mg/kg on day 28. BRG substantially increased TH when compared to STZ-induced neuropathic pain at doses of 50 and 100 mg/kg on day 28. When compared to STZ-induced neuropathic pain, the therapeutic effect of CAR at 10 and 20 ml/kg on day 28 was substantially more beneficial.

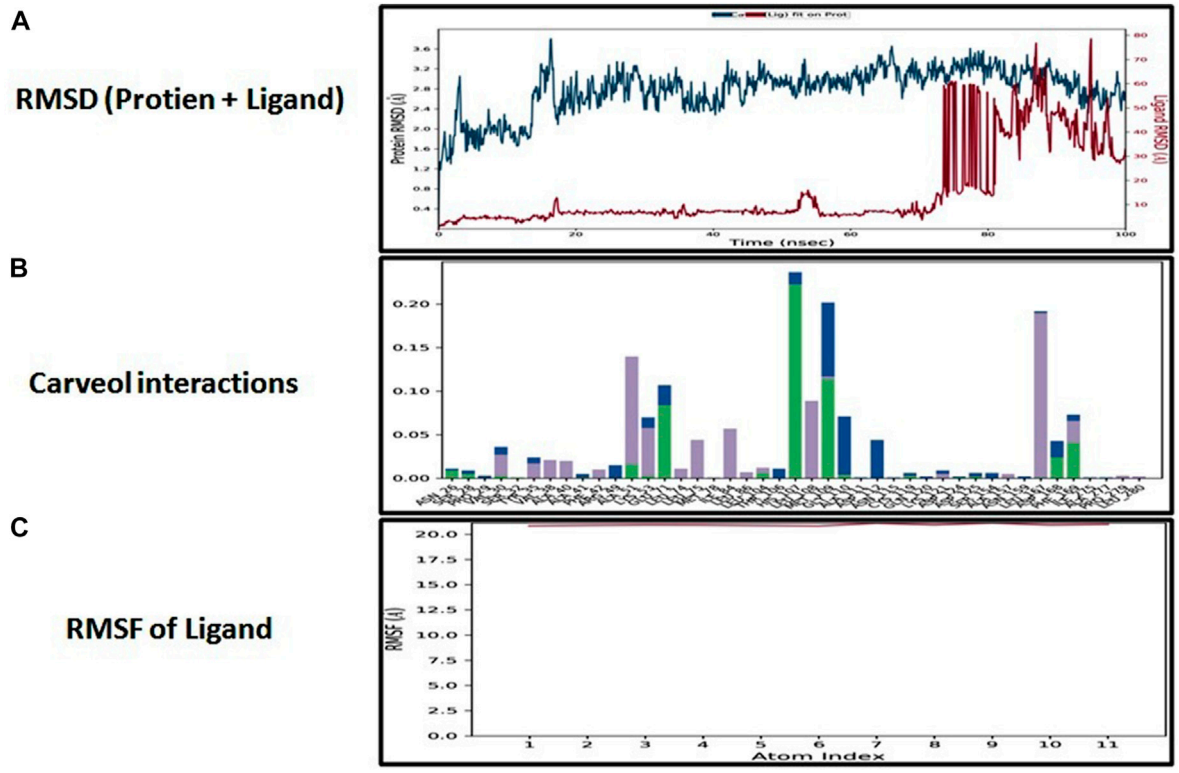

FIGURE 6
Represents root mean square deviation (RMSD) of mitogen-activated protein kinase (MAPK, PDB I.D: 5UOJ) and carveol **(A)**, interactions (hydrogen bonds, hydrophobic interaction ionic and water bridges) of carveol **(B)**, and root mean square fluctuation (RMSF) of carveol **(C)**, obtained through Desmond software package.

Effect on oxidative stress markers

Table 3 displays the effects of berbamine (BBM), bergapten (BRG), carveol (CAR), and pregabalin (PBN) on streptozotocin (STZ)-induced DN, as well as the expression of glutathione (GSH), glutathione s-transferase (GST), inducible nitric oxide (iNOS), and lipid peroxidase (LPO). Results show that BBM, BRG, and CAR enhanced the level of protective markers (GSH and GST), while decreasing the harmful oxidative markers (LPO and iNOS). This may be the reason why therapy was able to reduce the neuroinflammation and neuropathic pain associated with STZ-induced neuropathic pain.

Histopathological analysis (H&E staining)

The CCI group showed injury to the sciatic nerve and spinal cord, with various types of nerve damage, such as cellular spaces, edema formation, and jumbled cellular pattern because of CCI-induced pathological changes. Treatment with berbamine, bergapten, and carveol significantly improved disorientation of the sciatic nerve and spinal cord. Pregabalin also reversed the damage presented in Figures 7A,B.

Immuno-histochemistry evaluation

Results demonstrate that in collected tissue of the spinal cord there is marked elevation of the COX-2, TNF-α, and NF-κb in the STZ-induced diabetic group vs. saline group. Berbamine, bergapten and carveol attenuated over-expressed levels of COX-2, TNFα, and NF-κb significantly, as shown in Figures 8A,B. The standard drug (PBN) downregulated COX-2, TNF-α, and NF-κb in the spinal cord.

Effects on inflammatory markers (ELISA)

We investigated the effects of berbamine, bergapten, and carveol on the expression of COX-2, TNF-α, and NF-κb in the sciatic nerve and spinal cord as shown in Figures 9A–C. All three markers were overexpressed in the CCI group vs. the saline group in the sciatic nerve and spinal cord. Berbamine (5 and 15 mg/kg), bergapten (50 and 100 mg/kg), and carveol (10 and 20 ml/kg), decreased expression of COX-2 ($p < 0.01$) in the sciatic nerve and spinal cord. Berbamine (5 and 15 mg/kg), bergapten (50 and 100 mg/kg), and carveol (10 and 20 ml/kg),

TABLE 1 Effect of berbamine (BBM), bergapten (BRG), carveol (CAR), and pregabalin (PBN) on blood glucose level (mg/dl) of rats at 48 h, 14th day, and 28th day after streptozotocin (STZ)-induced diabetic neuropathy. One-way ANOVA followed by *post hoc* Tukey test. $^{###}p < 0.001$ vs. sham.

Plasma glucose level after 48 h

ND (10 ml/kg)	DM (65 mg/kg)	BBM (1 mg/kg)	BBM (5 mg/kg)	BBM (15 mg/kg)	PBN (30 mg/kg)
150.3 ± 3.1	450.1 ± 2.5 ###	480.3 ± 2.2	428.4 ± 2.2	433.3 ± 2.1	423.9 ± 1.8
ND (10 ml/kg)	DM (65 mg/kg)	BRG (25 mg/kg)	BRG (50 mg/kg)	BRG (100 mg/kg)	PBN (30 mg/kg)
150.3 ± 3.1	450.1 ± 2.5 ###	490.5 ± 1.6	440.2 ± 2.8	490.8 ± 1.5	423.9 ± 1.8
ND (10 ml/kg)	DM (65 mg/kg)	CAR (5 ml/kg)	CAR (10 ml/kg)	CAR (20 ml/kg)	PBN (30 mg/kg)
150.3 ± 3.1	450.1 ± 2.5 ###	410.2 ± 3.1	480.1 ± 1.0	455.7 ± 3.2	423.9 ± 1.8

Plasma glucose level after 14 days

ND (10 ml/kg)	DM (65 mg/kg)	BBM (1 mg/kg)	BBM (5 mg/kg)	BBM (15 mg/kg)	PBN (30 mg/kg)
153.2 ± 2.1	455.7 ± 4.0 ###	458.6 ± 1.8	468.4 ± 3.2	453.3 ± 2.1	442.3 ± 2.8
ND (10 ml/kg)	DM (65 mg/kg)	BRG (25 mg/kg)	BRG (50 mg/kg)	BRG (100 mg/kg)	PBN (30 mg/kg)
153.2 ± 2.1	455.7 ± 4.0 ###	472.2 ± 2.4	470.2 ± 4.8	420.8 ± 1.5	442.3 ± 2.8
ND (10 ml/kg)	DM (65 mg/kg)	CAR (5 ml/kg)	CAR (10 ml/kg)	CAR (20 ml/kg)	PBN (30 mg/kg)
153.2 ± 2.1	455.7 ± 4.0 ###	425.8 ± 2.8	445.1 ± 2.0	465.7 ± 3.2	442.3 ± 2.8

Plasma glucose level after 28 days

ND (10 ml/kg)	DM (65 mg/kg)	BBM (1 mg/kg)	BBM (5 mg/kg)	BBM (15 mg/kg)	PBN (30 mg/kg)
153.2 ± 2.1	446.6 ± 4.1 ###	465.4 ± 2.9	478.4 ± 1.2	420.3 ± 4.1	435.8 ± 3.5
ND (10 ml/kg)	DM (65 mg/kg)	BRG (25 mg/kg)	BRG (50 mg/kg)	BRG (100 mg/kg)	PBN (30 mg/kg)
153.2 ± 2.1	446.6 ± 4.1 ###	488.8 ± 4.3	480.2 ± 1.8	472.8 ± 1.8	435.8 ± 3.5
ND (10 ml/kg)	DM (65 mg/kg)	CAR (5 ml/kg)	CAR (10 ml/kg)	CAR (20 ml/kg)	PBN (30 mg/kg)
153.2 ± 2.1	446.6 ± 4.1 ###	460.1 ± 2.3	462.1 ± 1.5	485.7 ± 3.2	435.8 ± 3.5

TABLE 2 Effect of berbamine (BBM), bergapten (BRG), carveol (CAR), and pregabalin (PBN) on 14th day on mechanical allodynia and thermal hyperalgesia in streptozotocin (STZ)-induced diabetic neuropathy.

Mechanical allodynia (after 14 days of treatment)

ND (10 ml/kg)	DM(65 mg/kg)	BBM (1 mg/kg)	BBM (5 mg/kg)	BBM (15 mg/kg)	PBN (30 mg/kg)
38.4. ± 1.8	8.2 ± 2.5##	8.6 ± 2.0	15.4 ± 1.2*	31.4 ± 1.5**	28.8 ± 3.2**
ND (10 ml/kg)	DM(65 mg/kg)	BRG (25 mg/kg)	BRG (50 mg/kg)	BRG (100 mg/kg)	PBN (30 mg/kg)
38.4 ± 1.8	8.2 ± 2.5##	6.6 ± 1.5	14.2 ± 2.2*	29.30 ± 1.1**	28.8 ± 3.2**
ND (10 ml/kg)	DM(65 mg/kg)	CAR (5 ml/kg)	CAR (10 ml/kg)	CAR (20 ml/kg)	PBN (30 mg/kg)
38.4 ± 1.8	8.2 ± 2.5##	7.9 ± 2.4	16.8 ± 1.8*	30.9 ± 3.1**	28.8 ± 3.2**

Thermal hyperalgesia (after 14 days of treatment)

ND (10 ml/kg)	DM(65 mg/kg)	BBM (1 mg/kg)	BBM (5 mg/kg)	BBM (15 mg/kg)	PBN (30 mg/kg)
15.5 ± 1.5	3.8 ± 2.5###	4.0 ± 2.2	7.8 ± 1.6*	12.3 ± 1.1**	10.2 ± 1.0**
ND (10 ml/kg)	DM(65 mg/kg)	BRG (25 mg/kg)	BRG (50 mg/kg)	BRG (100 mg/kg)	PBN (30 mg/kg)
15.5 ± 1.5	3.8 ± 2.5###	4.6 ± 1.6	6.9 ± 1.2*	11.0 ± 1.8**	10.2 ± 1.0**
ND (10 ml/kg)	DM(65 mg/kg)	CAR (5 ml/kg)	CAR (10 ml/kg)	CAR (20 ml/kg)	PBN (30 mg/kg)
15.5 ± 1.5	3.8 ± 2.5###	4.3 ± 3.1	8.8 ± 2.2*	11.8 ± 1.2**	10.2 ± 1.0**

The data were expressed as mean ± SEM ($n = 6$). One-way ANOVA followed by *post hoc* Tukey test. $^{##}p < 0.01$, $^{###}p < 0.001$ vs. sham and $^{*}p < 0.05$, $^{**}p < 0.01$ vs. STZ-induced diabetic neuropathic pain.

TABLE 3 Represents the effect of berbamine (BBM), bergapten (BRG), carveol (CAR), and pregabalin (PBN) on streptozotocin (STZ)-induced diabetic neuropathy and expression of glutathione (GSH), glutathione s-transferase (GST), inducible nitric oxide (iNOS), and lipid peroxidase (LPO) in the sciatic nerve and spinal cord.

Sciatic nerve

Group	GSH (μmol/mg of protein)	GST (μmol CDNB conjugate/min/mg of protein)	iNOS (μmol/mg of protein)	LPO (nmol/TBARS/mg of protein)
ND (10 ml/kg)	48.22 ± 2.1	43.88 ± 1.5	34.22 ± 3.1	62.43 ± 1.8
DM (65 mg/kg)	22.32 ± 1.7##	28.42 ± 2.0#	65.32 ± 3.2##	112.81 ± 2.6###
BBM (15 mg/kg) + DM	40.29 ± 2.2**	36.10 ± 3.4**	55.21 ± 1.6*	92.36 ± 2.8*
BRG (100 mg/kg) + DM	42.34 ± 2.2**	38.24 ± 1.0**	41.04 ± 3.0**	85.16 ± 2.5**
CAR (20 ml/kg) + DM	46.44 ± 3.6**	40.11 ± 3.6**	48.97 ± 1.2**	84.22 ± 1.6**
PBN (30 mg/kg) + DM	42.17 ± 1.2**	38.30 ± 1.4**	53.21 ± 1.0*	88.42 ± 1.4*

Spinal cord

Group	GSH (μmol/mg of protein)	GST (μmol CDNB conjugate/min/mg of protein)	iNOS (μmol/mg of protein)	LPO (nmol/TBARS/mg of protein)
ND (10 ml/kg)	43.22 ± 1.8	35.71 ± 2.1	41.35 ± 1.2	62.33 ± 1.3
DM (65 mg/kg)	25.12 ± 2.1##	22.81 ± 2.8##	75.11 ± 2.1###	105.18 ± 3.1##
BBM (15 mg/kg) + DM	38.15 ± 2.8**	33.94 ± 1.2**	48.42 ± 3.5***	80.52 ± 1.8***
BRG (100 mg/kg) + DM	39.16 ± 1.6**	34.22 ± 4.2**	63.31 ± 1.8*	87.42 ± 2.6**
CAR (20 ml/kg) + DM	35.26 ± 2.6**	30.55 ± 1.2*	54.81 ± 2.6**	77.54 ± 3.2**
PBN (30 mg/kg) + DM	35.11 ± 2.5**	32.41 ± 1.8*	45.15 ± 2.2***	85.41 ± 2.1**

The data were expressed as mean ± SEM ($n = 6$). One-way ANOVA, followed by *post hoc* Tukey test. $^{\#\#\#}p < 0.001$ vs. sham $^{*}p < 0.05$, $^{**}p < 0.01$ vs. STZ-induced diabetic neuropathy.

down regulated TNF-α in the sciatic nerve and spinal cord. Berbamine (5 and 15 mg/kg), bergapten (50 and 100 mg/kg), and carveol (10 and 20 ml/kg) lowered the expression of NF-κb in the sciatic nerve and spinal cord. Pregabalin at 30 mg/kg minimized overexpression of all the inflammatory markers, including COX-2, TNF-α, and NF-κb in the sciatic nerve and spinal cord.

Effect on mRNA expression of TNF-α

We investigated the effect of BBM, BRG, and CAR on the mRNA expression of TNF-α in the spinal cord as shown in Figure 10. The result revealed that mRNA expression of TNF-α is significantly raised in the DN group. The treatment (BBM, BRG, and CAR) down regulated the mRNA expression of TNF-α as displayed. Pregabalin was used as standard, and found to decrease the mRNA expression of TNF-α.

Discussion

The STZ-induced diabetic model not only summarized the elevated PGLs and reduced body weight, but rats were also affected by neuropathic pain, making it a perfect model to examine DN. Diabetic neuropathy is one of most widely recognized complications of DM and has multi-factorial causes, and so its pathogenesis is debatable. Consequently, it is a serious challenge for scientists to explore the mechanisms of DN in addition to investigating and assessing new therapeutic alternatives. Nevertheless, oxidative stress and neuroinflammation have been identified as the leading pathophysiological cause in the development of DN (Kumar and Mittal, 2017).

In this study, a thermal-hyperalgesia test was used to confirm DM-induced DN in rats. The DN group had diminished body weight, significantly higher PGLs, and the nociceptive-threshold of thermal hyperalgesia and mechanical allodynia were

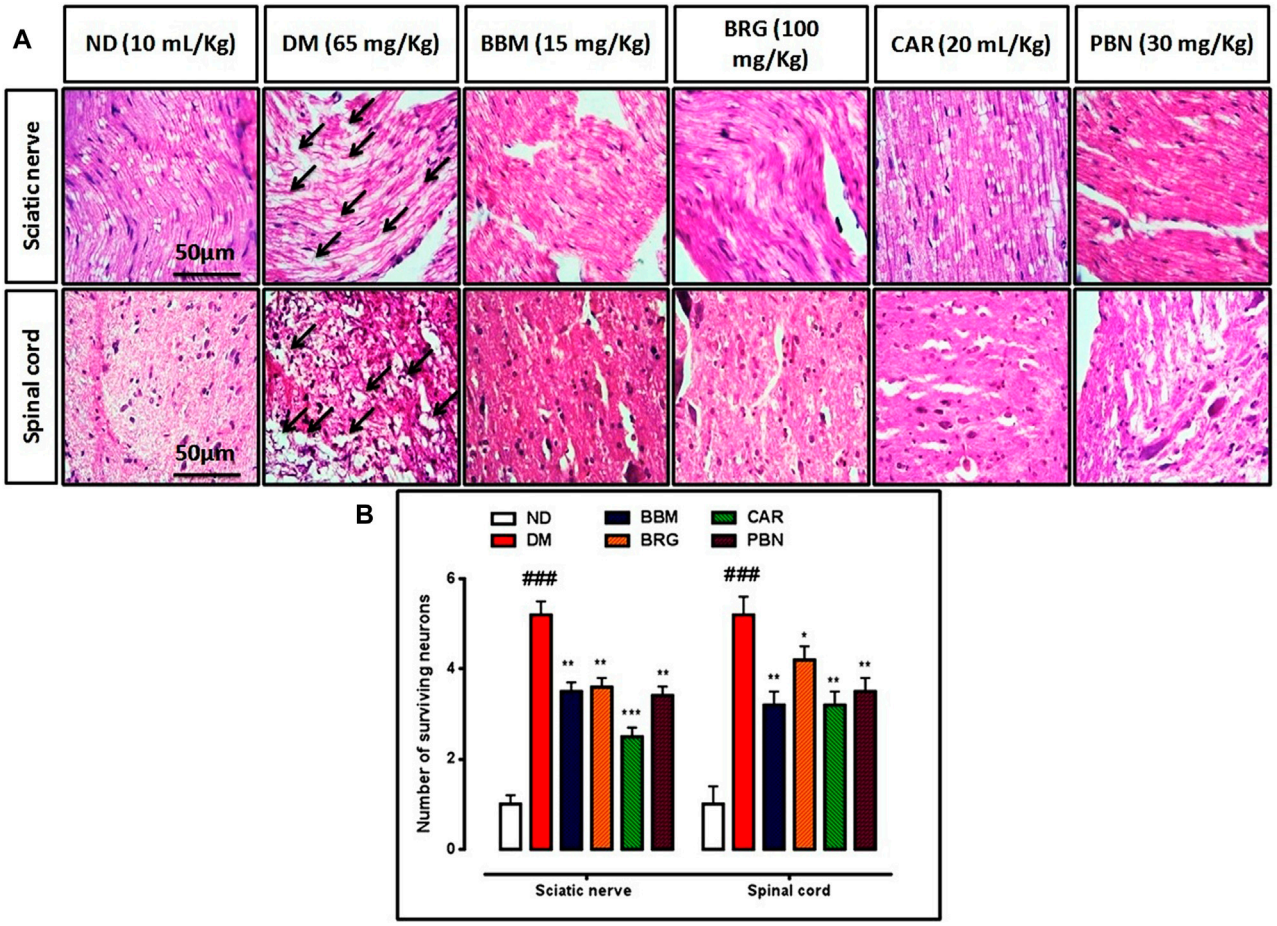

FIGURE 7
(A,B) represents hematoxylin and eosin (H&E) staining of the sciatic nerve and spinal cord and effect of berbamine, bergapten, carveol, and pregabalin on relative integrated density of the sciatic nerve and spinal cord. Images are analyzed by ImageJ software. Bar 50 µm, magnification 40x. GraphPad prism is used to plot the graph and probability value is calculated by Graph pad instate. Data expressed as mean ± SEM, (n = 6). One-way ANOVA followed by *post hoc* Tukey test. $^{###}p < 0.001$ vs. non-diabetic (ND),$^{*}p < 0.001$, $^{**}p < 0.001$,$^{***}p < 0.001$ vs. streptozotocin-induced diabetic neuropathy (DN).

significantly lower compared to the saline-group, indicating the development of DN. The current research explored neuro-protective effects in DN-induced neuro-inflammation through natural isolates. The outcomes of 14-days of therapy disclose that the behavioral deficit and raised expression of oxidative stress were significantly reversed, and the higher-expressions of inflammatory mediators, such as COX-2 and TNF-α were minimized. To explore the impact of berbamine, bergapten, and carveol on DN, various methods such as *in silico* studies, behavioral investigation, and molecular analysis were used.

In the *in silico* studies, BBM, BRG, and CAR were docked against target MAPKs. The DSV-2016 was used to attain molecular interactions by visualizing the docked ligand and target proteins. The ligand-target complex was significantly stabilized by molecular interaction in the form of hydrogen bonds and hydrophobic bonds (Al Kury et al., 2019). The interactions of BBM, BRG, and CAR constituted hydrogen bonding with binding affinity of 6.9 (BBM), 6.5 (BRG) and 5.8 (CAR) Kcal/mol against the MAPKs. The docking was further validated by utilizing a more advanced technique of molecular dynamic simulation and the results are best explained in Figures 3, 4, 5 and 6. The simulation study also explored how, in a physiological environment, the ligands interact with the target as reflected by their hydrogen bonds. Hence the docking studies are further validated with the simulation, with the compounds exhibiting hydrogen bonding with the same targets (ASP168).

Published research discloses that diminished sensory and motor nerve conduction velocities initiate different signs and symptoms related to peripheral DN, which include numbness induced by chronic hyperglycemia (Edwards et al., 2008).

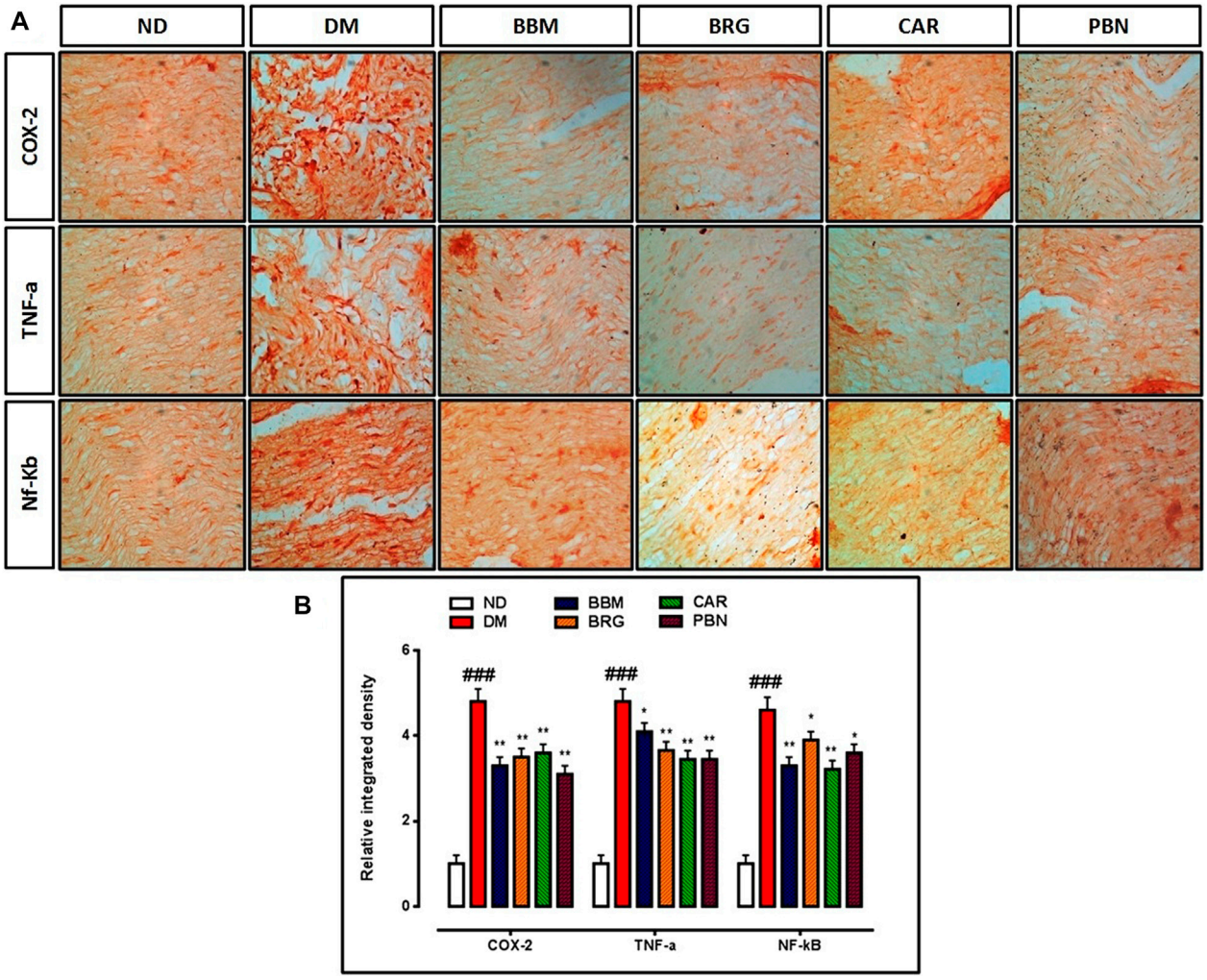

FIGURE 8
(A,B) represent the effect of berbamine, bergapten, carveol, and pregabalin on protein expression of cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), and nuclear factor kappa B (NF-κb) in the sciatic nerve. Images were analyzed by ImageJ software Bar 50 μm, magnification 40x. GraphPad prism is used to plot the graph and probability value was calculated by GraphPad instate. Data expressed as mean ± SEM, (n = 6). One way ANOVA followed by *post hoc* Tukey test. $^{###}p < 0.001$ vs. non-diabetic (ND), $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. streptozotocin-induced diabetic neuropathy (DN).

Oxidative stress is the principal pathophysiology associated with the worsening progression of many diseases (Chanchal et al., 2016). In DM, oxidative stress is recognized as a main risk factor for nerve injury, which results in the aggravation of pain impulses that characterize DN (Sandireddy et al., 2014). Diminished antioxidant capability and high oxygen utilization decrease nerve tissues prone to oxidative attack (Singh et al., 2014). It is well reported that oxidative stress in DM causes nerve conduction loss due to loss of myelination, and enormous myelinated fibers cause axonal damage, which decrease the nerve action potential, changing pain discernment, which can be estimated through behavioral investigation, such as thermal-hyperalgesia and mechanical allodynia (Said, 2007). Diabetic neuropathy in the rat model was reduced by minimizing oxidative stress to nerve tissue.

In the STZ-induced DN group, the levels of GSH and GST were significantly decreased and an enormous rise in iNOS and LPO was seen in the sciatic nerve and spinal cord. The sciatic nerve injury resulted from inflammation via the creation of a system of reactive oxygen species (ROS) and elevation of the level of inflammatory mediators. The spinal cord was also damaged due to direct association with the sciatic nerve (Shah et al., 2018). As an important antioxidant, GSH is also a significant controller of intra-cellular redox potential (Musaogullari et al., 2020). The values of GSH were considerably reduced within the sciatic nerves of the STZ-induced DN group (Kishore et al., 2017),

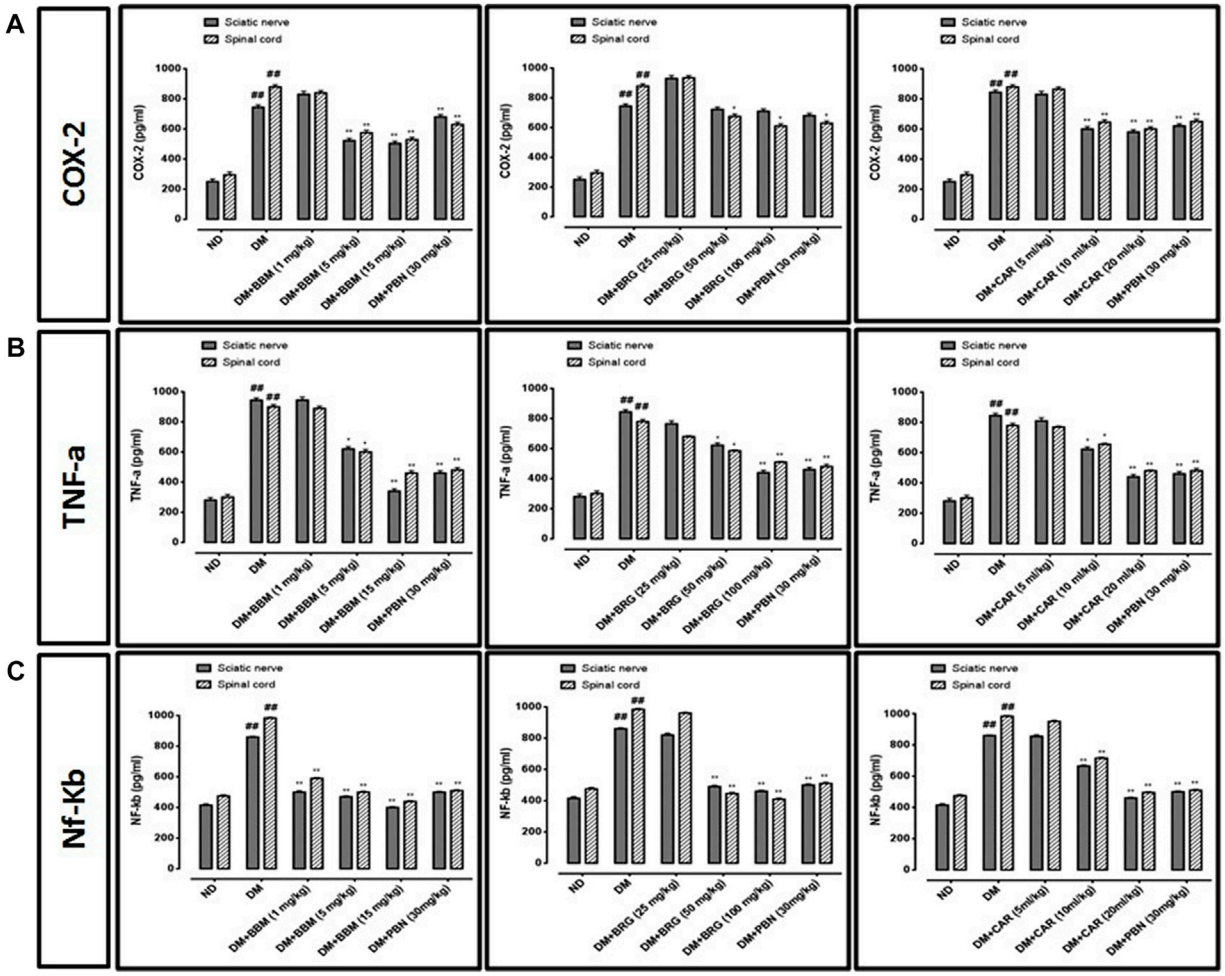

FIGURE 9
Represents the effect of berbamine, bergapten, carveol, and pregabalin on protein expression of **(A)** cyclooxygenase-2 (COX-2), **(B)** tumor necrosis factor-α (TNF-α), and **(C)** nuclear factor kappa B (NF-κb) in the sciatic nerve and spinal cord quantified by ELISA. Data expressed as mean ± SEM, (n = 6). One-way ANOVA followed by *post hoc* Tukey test. $^{###}p < 0.001$ vs. non-diabetic (ND), $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. streptozotocin-induced diabetic neuropathy (DN).

but were restored through treatment with BBM, BRG, and CAR. Consequently, the deficit in antioxidant capability in the STZ-induced DN group stimulated free radicals to assault cell membranes, initiate lipid peroxidation, and augment the level of inflammatory nitric oxide, thus damaging the tissue as a consequence of neuropathy (Hosseini and Abdollahi, 2013). The lipid peroxidation augmented by free radicals, which may be generated through glucose-auto-oxidation and protein-glycation (Metz et al., 2003), results in disruption of the antioxidant defense system (Faheem et al., 2021). The development of LPO and iNOS, which is especially associated with diminishing defensive endogenous antioxidants of the body and increasing ROS production, in turn induced inflammation in the spinal cord (Hains and Waxman, 2006). The levels of antioxidants (GSH and GST) were significantly reduced, whereas levels of LPO and iNOS were elevated in the STZ-induced DN group as compared to the saline group. Administration of different doses of BBM, BRG, and CAR ameliorated lipid peroxidation and improved antioxidant status in the sciatic nerve and spinal cord of DN rats *via* increasing levels of antioxidant enzymes.

The principle reason for painful DN is neuro-inflammation, which diminishes the peripheral nerves both structurally and functionally (Calvert et al., 2009). Multiple lines of proof acquired from experimentation and medical studies confirm that neuroinflammation is one of the major reasons for several deficiencies found in DN. In such manner, it was properly reported that peripheral neurons in DM activate *p*-NF-κB that mediates the improved inflammatory response (Wang et al., 2008). Many reviews have proven that in rat

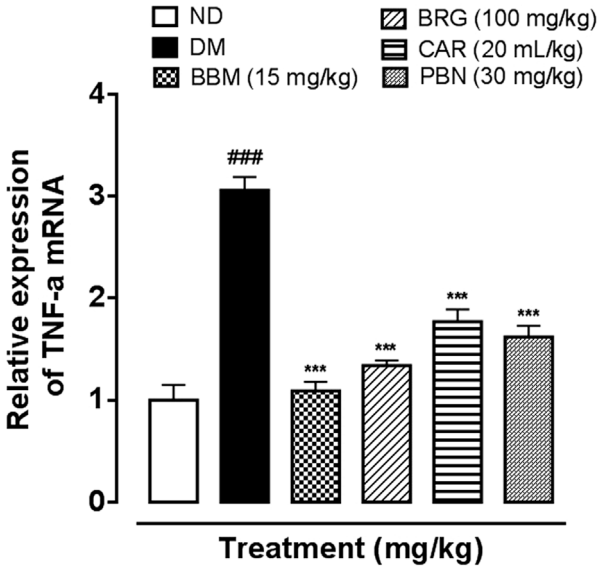

FIGURE 10
Represents the effect of berbamine, bergapten, carveol, and pregabalin on protein expression of mRNA of tumor necrosis factor-α (TNF-α) in the spinal cord quantified by polymerase chain reaction. Data expressed as mean ± SEM, (n = 6). One-way ANOVA followed by *post hoc* Tukey test. $^{###}p < 0.001$ vs. non-diabetic (ND), $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. streptozotocin-induced diabetic neuropathy (DN).

tissues of DM, hyperglycemia results in aggregation of advanced glycation end products, which bind with a cellular-receptor and stimulate the progression of *p*-NF-κB. This in turn triggers the production of pro-inflammatory cytokines (TNF-α and COX-2) and the elevation of oxidative stress markers (Schmidt et al., 1999).

Similarly, elevated levels of TNF-α were also observed within the sciatic nerves and spinal cords of the STZ-induced DN group, and suppression of these proinflammatory cytokine levels was accompanied by mitigation of neuropathic pain (Leng et al., 2020). COX-2 has been shown to be involved in the initiation and maintenance of DN (Kellogg et al., 2008). Several examinations have confirmed that TNF-α is an important pro-inflammatory cytokine in the STZ-induced DN group, and might stimulate the nuclear transcription factor NF-κB, which in turn initiates the cellular viability deterioration and stimulates demyelination of nerves (Evangelista et al., 2018). The NF-κB significantly stimulates the overexpression of proinflammatory cytokines and results in nerve damage (Sandireddy et al., 2014). Cyclooxygenase 2 (COX-2), and other inflammatory cytokines, have been regulated by direct involvement of the principle nuclear transcription factor, *p*-NF-κB.

The elevated levels of COX-2 are one more marker associated with the arbitration and progression of thermal hyperalgesia and mechanical allodynia (Boccella et al., 2019). Stimulation of COX-2 *via p*-NF-κB prompts changes to the osmolyte levels in nerves and augmented vasoconstrictors, together with thromboxane accumulation (Pop-Busui et al., 2002). The higher levels of COX-2 can result in an inequality of TXA2/PGI2, which may additionally stimulate hypoxia in tissues, and impairment of nerve tissues both structurally and functionally (Kellogg et al., 2007). Previous studies confirm that in nerve tissues of rats, COX-2 was detected to regulate the Na^{+}- K^{+} activity and augment the production of vasoconstrictor thromboxane (Pop-Busui et al., 2002). A significant event in the whole series of changes is represented by the stimulation of the *p*- NF-κB pathway and elevated levels of proteins consisting of TNF-α and COX-2. However, the progression of DN is stated by overexpression of inflammatory markers, such as COX-2 and TNF-α. Inhibition of these inflammatory mediators results in significant decrease in severity of pain (Cheng et al., 2010). In treatment groups, immuno-histochemical assessments showed that elevated expression of COX-2 and TNF, and *p*- NF-κB in the STZ-induced DN group, decreased markedly after treatment with BBM, BRG, and CAR. The morphology of the sciatic nerves in DN rats was also severely destroyed.

In this study, we confirmed STZ-induced inflammatory responses with ELISA, analyzing the NF-κb, COX-2, and TNF-α protein levels in the sciatic nerve and spinal cord of the STZ-induced DN group, and our results confirmed that these proinflammatory cytokines were up regulated in response to STZ administration. Hence, to find out the expression of NF-κb, COX-2, and TNF-α in the saline, DN, and treatment groups, tissue samples of the sciatic nerve were processed for ELISA. The overexpression of NF-κb, COX-2, and TNF-α in the STZ-induced DN group were significant in pro-inflammatory cytokines. PG was also studied for its potential in bringing down the higher expression of NF-κb, COX-2, and TNF-α in DN rats, and for inhibiting the development of disease (Figures 8D,E). Importantly, our study showed that DM-induced NF-κb, COX-2, and TNF-α protein expression in the sciatic nerve was significantly suppressed by treatment, indicating a role for BBM, BRG, and CAR in the suppression of neuroinflammation in the STZ-induced diabetic group. Our findings are indeed consistent with known roles of IO in the inhibition of oxidative stress and inflammation.

In the present study, treatment significantly attenuated thermal hyperalgesia, and mechanical allodynia, but had no significant effect on PGLs in comparison to the DN group. Treatment reduced oxidative stress, promoting neurodegeneration by down regulation of *p*-NF-κB, COX-2, and TNF-α expression towards normal levels, showing that BBM, BRG, and CAR can decrease neuroinflammation. Therefore, focusing on oxidative stress and inflammation pathways will possibly be significant therapeutic methods in the future to diminish the prevalence of DN. In order to find out the impact of BBM, BRG, and CAR in DN, several approaches, along with *in silico*, molecular dynamic simulation, behavioral, and molecular techniques were applied. It was demonstrated that behavioral deficits, and biochemical and

inflammatory changes in the rat model of STZ-DM-induced DN were significantly ameliorated with BBM, BRG, and CAR.

Conclusion

The current research explored the therapeutic potential of BBM, BRG, and CAR in STZ-induced DN. The result revealed that the abovementioned compounds halt neuroinflammation and neurodegeneration by down regulating NF-κB, which in turn minimizes overexpressed levels of proinflammatory cytokines, including TNF-α and COX-2. These findings were confirmed by various molecular investigations, such as IHC, ELISA, and PCR. Thus, BBM, BRG, and CAR may offer a new remedial option for management of neuropathic pain, and for prevention of oxidative stress and neuro-inflammation in STZ-mediated DM-induced neuropathic pain.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved, and all experimental work was executed in accordance with the guiding principles of the Institute of Laboratory Animal Resources, Commission on Life Sciences University, National Research Council (1996), accepted through the Ethical Committee of Riphah Institute of Pharmaceutical Sciences (Ref. No. REC/RIPS/2019/28). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Parthenolide promotes expansion of Nestin+ progenitor cells *via* Shh modulation and contributes to post-injury cerebellar replenishment

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Background: Regeneration of injuries occurring in the central nervous system is extremely difficult. Studies have shown that the developing cerebellum can be repopulated by a group of Nestin-expressing progenitors (NEPs) after irradiation injury, suggesting that modulating the mobilization of NEPs is beneficial to promoting nerve regeneration. To date, however, effect of exogenous pharmaceutical agonist on NEPs mobilization remains unknown. Parthenolide (PTL), a sesquiterpene lactone isolated from shoots of feverfew. Although it has been shown to possess several pharmacological activities and is considered to have potential therapeutic effects on the regeneration of peripheral nerve injury, its efficacy in promoting central nervous system (CNS) regeneration is unclear. In this study, we aimed to elucidate the role and possible mechanism of PTL on regeneration in injured CNS after irradiation using a developing cerebellum model.

Methods: We investigated the radioprotective effects of PTL on the developing cerebellum by immunoblotting as well as immunofluorescence staining and ROS detection *in vivo* and *in vitro* experiments, and then determined the effects of PTL on NEPs in Nestin CFP and Nestin GFP fluorescent mice. Inducible lineage tracing analysis was used in Nestin-CreERT2×ROSA26-LSL YFP mice to label and track the fate of NEPs in the cerebellum after irradiation. Combined with cell biology and molecular biology techniques to determine changes in various cellular components in the cerebellum and possible mechanisms of PTL on NEPs mobilization in the injured developing cerebellum.

Results: We found that PTL could attenuate radiation-induced acute injury of granule neuron progenitors (GNPs) in irradiated cerebellar external granule layer (EGL) by alleviating apoptosis through regulation of the cells' redox state. Moreover, PTL increased cerebellar Shh production and secretion by inhibiting the PI3K/AKT pathway, thus promoting expansion of NEPs, which is the compensatory replenishment of granule neurons after radiation damage.

Conclusion: Collectively, our results indicate that activation and expansion of NEPs are critical for regeneration of the injured cerebellum, and that PTL is a promising drug candidate to influence this process.

KEYWORDS

parthenolide, cerebellum, NEPs, Shh, irradiation

Introduction

It is widely acknowledged that regeneration of injury to the central nervous system (CNS) is exceptionally rare, and that it causes irreversible and permanent damage. Over half of the human brain's mature neurons are located in the cerebellum, although it represents only 10% of the total brain volume (Manto et al., 2020; De Zeeuw et al., 2021). Recent works have shown that the cerebellum plays a major role not only in maintaining motor coordination, but also in processing perception, cognition, and emotion (Van Overwalle et al., 2020). Consequently, it has become an excellent model for studying neurogenesis and circuit assembly, thereby attracting numerous research interest as a locus for a range of disorders and diseases (Beckinghausen and Sillitoe, 2019). Cerebellar growth occurs mainly after birth in mice and is due to rapid proliferation of Shh-driven external granule layer (EGL). Proliferation of cerebellar granule neural precursors (GNPs) in the EGL peaks at postnatal day 7 (P7) and ends at P15 as the GNPs exit the cell cycle and migrate to the internal granular layer (IGL), and differentiate into cerebellar granule neurons (Consalez et al., 2020). Studies have shown that although repeated of X-ray doses may cause cerebellar malformation at the proper time of pregnancy and during the early postnatal period, some granule neuron progenitors (GNPs) can survive, proliferate and reconstruct EGL under the action of a single X-ray dose (Altman et al., 1969). Recent experimental observations have revealed that a population of cerebellar Nestin-expressing precursors (NEPs) is responsible for regeneration of damaged developing cerebellum. NEPs can convert their differentiation capacity to produce mature granule neurons after irradiation injury, a process regulated by Shh signaling in the microenvironment. (Wojcinski et al., 2017). Correspondingly, in our previous study, a population of radiation-resistant precursor cells characterized by Nestin expression was also identified in the developing cerebellar EGL (Li et al., 2013), results that indicated that therapeutic strategies targeting NEPs have potential for regeneration of the developing cerebellum These results suggest that the development of therapeutic strategies for targeting NEPs is potentially valuable for regeneration of the developing cerebellum after injury. Despite its potential as a drug, reports have shown that the Shh protein has a neuroprotective effect, as evidenced by reduction of behavioral defects in a Parkinson's disease rat model and induction of differentiation of dopaminergic neurons (Patel et al., 2017). However, Shh has a relatively short half-life in serum, and its curative effect is difficult to evaluate *in vivo*. In addition to playing a crucial role in cerebellar patterning, Shh also regulates normal cell development (Brady and Vaccarino, 2021). In particular, aberrant activation of Shh signaling can cause hyperproliferation and malignancy (Northcott et al., 2019). During neurodevelopment, in addition to the classical pathway, Shh signaling can support rapid cell growth by activating aerobic glycolysis and lipogenesis. This process is mediated by various genes including hexokinase 2 (HK2) and pyruvate kinase M2 (PKM2), and the upregulation of aerobic glycolysis is a distinctive feature of the developing cerebellum (Gershon et al., 2013; Tech et al., 2017). From a clinical perspective, there is need to prospect for agents that can cross the human blood-brain barrier, accurately and effectively control Shh signals in the microenvironment within a certain range, and also exert multiple protective effects for NEPs expansion through various pathways.

PTL, a sesquiterpene lactone mainly derived from the medicinal herb Feverfew (*Tanacetum parthenium*), has been widely utilized for treatment of high fever, headache, stomach pain, toothache, rheumatoid arthritis, irregular menstruation and other inflammatory diseases (Ghantous et al., 2013). Its rapid interaction with biological targets, anti-inflammatory, redox-modulating, and epigenetic activities, as well as selective cytotoxicity to cancer stem and progenitor cells, is regulated by the nucleophilic properties of methylene γ-lactone ring and epoxy group (Freund et al., 2020). Numerous studies have shown that PTL also has a protective effect on the nervous system, and can cross the blood-brain barrier to reduce brain inflammation (Zhang et al., 2022). The experimental results showed that PTL significantly improved neurological deficits, brain water content and infarct volume in a permanent middle cerebral artery occlusion (MCAO) model. (Dong et al., 2013). In an intracranial hemorrhage (ICH) rat model, PTL was also found to increase the number of surviving neurons to the improved neurological deficit and brain edema (Wang et al., 2020). Another study showed that PTL ameliorated oxygen-glucose deprivation ischemia/reperfusion-evoked neuronal injury and oxidative stress in PC12 cells (Zhang et al., 2017). Earlier researches focused on PTL's protective effects on cytotoxicity by inhibiting NF-κB. Interestingly, Gobrecht and colleagues (Gobrecht et al., 2016) demonstrated that a single low-dose of PTL also significantly promoted regeneration of injured sciatic nerve axonal by interfering with detyrosination of alpha-tubulin

in an NF-κB-independent manner. Furthermore, PKM2, a master regulator of aerobic glycolysis, is highly expressed in undifferentiated cerebellar GNPs, and aerobic glycolysis is involved in cerebellar development (Tech et al., 2017). Recent studies reveal that PTL and its derivatives as activators of PKM2 can promote tetramer formation of PKM2, prevent nucleus translocation of PKM2 dimer, and inhibited PKM2/STAT3 signaling pathway *in vitro* and *in vivo* (Ding et al., 2020; Liu et al., 2021). Collectively, these data indicate that PTL has multiple actions and a pharmacological potential for promoting nerve regeneration. Although PTL can increase the radiosensitivity of prostate cancer cells while preventing radiation damage to normal prostate ones (Morel et al., 2017), its neuroprotective effects in Irradiation-induced CNS injury, especially in regeneration of developing cerebellum, remains unclear.

Here, we report that PTL is a promising neuroprotectant that can protect GNPs from acute radiation injury by regulating oxidative stress levels of GNPs in EGL after radiation. Our results further show that PTL can also inhibit PI3K/AKT phosphorylation and increase secretion of Shh by cerebellar irradiated astrocytes to promote NEPs expansion. Collectively, our findings demonstrate that PTL is a potential alternative drug for regeneration of the developing cerebellum after injury.

Materials and methods

Animals and treatments

Five strains of mice were used in the experiment. Nestin-CFP mice were a kind gift from Yang Lab (The Fox Chase Cancer Center, Philadelphia, United States). Nestin-GFP mice were obtained from Cyagen (Suzhou, China). Nestin-CreERT2 and ROSA26-LSL YFP reporter mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J mice were purchased from the Experimental Animal Center of the Army Medical University (AMU, Chongqing, China). The purchased mice were mated and bred, and newborn mice were obtained from their offspring for experiments. Juvenile mice were bred from these above. The adult mice used in the experiment were half males and half females. While some of the mice used were too young to distinguish between males and females. Mice receiving brain tissue died after perfusion, and non-perfused mice were killed by carbon dioxide inhalation after completion of the experiment. Animal was divided into several groups (Supplementary Table S1). All animal experiments were performed in compliance with institutional guidelines and had been approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (approval number: AMUWEC20211295). All mice were raised in pathogen-free conditions, maintained at a temperature of 22°C–25°C, with a 12-h light/dark cycle, regular ventilation and free access to water and food, which approved all animal care and experimental procedures of the Experimental Animal Center in AMU. Animals that reached the end point were sacrificed by CO_2 anesthesia.

Tamoxifen (TM; Sigma T5648) was dissolved in corn oil at 20 mg/ml. Nestin-CreERT2 × ROSA26-LSL YFP pups received intraperitoneal injections of 100 μg of TM on postnatal days P4 to induce Cre-mediated recombination. Parthenolide (PTL) was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/ml. P4 mice were received one 40 mg/kg dose of PTL *via* intraperitoneally injection, 2 h later, mice were anesthetized by hypothermia and irradiated only in cerebellum (CB) for 4Gy using an X-RAD RS 2000 (Rad source Technologies) in the central Lab of AMU. To specifically target the CB, the rest of the body was covered with the lead plate. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Tissue processing and immunofluorescence

During anesthesia, pentobarbital (50 mg/kg) was injected intraperitoneally, and ice-cold PBS and 4% paraformaldehyde were perfused transcardially to the animals. As described previously, brains were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and then submerged in 30% sucrose until sinking (Li et al., 2013). Sagittal sections of 8 μm thicknesses were prepared using a cryostat (Thermo Fisher Scientific) at −20°C and stored at −80°C until use. For staining, Sections and cells were blocked with 10% goat serum (BeyotimeBiotech) in PBS containing 0.05% TritonX-100 for 1 h, incubated with various primary antibodies (Supplementary Table S2) overnight at 4°C, washed three times with PBST, and added with secondary antibodies (Supplementary Table S3), protected from light at room temperature Incubate for 2 h and then counterstain with DAPI (Solarbio). Images were captured using a Leica fluorescence microscope at 10X and 40X magnification (Leica, Germany). Images were analysed using Adobe Photoshop and ImageJ software.

Granule neuron precursors isolation and cell culture

For GNPs isolation, we followed the previously described protocol (Li et al., 2013), cerebella were harvested from C57BL/6J mice at P4-P7 then digested in a solution containing 10 units/ml papain (Worthington), 25 U/ml DNase I (Solarbio) and 2 mg L-cysteine (Solarbio) at 37°C for 30 min to obtain a single-cell suspension. After filtered with 70 μm strainer, cells were centrifuged through a 35%–65% Percoll gradient (GE Healthcare). Cells from the 35–65% interface were suspended in Neurobasal Medium with B27 supplement. Then GNPs were

suspended in NB-B27 and plated on Poly-L-omithine hydrobromide (PLO) and Laminin (all from Sigma)-coated coverslips. GNPs was treated with 5 μM PTL, 50% sonic hedgehog conditional medium (Shh-CM) and Shh-CM adding 5 μM PTL for 24 h. EDU (Proteintech) was added 20 μM and incubated GNPs for 1 h. Then stained according to the instructions.

Primary astrocytes isolation and culture

Embryonic day 14.5 (E14.5) cerebella of C57BL/6J mice were used to prepare primary astrocytes. Isolation and culture of primary astrocytes were performed according to previous instructions (Liu et al., 2017). Briefly, cerebella were digested using 0.125% trypsin at 37°C for 15 min to obtain a single cell suspension, then plated on a 75 cm^2 flask coated with poly-D-lysine (Solarbio) at a density of 20,000 cells/cm^2. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, BI) containing 1% N2-Supplement-A (Stem cell), 1% fetal bovine serum (FBS, BI), 2 mM L-glutamine (Gibco), 100 U/mL penicillin and 0.1 mg/ml streptomycin (BI). For the following experiments, pure passage 2 to 5 was used. Astrocyte cell line C8-D1A was purchased from China Center for Type Culture Collection (Wu Han, China). C8-D1A were cultured in Dulbecco's modified Eagle's medium (DMEM, BI) containing 10% fetal bovine serum (FBS, BI), 100 U/mL penicillin and 0.1 mg/ml streptomycin (BI). Media was changed twice weekly.

Determination of reactive oxygen species production

The commercial kit (Nanjing Jiancheng Institute of Biological Engineering) was used to determine the content of hydrogen peroxide in cerebellum. H_2O_2 combines with molybdic acid to form a complex, which was determined at the 405 nm wavelength, and the content of H_2O_2 was calculated according to the instructions.

The intracellular ROS levels were detected by Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, China) according to the manufacturer's protocol. Briefly, the cells were seeded in 96-well plates. After the treatment, the cells were incubated with 10 μM DCFH-DA for 20 min at 37°C. Then cells were measured at 488 nm excitation and 525 nm emission by a fluorescence spectrophotometer (BMG LABTECH, GRE).

RNA extractions and quantitative RT-PCR

In accordance with the manufacturer's protocol, total RNAs were extracted from cultured cells using Trizol reagent (DingGuo). cDNA was prepared using 5x HiScript II RT SuperMix (Vazyme). Quantitative PCR was performed using 2x AceQ qPCR SYBR Green Master Mix (Vazyme). In this study, fold-changes in expression were calculated based on the ΔΔCt method. Results were normalized using the GAPDH gene. The primer pairs were listed in Supplementary Table S4.

Western blot

Cells or tissues were lysed in cell lysis buffer (Beyotime Biotechnology) on ice or liquid nitrogen to obtain protein lysate and the concentration measured using an enhanced BCA protein assay kit (Beyotime Biotechnology). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore), after blocking with 5% non-fat milk in Tris-buffered saline membranes were incubated with primary antibodies (Supplementary Table S5) overnight at 4°C, then incubated with secondary antibodies (Supplementary Table S6) at room temperature. To detect immunoreactivity, blots were incubated with Immobilon western chemilum hrp substrate (Millipore, WBKLS0100) and imaged on Fluorescence Chemiluminescence Imaging System (CLiNX). Relative intensity of blots was quantified using ImageJ software.

Quantifications and statistical analyses

The area near the midline of the cerebellum (μm^2) was measured using ImageJ software. For all IF staining, cell counts were obtained using ImageJ and Photoshop CS6. Certainly, three sections per animal, and at least 3 animals per group, were analyzed. Results were expressed as mean ± SEM. The statistical analyses were conducted using Prism version 6 (GraphPad Software) and SPSS version 20 (IBM Corp.). Two-tailed Student's T tests were used to analyze the data in relation to vehicles or scrambled controls. When more than two groups were compared, Kruskal–Wallis nonparametric one-way ANOVA was used to detect group effects, followed by Dunn's multiple comparison post hoc test. Significance was determined at $p < 0.05$. ns, not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

NEPs exhibit differential spatiotemporal distribution in neonatal mouse cerebellum

Wojcinski et al. (2017) used *in vivo* lineage-tracing Cre/loxP technologies to reveal that NEPs switched their fate from producing astroglia and interneurons during mid-embryogenesis to generate

granule neurons in mice. Their concern, however, was how the existing NEPs could regenerate GNPs after irradiation damage. In the present study, we focused on the role of the newly produced NEPs in this process. To investigate distribution patterns of NEPs in the developing postnatal cerebellum, we studied Nestin-CreERT2 mice crossed with ROSA26-LSL YFP mice to obtain Nestin-CreERT2; ROSA26-LSL YFP (NR) mice, which expressed GFP preceded by a loxP-flanked stop sequence. We intraperitoneally injected tamoxifen into P0, P2, and P3, respectively, then analyzed distribution of NEPs cells in the cerebellum *via* GFP immunofluorescence staining. Results revealed that the number and distribution of fluorescently labeled cells (NEPs) in the cerebellum differed dramatically when Tamoxifen was given at different developmental stages. Most cells were labeled at P0, and positive cells were found in the cerebellar EGL, Purkinje cell layer (PCL), and IGL, of which PCL represented the site with the highest abundance (Supplementary Figure S1A). Delayed TAM administration resulted in a sharp decrease in number of fluorescently labeled NEPs, and the labeled cells in P2 and P3 pups were scattered in the EGL and PCL (Supplementary Figure S1B,C). These results suggest that NEPs gradually lose the ability to express Nestin with development and differentiation of the cerebellum, thus may be transformed into other cell types. Next, we investigated whether radiation affects the distribution of labeled cells induced by TAM by comparing expression patterns of GFP positive cells labeled by TAM before and after irradiation. Results showed that the number of TAM-labeled NEPs at P3 was significantly lower after irradiation than before, although both of them were irradiated in the cerebellum at P1, which indicated that there were not many new NEPs produced after irradiation (Supplementary Figure S1D,E). Furthermore, we explored new generation of NEPs in the developing cerebellum after irradiation at different stages. Interestingly, we found that a significantly higher number of new NEPs emerged in the P4-irradiated cerebellum compared to other periods (Supplementary Figure S1F). In order to have enough newly generated NEPs for the study at a later stage, we employed the irradiation strategy in P4 pups in the follow-up experiment.

PTL protected the cerebellum from acute radiation injury by modulating cerebellar ROS levels

To exclude possible TAM effects on neuroprotection, we first confirmed the protective effect of PTL using wild-type mice. To this end, we employed PTL acute toxicity studies using a series of doses based on the *in vitro* results and previous literature (Freund et al., 2020; Wang et al., 2020) Firstly, weassessed thee effects of various PTL concentrations on proliferation of GNPs *in vivo*. Results from Ki67 immunofluorescence staining revealed that PTL had no significant effect on the proliferation of GNPs in the cerebellar EGL relative to the control sample even at the highest concentrations (i.e., nearly 40 mg/kg) (Supplementary Figure S2A,B). Next, we compared the effect of PTL administration schedule. To this end, we determined PTL's protective effect before or after irradiation on the EGL of irradiated cerebellum, which has implications for application of PTL as a prophylactic or protective agent. Considering the single dose and the blood concentration across the blood-brain barrier (BBB), we used 40 mg/kg as the fixed dose for *in vivo* assay in our experiment. Analysis of IF results, 24 and 48 h after IR, showed that mice cerebellum pre-treated with PTL had more Ki67 + cells in the EGL than the vehicle after IR ($p < 0.01$, Supplementary Figure S2C–F). To confirm if high PTL concentrations were safe for the GNPs, we again subjected cerebellar sections to γ-H2AX staining after low (10 mg/kg) and high (40 mg/kg) dose administration and assessed for possible cellular DNA damage. Results showed that PTL did not increase γ-H2AX positive cells 4 h after irradiation, with high PTL concentrations associated with a decrease in the number of positive cells to some extent (Supplementary Figure S2G,H). Therefore, we selected 40 mg/kg PTL for 2 h before IR as the dosage in all subsequent experiments. Subsequently, we performed MTS assays to assess PTL's cytotoxicity on primary GNPs, and found an IC50 value of 4.36 ± 0.68 μM (data not show). Consequently, we chose 1 μM PTL, which was lower than the IC50, for subsequent *in vitro* GNP experiments.

We investigated the effect of PTL pretreatment on EGL GNPs damage and apoptosis after irradiation by analyzing levels of γ-H2AX and cleaved-caspase3 (Cl-cas3) expression. IF assay results showed that PTL IR mice had a lower proportion of γ-H2AX + cells (2.30 ± 0.62%) than their IR counterparts (12.33 ± 2.52%) ($p < 0.01$, Figures 1A,B), as well as lower proportions of Cl-cas3-positive cells (IR = 30.33 ± 1.53% vs. PTL IR = 21.00 ± 1.00%) ($p < 0.01$, Figures 1C,D). These results indicated that PTL could protect GNPs from acute radiation injury to a certain extent. Previous studies have shown that radiation-induced ROS production is an important factor determining radiation damage (Wozny et al., 2019). Next, we measured ROS levels in the cerebellum through H_2O_2 detection, and found that PTL pretreatment induced a 2.8- and 1.9-fold reduction in H_2O_2 production in PTL and PTL IR mice, respectively, relative to that in the control groups (Figure 1E). Since primary GNPs in the undifferentiated state cannot be cultured *ex vivo* for a long time, we used cell lines to study PTL's effect on cell ROS production. Previous studies have demonstrated that PC12 cells derived from rat adrenal glands are an excellent model for studying differentiation of neuron precursors (He et al., 2020). In the present study, we performed DCFH-DA assay to detect ROS production in PC12 cells and found that irradiation mediated a 2.8-fold increase in cells. Notably, exposure to elevated PTL markedly reversed the observed increase in ROS production. ROS levels decreased to 64 and 21% in 5 μM and 10 μM PTL pretreatment groups, respectively. (Figure 1F). Next, we performed Western Blot assay to determine the rate of apoptosis in cells within 4 h of

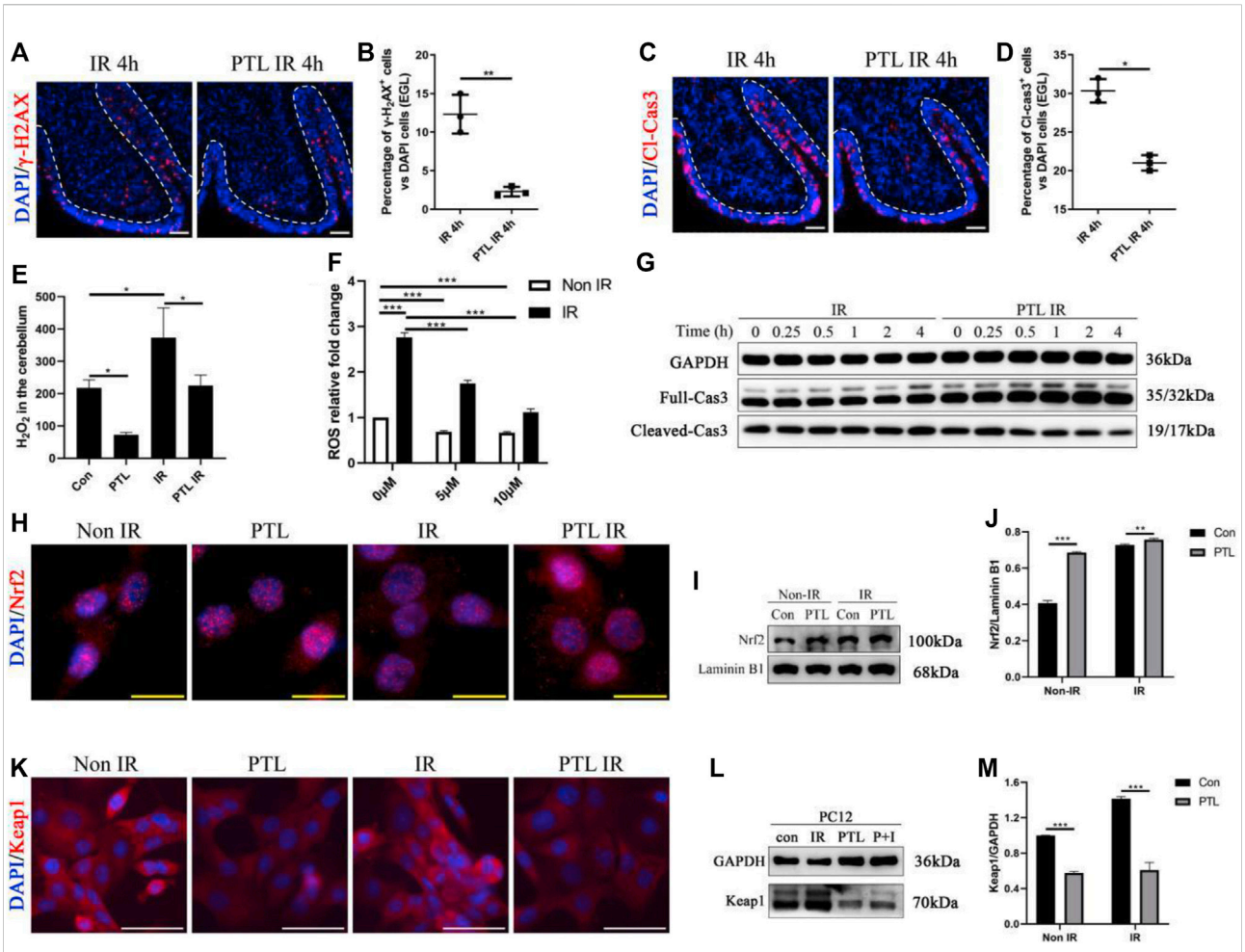

FIGURE 1
Pretreatment with PTL reduces cerebellar EGL loss after P4 irradiation by modulating cerebellar ROS levels. Sagittal sections, and stained for a marker of DNA damage (γ-H2AX; red), a marker of cell apoptosis (cleaved-caspase3, Cl-cas3; red), a marker of cell proliferation (Ki67; red) or nuclei (DAPI; blue). **(A)**. The γ-H2AX immunofluorescence detection of DNA damage (×100 magnification) on midsagittal sections (~8 μm) of the cerebellum were taken from irradiated (IR) mice and PTL IR (PTL pretreated before irradiation) mice at corresponding time at P4 with red showing γ-H2AX foci and blue showing DAPI stained nuclei. The external granule cell layer (EGL) painted by white dotted line. **(B)**. Graph represents the quantifications of **(A)**. Each scatter points represented individual measurement of the percentage of γ-H2AX-positive cells in EGL of IR, PTL IR and IR PTL mice by 4 h after IR at P4 (n = 3). **(C)**. The Cl-cas3 immunofluorescence detection of apoptosis (×100 magnification) on midsagittal sections of IR and PTL IR mice by 4 h after IR at P4 with red showing Cl-cas3$^+$ cells and blue showing DAPI stained nuclei. The external granule cell layer (EGL) painted by white dotted line. **(D)**. Graph represents the quantifications of **(A)**. Each scatter points represented individual measurement of the percentage of Cl-cas3-positive cells in EGL of IR, PTL IR and IR PTL mice by 4 h after IR at P4 (n = 3). **(E)**. Bar graphs represent the concentration of hydrogen peroxide (H_2O_2) in the cerebellum among Control (Con) mice, PTL treatment (PTL) mice, IR and PTL IR mice (n = 3). **(F)**. Bar graphs represent the DCFH-DA detection of ROS in PC-12 cells without radiation (non-IR, white) and with 4Gy X-ray radiation (IR, black) after pretreatment with 0 μM, 5 μM, and 10 μM PTL for 4 h (n = 3). **(G)**. PC-12 cells pretreated with or without 10 μM PTL for 1 h before 4 Gy X-ray exposure and were then irradiated. Representative Western blot images of GAPDH (top) and markers of apoptosis, total (middle) and cleaved Cas3 (down) at different time points after irradiation. **(H)**. Immunofluorescence analysis of the effect of 10 μM PTL preconditioning on changes of Nrf2 fluorescence (red, ×400 magnification) of PC-12 cells after 2 h of IR injury. Overlaying the images of Nrf2 and DAPI (blue fluorescence, used as a nucleus indicator) confirmed the nuclear location of Nrf2. **(I)**. Representative Western blot images of Nrf2 (top) and laminin B1 (for nuclear protein internal reference, down) in cells after 4Gy X-ray 2 h irradiation, non-PTL pretreated cells as control. **(J)**. Bar graphs represents the quantifications of **(I)**. Nrf2 was normalized to Laminin B1. Black bar, control; Gray bar, PTL pretreatment group (n = 3). **(K)**. Immunofluorescence analysis of the effect of 10 μM PTL preconditioning on expression of KEAP1 fluorescence (red, ×200 magnification) of PC-12 cells after 2 h of IR injury. DAPI (blue fluorescence) used as a nucleus indicator. **(L)**. Western blot analysis of GAPDH (top) and KEAP1 protein (down) in PC-12 cells pre-treated with 10 μM PTL or not after IR at 2 h **(M)**. Bar graphs represents the quantifications of (l). KEAP1 was normalized to GAPDH. Black bar, control (con, IR); Gray bar, PTL pretreatment (PTL, P + I) group (n = 3). All graphical data were presented as mean ± s.e.m., and significance was determined by two-tailed Student's t test or Kruskal–Wallis nonparametric one-way ANOVAs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. White scale bar, 50 μm. Yellow scale bar, 20 μm.

irradiation. Results showed that cleave-caspase 3 was significantly down-regulated in the PTL pretreatment group, although this change was not obvious in the single radiation group, possibly due to the short duration of exposure. These results suggest that PTL-mediated inhibition of apoptosis acts independently and are not initiated by irradiation (Figure 1G). Previous studies have shown that PTL can play an antioxidant role by regulating the Keap1-Nrf2 pathway (Xu et al., 2013). To assess the involvement of Keap1/Nrf2 in our model, we analyzed protein expression and intracellular distribution of Nrf2 and Keap1 after exposure to PTL. To this end, we used immunofluorescence (IF) and western blot assays to evaluate the capability of PTL to activate NRF2 in PC12. Staining results showed that Nrf2 was mainly expressed in the cytoplasm of control cells. However, irradiation induced its nuclear translocation to some extent, with PTL treatment further promoting this translocation (Figure 1H). Western blots revealed that PTL significantly upregulated Nrf2 expression, as evidenced by a 1.6-fold increase in the nucleus of PC12 cells treated with PTL for 2 h. Notably, PTL still generated a slight increase even under irradiated conditions (Figures 1I,J). On the other hand, Keap1 protein was mainly expressed in cytoplasm of PC12 cells, and KEAP1 staining was markedly diminished following treatment with PTL (Figure 1K). Western blots revealed a consistent pattern with IF results, relative to the untreated group. Notably, PTL downregulated KEAP1 expression by 48% in PTL treatment group and 61% in control group, respectively (Figure 1M). Collectively, these results indicated that PTL can protect the cerebellum from acute radiation injury by modulating cerebellar ROS levels through the KEAP1/NRF2 pathway.

PTL attenuated cerebellar hypoplasia after irradiation injury

Cerebellum development is a highly coordinated process. After a short period of proliferation, GNPs in EGL migrate inward under the guidance of Bergman's radial glial fibers and penetrate the internal granular layer (IGL), where they exit the cell cycle and complete cell differentiation. Restoration and maintenance of GNPs' proliferative ability in EGL are crucial for regeneration of the damaged developing cerebellum (van der Heijden and Sillitoe, 2021). We first performed Ki67 staining to assess the effect of PTL pretreatment on GNPs proliferation at the different time points (P5, P8, P14) after IR on P4, and found that proliferating cells were stained red. Proliferating GNPs were mainly localized in the EGL on the cerebellar surface, and as the cerebellum developed, the cells exited the cell cycle and migrated inward, with the EGL layer becoming markedly thinner by P14, consistent with our earlier findings. Irradiation can cause destruction of EGL. After 24 h of irradiation, the EGL almost dissipated and only some scattered cells remained. After 4 days, the EGL recovered to some extent, but still did not return to normal levels. However, by P14, EGL had significantly thickened compared to the control group. In contrast, PTL pretreatment dramatically improved the damage to EGL at all stages of irradiation (Figure 2A). Next, we quantify cell proliferation by determining the percentage of Ki67-positive cells. Results showed that the rate of GNPs proliferation markedly decreased after 24 h, from 76.00 ± 4.00% to 23.33 ± 1.53% with IR, while PTL pretreatment restored it to 48.00 ± 2.00%. By P8, the proliferation rate declined from 59.00 ± 3.61% in the control to 41.00 ± 3.61% in the IR group and 66.00 ± 5.29% in PTL pretreatment. The remaining cells on the surface of normal cerebellar EGL showed a significant decrease at P14, although most of them kept proliferating. The percentage of Ki67 + cells in the IR group dropped to about 28.00 ± 2.00%, compared to about 84.67 ± 4.51% in normal mice. Pretreatment with PTL caused a considerable increase in the number of cells on the surface of EGL, while proliferation rates (86.00 ± 5.29%) returned to match those in untreated control cells. Furthermore, we compared the EGL thickness at P8 and P14, and found that PTL pretreatment could promote EGL replenishment after IR (Figure 2B). Notably, we found although irradiation caused a dramatic reduction in the thickness of cerebellar EGL at P8, nevertheless, the pretreatment of PTL (30.70 ± 5.61 μm) still performed slightly better than the pretreatment of IR (20.33 ± 3.59 μm). IR mice exhibited a thicker EGL at P14 (28.21 ± 2.59 μm) than their Non-IR (17.42 ± 2.49 μm) and PTL IR (26.96 ± 3.80 μm) counterparts. Collectively, these results indicated that IR disrupted proliferation and differentiation of GNPs, while PTL significantly ameliorated the proliferation inhibition of IR-induced GNPs. Previous studies had demonstrated that although the developing cerebellum can accomplish self-repair after IR, the volume of the repaired cerebellum after maturation is significantly smaller than that of a normal cerebellum (Wojcinski et al., 2017). In the present study, we measured the area of the sagittal section of the mature cerebellum (P30) and found that IR decreased the size of the cerebellum (7.30 ± 0.50 mm^2) relative to the Non-IR (11.27 ± 0.64 mm^2) group. However, PTL pretreatment rescued the cerebellar size (PTL IR = 8.43 ± 0.21 mm^2) (Figures 2C–E). Taken together, these findings indicate that PTL sustained the proliferation of GNPs in EGL after IR, thus enhancing replenishment of the injured cerebellum.

PTL promoted NEP reprogramming in both EGL and PCL of the cerebellum after IR

Considering the plasticity of NEPs and the cell compensation mechanism after IR, which enhances recovery of the damaged cerebellum (Li et al., 2013; Wojcinski et al., 2017), we utilized Nestin-CFP transgenic mice to test the effect of PTL on NEPs.

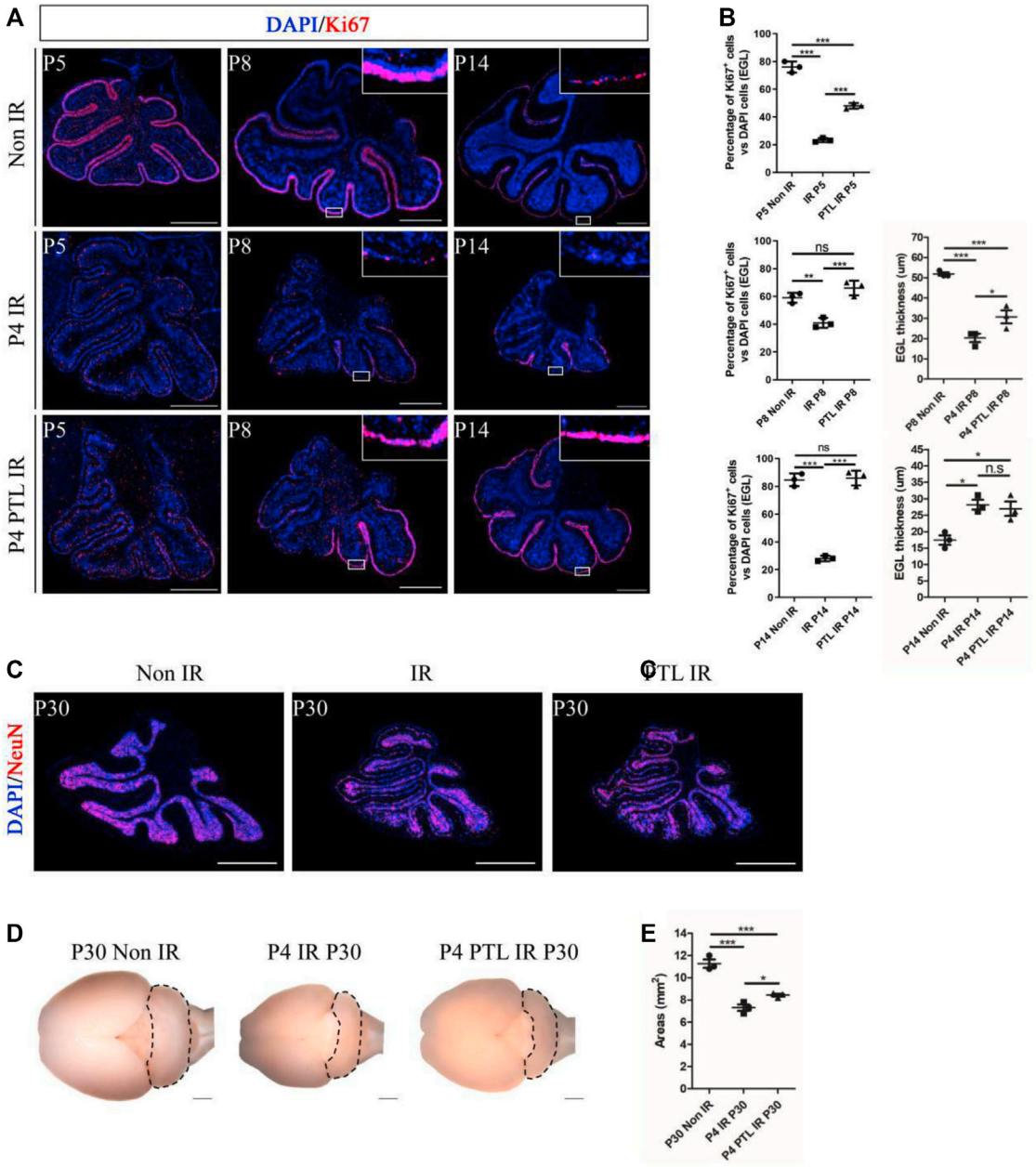

FIGURE 2
PTL promoted replenishment of the cerebellar EGL after irradiation damage. **(A)**. The Ki67 (red) immunofluorescence detection of proliferation (×50 magnification) on midsagittal sections of Non IR, IR and PTL IR mice at P5, P8, P14 with red showing Ki67$^+$ cells and blue showing DAPI stained nuclei. High-power images were shown of the areas indicated by white rectangles. White scale bar, 500 μm. **(B)**. Graph showing the percentage of Ki67$^+$ cells in EGL of Non IR, IR and PTL IR mice at P5, P8, P14. EGL Ki67$^+$ cells decline significantly during IR, and PTL can relatively slow this decline at P5 (left/top, n = 3), P8 (left/middle, n = 3) and P14 (left/down, n = 3). Quantification of the EGL thickness showing thicker EGL in IR mice and lesser magnitude of thinning in PTL IR mice at P8 (right/top, n = 3). IR and PTL IR mice cerebella (right, n = 3). By P14, the thickness of EGL in IR mice and PTL IR mice was significantly higher than that in the Non IR mice (right/down, n = 3). **(C)**. IF detection of NeuN and DAPI on midsagittal sections (red, ×50 magnification) of Non IR, IR and PTL IR mice at P30. White scale bar, 500 μm. **(D)**. Bright field images of the cerebella of Non IR, IR and PTL IR mice cerebellum at P30. The cerebellum painted by black dotted line. **(E)**. Graph represents the quantifications of **(C)**. Each scatter points represented individual measurement of the areas of midline sections of the cerebella of Non IR, IR, and PTL IR mice at P30 (n = 3). All graphical data were presented as mean ± s. e.m., and significance was determined by two-tailed Student's t test or Kruskal–Wallis nonparametric one-way ANOVAs. ns, nonsignificant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

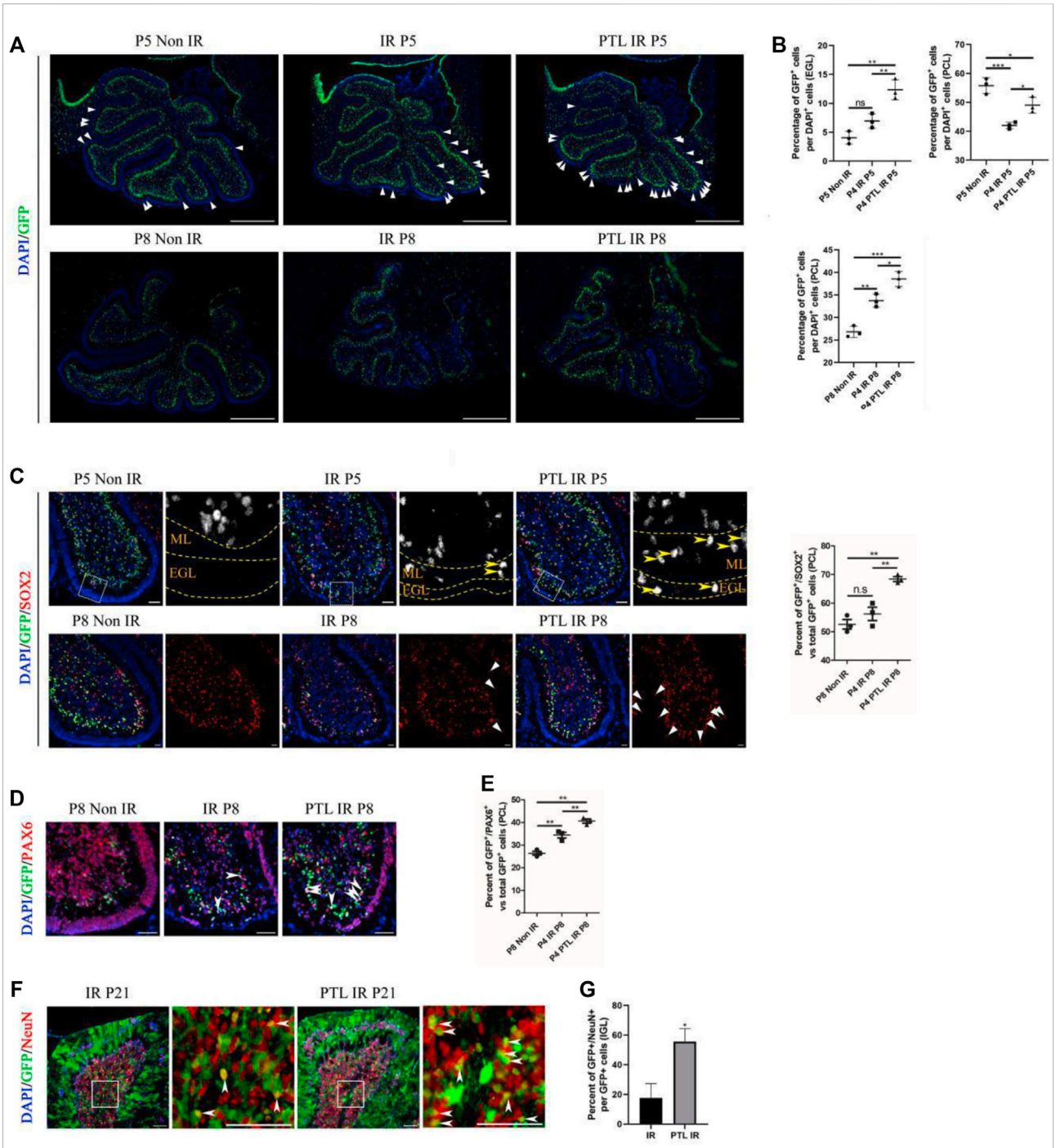

FIGURE 3
PTL augmented NEPs in both EGL and PCL of the cerebellum after IR Midsagittal sections (8 μm) of cerebellum were taken from Non IR, IR and PTL IR mice at indicated ages with the indicated transgene mice, and stained for GFP (green), Ki67 (red), a neuron stem cell marker (SOX2; red), a granule neuron precursors marker (PAX6; red) and DAPI (blue). a-e were Nestin-CFP mice. f-g were Nestin creER^{T2}×Rosa26 LSL YFP (NR) mice injected with Tm at P5. **(A)**. The GFP immunofluorescence detection of NEPs (×50 magnification) on midsagittal sections of Non IR, IR and PTL IR mice at P5 (top) and P8 (down). The triangle indicated GFP$^+$ cells in EGL. White scale bar, 500 μm. **(B)**. Graph represents the quantifications of **(A)**. Each scatter points represented individual measurement of the percentage of GFP + cells in EGL (top/left) and PCL (top/right) of Non IR, IR and PTL IR mice at P5 (n = 3). Scatter plot of the percentage of GFP + cells in PCL of Non IR, IR and PTL IR mice at P8 (down/left = 3). **(C)**. IF detection of GFP, SOX2 and DAPI on midsagittal sections (×100 magnification) of Non IR, IR and PTL IR mice at P5 and P8. High-power images were shown of the areas indicated by white dotted rectangles. The GFP$^+$/SOX2$^+$ cells are represented in grayscale for better visualization. Yellow arrowheads indicated GFP$^+$/SOX2$^+$ cells in EGL and ML (left/top). The left/down panel showing the staining at P8. White triangles indicated SOX2$^+$ cells in EGL. White scale bar, 50 μm. Scatter plot of represents the percentage of GFP$^+$/SOX2$^+$ cells vs. all GFP$^+$ cells in PCL of Non IR, IR and PTL IR mice at P8 (right, n = 3). **(D)**. IF

(*Continued*)

FIGURE 3 (Continued)
detection of GFP, PAX6 and DAPI on midsagittal sections (×200 magnification) of Non IR, IR and PTL IR mice at P8. White arrowheads indicated $GFP^+/PAX6^+$ cells in PCL. White scale bar, 50 μm. **(E)**. Graph represents the quantifications of **(D)**. Each scatter points represented individual measurement of $GFP^+/PAX6^+$ cells vs. all GFP^+ cells in PCL of Non IR, IR and PTL IR mice at P8 (n = 3). **(F)**. Nestin $creER^{T2}$ postnatal lineage tracing of IR and PTL IR mice. IF detection of GFP, NeuN and DAPI on midsagittal sections (×200 magnification) of mice at P21. High-power images were shown of the areas (IGL) indicated by white dotted rectangles. White arrowheads indicate $GFP^+/NeuN^+$ cells in IGL. White scale bar, 50 μm. **(G)**. Graph represents the quantifications of **(F)**. Each scatter points represented individual measurement of percentage of $GFP^+/NeuN^+$ cells vs. all GFP^+ cells in IGL of IR and PTL IR mice at P21 (n = 3). All graphical data were presented as mean ± s. e.m., and significance was determined by two-tailed Student's t test or Kruskal–Wallis nonparametric one-way ANOVAs. ns, nonsignificant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

This choice was determined empirically in our previous studies (Li et al., 2013). Here, we selected P5 and P8 (24 and 96 h after IR) as phases for determining the amount of NEPs in EGL and PCL, respectively (Figures 3A,B). Firstly, we analyzed differences in CFP-positive cells in cerebellar EGL after about 24 h of irradiation (P5), and found that there were only a few CFP-positive cells in the residual EGL after irradiation, and although there was an increase in the proportion, it was not statistically significant (Non-IR = 4.05 ± 1.10% vs IR = 6.96 ± 1.24%). We attributed this phenomenon to a severe damage of EGL, which induced death of many cells whose nuclei subsequently stained positive for DAPI, thus lowering the ratio. PTL pretreatment promoted the increase in CFP positive cells in EGL after IR (PTL IR = 12.35 ± 1.75%). Next, we analyzed changes in CFP-positive cells in PCL, and found that IR decreased GFP expression in PCL (42.03 ± 1.16%) relative to non irradiated controls (55.79 ± 2.72%). PTL slightly reduced this IR-induced decrease (49.05 ± 2.78%). Comparison between CFP-positive cells in EGL and PCL of P8 mice revealed that CFP positive cells were scattered in all three groups of mouse EGL, albeit at no statistically significant difference. To this end, we hypothesized that NEPs would no longer express Nestin after differentiating into other types of cells, and its CFP fluorescence could not be detected. However, IR increased the number of CFP-positive cells in PCL (IR = 33.73 ± 1.43%) relative to the non-IR group (26.81 ± 1.25%). PTL contributed to the rise of CFP-positive cells in irradiation-induced PCL (PTL-IR = 38.54 ± 1.68%). Overall, these results indicated that IR can activate the mobilization of NEPs, a process that is accelerated by PTL pretreatment.

Studies had shown that NEPs can only differentiate into GABAergic interneurons and astrocytes under normal conditions, but cannot transform glutamatergic neurons (like granule neurons). Moreover, irradiation has been shown to induce NEP reprogramming in the cerebellum thus conferring them with the ability to differentiate into GNPs and mature granule neurons (Wojcinski et al., 2017; Andreotti et al., 2018). To determine whether PTL can promote NEPs reprogramming and enhance their ability to differentiate into GNPs, we stained GFP/SOX2 and GFP/PAX6 in Nestin-CFP mice. SOX2, a transcription factor of neural stem cells that regulates vertebrate neurodevelopment and adult neurogenesis (Wakamatsu and Uchikawa, 2021), is one of the key genes used to reprogram differentiated cells into induced pluripotent stem cells. Previous studies have also shown that SOX2-positive cells are involved in repairing damaged cerebellar EGL (Takahashi and Yamanaka, 2016; Wojcinski et al., 2017). In the present study, immunofluorescence staining results revealed that SOX2-positive cells were mainly distributed in PCL and WM under normal conditions. However, the number of CFP-positive cells in PCL decreased after 24 h of irradiation, while there were no significant differences in the number of SOX2 and CFP double-positive cells between the IR and PTL-IR groups. Moreover, irradiation induced appearance of SOX2 and CFP double-positive cells in the molecular layer, which is consistent with the previous report. Pretreatment with PTL resulted in a significant increase in the number of SOX2+/CFP + cells in the ML and EGL. By P8, there were about 51.4% SOX2+/CFP + cells in the PCL of control mice (non-IR) and 54.6% in IR mice, although there was no statistically significant difference between them. However, PTL pretreatment significantly increased double-positive cells in the PCL of PTL pretreated mice, reaching 67.8% (Figure 3C). Next, we evaluated the ability of NEPs cells to differentiate into granule neuronal cells by detecting PAX6 expression, a marker of granule neuron precursors (Park et al., 2018) in NEPs. At P5, we found no significant difference in expression of CFP+/PAX6+ cells among mice in Non-IR, IR, and PTL IR groups (Supplementary Figure S3A). In P8 Nestin-CFP transgenic mice, IR mediated upregulation of CFP+/PAX6+ cells in PCL (IR = 34.40 ± 2.12%), relative to Non-IR (Non-IR = 26.41 ± 1.51%) group (Figures 3D,E). In addition, we verified that PTL could promote NEPs mobilization in two other animal models and obtained similar results in Nestin-GFP (Supplementary Figure S3B) and Nestin-CreERT2/ROSA26-LSL YFP (NR) mice. In addition, we verified the fate of newly produced NEPs by activating Cre activity in NR mice with tamoxifen (Tm) 24 h after irradiation of P4 mice, and found pretreatment with PTL increased GFP+/PAX6+ cells in PCL after IR (IR = 10.67 ± 3.06%, PTL = 72.00 ± 8%) (Supplementary Figure S3C,D). These results indicated that PTL accelerated reprogramming of NEPs in EGL and PCL after irradiation.

To further understand the fate of NEPs affected by PTL, we crossed Nestin-CreERT2 mice to those harboring R26R-YFP to lineage-trace NEPs in the postnatal cerebellum. Next, we performed double staining of GFP and NeuN on cerebellar sections, at P21 (maturation stage of the cerebellum) and found

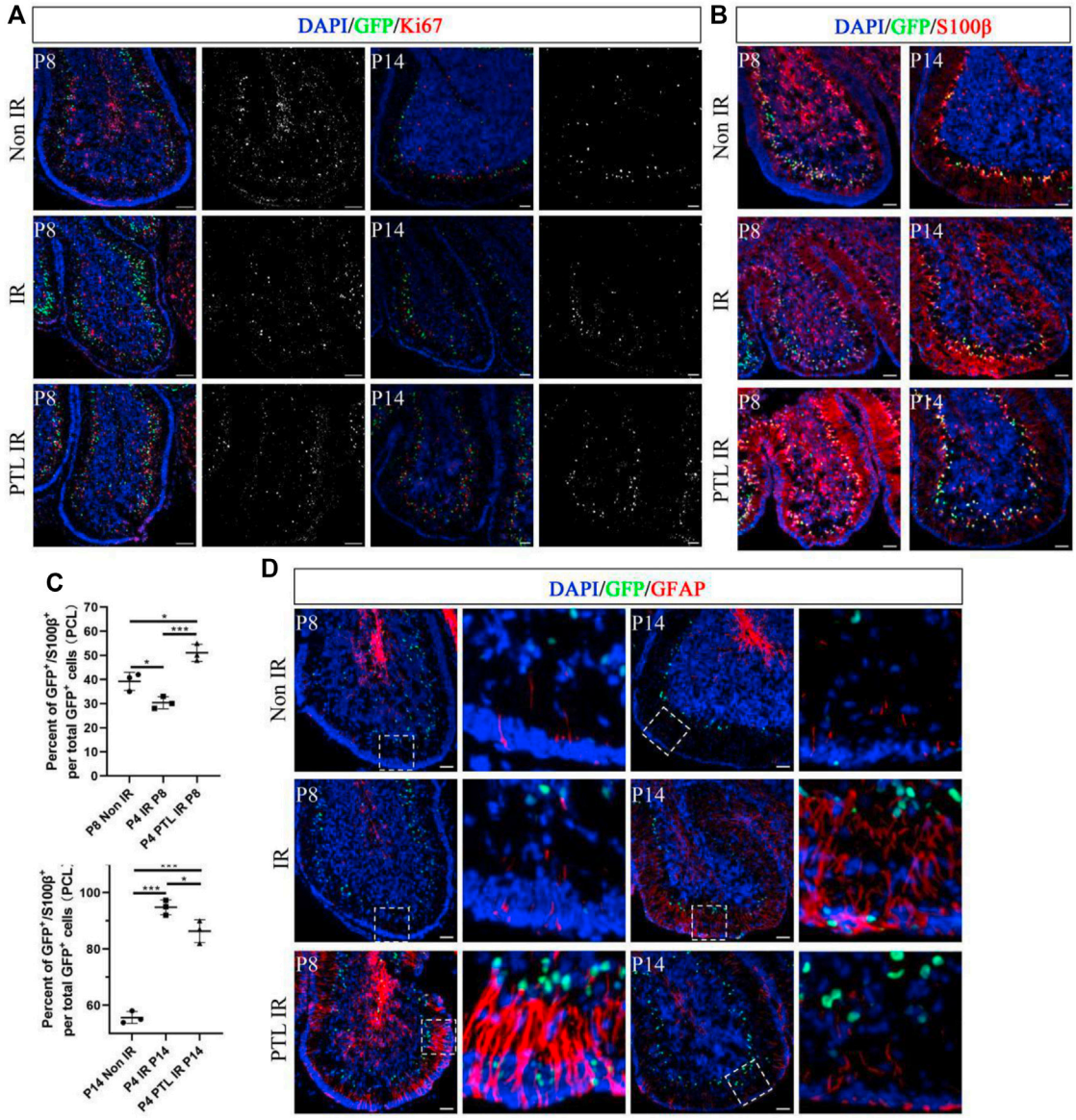

FIGURE 4
PTL facilitates the increase of cerebellar interneurons and glial cells after irradiation Midsagittal sections (8 μm) of cerebellum were taken from Non IR, IR and PTL IR mice at P8 and P14 with Nestin-CFP transgene mice. Stained for GFP (green), interneuron marker (PAX2; red), astrocytes marker (S100β; red) and glial filaments marker (GFAP; red) or DAPI (blue). **(A)**. IF detection of GFP, PAX2 and DAPI on sagittal sections (×100 magnification) of Non IR, IR and PTL IR mice at P8 (left two columns) and P14 (right two columns). Grayscale images indicated PAX2 positive cell. White scale bar, 50 μm. **(B)**. IF detection of GFP, S100β and DAPI on sagittal sections (×100 magnification) of Non IR, IR and PTL IR mice at P8 and P14. White scale bar, 50 μm. **(C)**. Graph represents the quantifications of **(B)**. Scatter plot of represents the percentage of $GFP^+/S100\beta^+$ cells vs. all GFP^+ cells in PCL of Non IR, IR and PTL IR mice at P8 (left column, n = 3) or P14 (right column, n = 3). **(D)**. IF detection of GFP, GFAP and DAPI on sagittal sections (×100 magnification) of Non IR, IR and PTL IR mice at P8 (left two columns) and P14 (right two columns). Grayscale images indicated PAX2 positive cell. High-power images were shown of the areas indicated by white dotted rectangles. White scale bar, 50 μm. All graphical data were presented as mean ± s.e.m., and significance was determined by two-tailed Student's t test or Kruskal–Wallis nonparametric one-way ANOVAs. ns, nonsignificant; $*p < 0.05$; $***p < 0.001$.

that the cerebellar surface had abundant fiber-like green fluorescence-positive cells with a morphology similar to that of radial glial cells. The cerebellum's internal IGL was filled with NeuN-positive cells, and the red fluorescence was mainly localized in the nucleus, which also had scattered green fluorescence-positive cells, both of which were able to co-localize. Results of the IF assay showed that PTL pretreatment promoted the expression of GFP+/NeuN + cells

in IGL (IR = 18.23 ± 8.54% vs. PTL = 53.09 ± 6.74%) (Figures 3F,G). Taken together, our results demonstrated that PTL can not only induce mobilization but also contribute to differentiation of NEPs into neuronal lineage cells for the recovery of the injured cerebellum.

Effects of PTL on interneurons and glial cells in irradiated cerebellar microenvironment

The appropriate ratio of different types of cells in the cerebellum correlates with its function. Normally, the initial NEPs population in EGL mainly produces interneurons and astrocytes (Wojcinski et al., 2017). Previous results also confirmed that PTL pretreatment enhanced the production of radial glial cell-like cells from NEPs. Therefore, we investigated whether PTL had an effect on interneurons and glial cells of irradiated cerebellum. A comparison of the immunofluorescence expression profile of the interneuron marker PAX2 in the cells of NEPs at P8 and P14 revealed that CFP+/PAX2+ cells were scattered in the PCL of all three groups of mice, but their proportions were not significantly different. However, for PAX2+ cells, irradiation could reduce PAX2+ cells at P8. Although normal PAX2+ cells levels could not be restored, PTL pretreatment attenuated the reduction. At P14, the number of PAX2+ cells were significantly more than that in the control group, and the increase in positive cells in the cerebellum of PTL-pretreated mice was more pronounced compared with irradiation alone mice (Figure 4A). Ptf1a is a key transcription factor in the genesis of Purkinje cells and cerebellar GABAergic interneurons (Jin and Xiang, 2019). Quantitative PCR (qPCR) results revealed that PTL promoted the expression of Ptf1a, suggesting its role in differentiation of interneurons (Supplementary Figure S4A). We further investigated the change of astrocytes after treatment with PTL. IF assay showed that IR decreased the number of GFP+/S100β+cells in PCL (Non-IR = 39.26 ± 3.74%, IR = 30.36 ± 2.51%), but these were then increased by PTL treatment (PTL-IR = 51.04 ± 3.59%). At P14, IR increased the number of GFP+/S100β+cells in PCL (Non-IR = 55.63 ± 2.13%, IR = 94.76 ± 2.64%), but these were then slightly decreased by PTL (PTL-IR = 86.32 ± 4.04%). At this moment, astrocytes in PTL-IR mice and Non-IR mice showed similar morphology (Figures 4B,C). We tested the expression of the glial fibrillary acidic protein (GFAP), an important astrocyte marker expressed in the glial filaments. At P8, after IR treatment, filaments in EGL and ML were short and small, whereas PTL pretreatment induced the expression of GFAP and produced long and orderly filaments. At P14, IR still made the filaments disorderly, and PTL pretreatment made them perpendicular to the surface of EGL (Figure 4D). These results showed that PTL maintained the integrity of the filaments and may promote migration of GNPs in EGL after IR. We evaluated the effect of PTL on Purkinje cells utilizing Calbindin staining, a specific marker for Purkinje cells. At P8, Purkinje cells (PCs) from non-IR mice aligned in a monolayer, IR misaligned the PCs, but PTL pretreatment made them to align in a monolayer with richly arborizing planar dendrites. P14 showed that Purkinje cells were disordered in IR mice, whereas Purkinje cells were well-ordered in non-IR mice and PTL-IR mice (Supplementary Figure S4). These results suggest that PTL potentially has the ability to promote the maturation of post-IR Purkinje cells and maintain the orderly arrangement of post-IR Purkinje cells.

PTL promotes the secretion of Shh in the cerebellum after IR

Considering the crucial role of Shh signaling in the development of normal cerebellum and the repair of damaged cerebellum, especially for the initial step in NEP reprogramming, we speculated that PTL might be a suppressor or activator of the Shh pathway. To test this hypothesis, we performed EdU proliferation incorporation assay to compare the proliferation of primary GNPs cultured under Shh-conditioned culture medium with or without PTL. If PTL had an effect on the SHH pathway, it would significantly affect the proliferation of Shh-induced GNPs cells *in vitro*. The proliferating cells, differentiated cells and nuclei were labeled with EdU (red), TUJ1 and DAPI (blue), respectively (Figure 5A). The results showed that about 11.3% of GNPs maintained their proliferation *in vitro* under normal conditions, and PTL had no significant effect on cell proliferation rate, which was about 10.5%. Stimulation of the Shh pathway increased the proportion of Edu-positive cells in GNPs to 82.6%, which was not significantly affected by PTL, with a rate of about 81.4% (Figure 5B). Our results showed that PTL had no apparent role on Shh signal transduction. Subsequently, we investigated the effects of time of PTL pretreatment on Shh protein expression in irradiated cerebellum. Interestingly, WB results showed that Shh was upregulated in the cerebellum after irradiation, and more significantly upregulated after PTL treatment in a time-dependent manner. There was no significant difference in Shh expression in the PTL pretreatment group compared with the irradiated group at 2 h. At 4 h, 6 h and 8 h, Shh expression level in the PTL pretreatment group was 1.6, 1.8 and 1.4 times higher than that in the irradiated group alone, respectively (Figure 5C). The same conclusion was reached using qPCR to observe Shh mRNA level. At 4 h and 8 h, the expression of Shh mRNA in PTL pretreatment group was 5.7 and 2.8 times higher than that in the irradiation group, respectively. DISP1 is required for secretion of lipid-modified Shh (Wang et al., 2015). PTL pretreatment increased DISP1 mRNA expression 9.6-fold and 5.8-fold in the

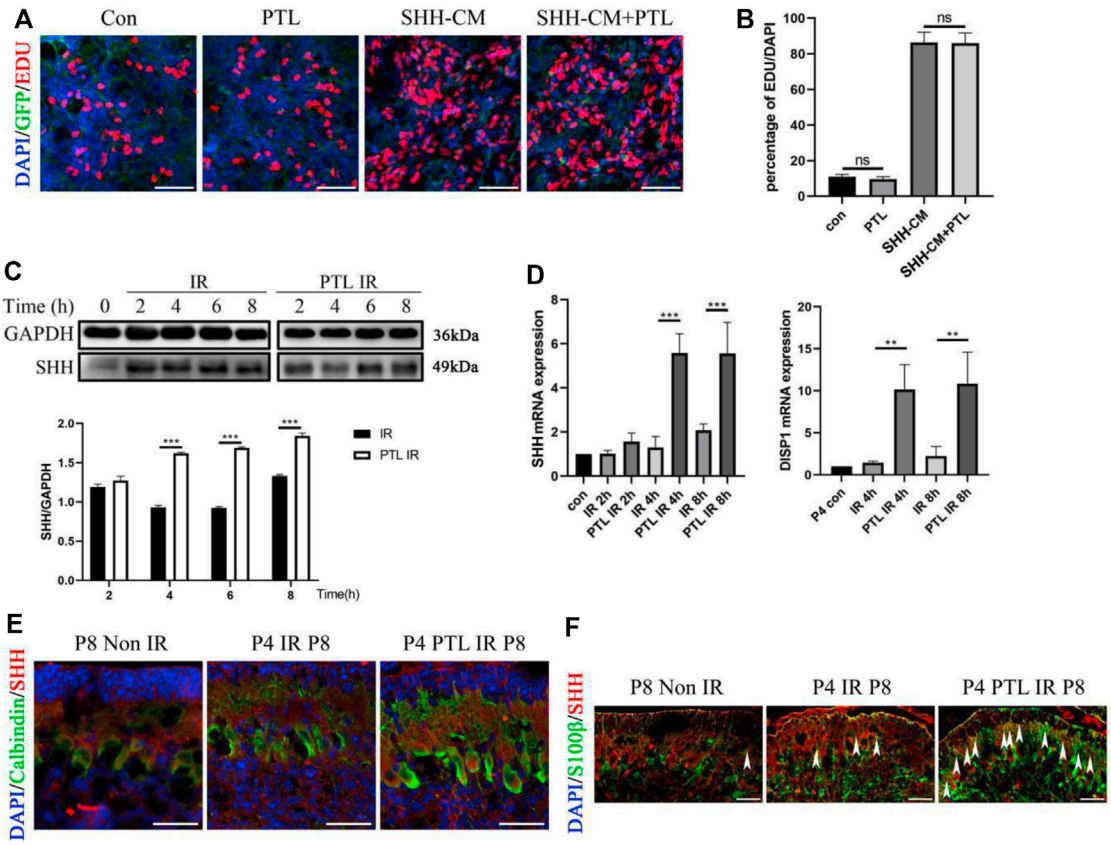

FIGURE 5
PTL promote Irradiation-Induced Production and Secretion of Shh in cerebellum **(A)**. Representative pictures (×100 magnification) of EdU and Tuj-1staining of GNPs. GNPs from wild type (WT) mice treated with 1 μM PTL, 50% Shh-CM, 50% Shh-CM adding 1 μM PTL *in vitro* for 24 h, then stained by β3-Tubulin (Tuj1; green) for neuron differentiation, EDU for proliferation (red) and DAPI (blue) for nuclei. White scale bar, 50 μm. **(B)**. Graph represents the quantifications of **(A)**. Scatter plot of represents the percentage of EDU$^+$ cells vs. all DAPI cells of WT GNPs treated with 1 μM PTL, 50% Shh-CM, 50% Shh-CM adding 1 μM PTL (n = 3). **(C)**. Representative Western blot images of GAPDH and Shh from the wild type mice cerebella protein pre-treated with 40 mg/kg PTL or not at 2, 4, 6, 8 h after irradiation with 4Gy X-ray (top). Bar graphs represents the quantifications of Western blotting results. Shh was normalized to GAPDH. Black bar, IR; white bar, PTL IR group (down, n = 3). **(D)**. QRT-PCR quantification of Shh (left) and DISP1 (Dispatched-1, a membrane protein facilitates the release of Shh,right) from the cerebella of wild type mice pre-treated with 40 mg/kg PTL or not at 2, 4, 8 h after irradiation with 4Gy X-ray at P4 (n = 3). **(E)**. IF detection of Calbindin (Green), Shh (red) and DAPI (blue) on sagittal sections of Non IR, IR and PTL IR mice at P8 (×200 magnification). White scale bar, 20 μm. **(F)**. IF detection of S100β (Green), Shh (red) and DAPI (blue) on sagittal sections of Non IR, IR and PTL IR mice at P8 (×200 magnification). White arrowheads indicated S100β$^+$/Shh$^+$ cells present in PCL. White scale bar, 20 μm. All graphical data were presented as mean ± s. e.m., and significance was determined by two-tailed Student's t test or Kruskal–Wallis nonparametric one-way ANOVAs. ns, nonsignificant; $^{**}p < 0.01$; $^{***}p < 0.001$.

4 h and 8 h groups, respectively (Figure 5D). It was demonstrated that Shh is mainly made by Purkinje cells in the cerebellum (van der Heijden and Sillitoe, 2021). Therefore, we detected the expression of Shh in Purkinje cells by staining with Calbindin and Shh. IF assay showed that PTL IR mice had a stronger fluorescence of Shh in Purkinje cells at P8 (Figure 5E). Recently, many of researchers have found that astrocytes could also secrete Shh to support brain development (Liu et al., 2017; Petrov et al., 2017). Therefore, we also stained the astrocyte marker S100β and Shh in the frozen section. The results showed that PTL pretreatment increased the number of S100β+/Shh + cells after IR at P8 (Figure 5F). In general, PTL could promote Shh secretion in the cerebellum after IR.

PTL facilitates secretion of Shh in astrocytes and protects cells from irradiation *via* the AKT pathway

In cerebellar radiation damage, the most affected cells by irradiation are granule neurons and astrocytes. Astrocytes were considered another potential primary cellular source of Shh (Alvarez et al., 2011). Therefore, we explored the effect of PTL on astrocytes. To test the effect of PTL on astrocytes *in vitro*, we used primary astrocytes from wild-type mice embryonic day 14.5 (E14.5). After Shh staining in comparison with the control group without PTL treatment, stronger red fluorescence (Shh) was found in primary astrocytes treated with PTL for 24 h.

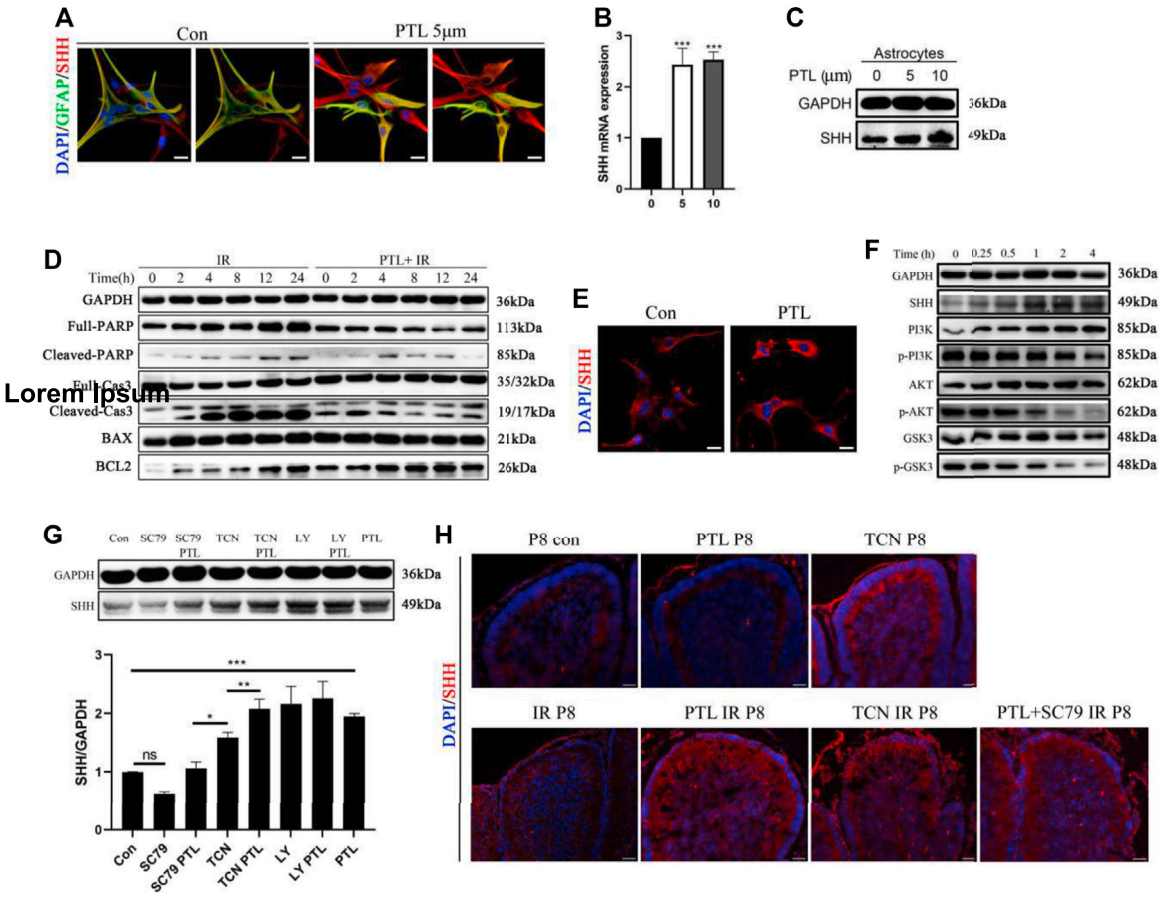

FIGURE 6
PTL increases the secretion of Shh in astrocytes through AKT pathway inhibition **(A)**. IF detection of GFAP (Green), Shh (red) and DAPI (blue) in primary astrocytes (×200 magnification) treated with or without 5 μM PTL for 24 h. Left two images are control, and the image on the right is without DAPI. Right two images are PTL treatment group, and the image on the right is without DAPI. White scale bar, 50 μm. **(B)**. QRT-PCR of Shh from primary astrocytes treated with or without 5 μM or 10 μM PTL for 24 h. Shh was normalized to β-actin. Black bar, control (0); black bar, 5 μM PTL pretreatment (5) Gray bar, 10 μM PTL pretreatment (10) (n = 3). **(C)**. Representative Western blot images of GAPDH and Shh from primary astrocytes treated with or without 5μM and 10 μM PTL for 24 h **(D)**. Astrocyte cell line (C8-D1A) pretreated with or without 10 μM PTL for 1 h before 4 Gy X-ray exposure and were then irradiated. Representative Western blot images of GAPDH and markers of apoptosis, PARP/p-PRAP, total Cas3/cleaved Cas3, BAX, BCL2 at different time points after irradiation. **(E)**. IF detection of Shh and DAPI in C8-D1A (×200 magnification) treated with or without 5 μM PTL for 24 h. White scale bar, 20 μm. **(F)**. Representative Western blot images of GAPDH, Shh, PI3K, p-PI3K, AKT, p-AKT, GSK3, p-GSK3 in astrocyte cell line (C8-D1A), treated with 10 μM PTL for 0, 0.25, 1, 2, 4 h **(G)**. Western blot of Shh in C8-D1A treated with 10 μM SC-79 (AKT activator), 10 μM SC-79 adding 5 μM PTL, 0.5 μM Triciribine (TCN, AKT inhibitor), 0.5 μM TCN adding 5 μM PTL, 1 μM LY294002 (LY, PI3K inhibitor), 1 μM LY adding 5 μM PTL for 24 h (top). Bar graphs represents the quantifications of Western blotting results. Shh was normal ized to GAPDH. (Down, n = 3). **(H)**. IF detection of Shh (red) and DAPI (blue) on sagittal sections of Con, PTL, TCN, IR, PTL IR, TCN IR and PTL adding SC-79 IR mice at P8 (×200 magnification). White scale bar, 20 μm. All graphical data were presented as mean ± s.e.m., and significance was determined by two-tailed Student's t test or Kruskal–Wallis nonparametric one-way ANOVAs. ns, nonsignificant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Astrocytes were labelled with immunofluorescent green for GFAP. Although GFAP expression in primary astrocytes was heterogeneous, including weak, moderate and strong positive staining, we could still observe co-localization of GFAP with Shh (yellow) (Figure 6A). qPCR results showed that 5 μM and 10 μM PTL treatments increased Shh mRNA expression in primary astrocytes approximately 2.3 and 2.5-fold, respectively, which was consistent with WB result (Figures 6B,C). To study the mechanism by which PTL protects astrocytes, we utilized the astrocyte cell line C8-D1A for this experiment. First, to determine whether PTL could protect astrocytes after irradiation *in vitro*, we irradiated C8-D1A cells at 4Gy X-ray pre-treated with 10 μm PTL for 2 h. After IR-activated cell apoptosis, the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) was cleaved to p-PARP by Caspase 3 (Cas3), which was simultaneously transformed to Cl-cas3. BCL2 plays an antiapoptotic role in this process, whereas BAX not only antagonizes the inhibitory effect of BCL2, but also promotes apoptosis. WB assay showed that pretreatment with PTL significantly inhibited the expression of PARP,

p-PARP, Cl-cas3, and BAX, but promoted the expression of BCL2 (Figure 6D). These results suggest a protective role of PTL for irradiated astrocytes *in vitro*. Immunofluorescence assay showed that PTL also increased the expression of Shh in C8-D1A cells (Figure 6E). Acute neuronal molecules glycogen synthase kinase-3β (GSK-3β) and phosphatidylinositol-3 kinase/AKT (PI3K/AKT) played a key role in the establishment and maintenance of neurite polarity (Gobrecht et al., 2016; Ranea-Robles et al., 2018). WB results showed that PTL prominently increased the expression of Shh and inhibited PI3K/AKT/GSK3 signaling in a time-dependent manner in C8-D1A cells (Figure 6F). GSK phosphorylation, which depended on PI3K/AKT signaling, inhibited most of these microtubule-associated proteins (MAPs), which regulated axon growth (Chiang et al., 2009).

These results suggested that PTL might affect Shh expression *via* the PI3K/AKT/GSK3 pathway, promoting the mobilization of NEPs after irradiation. Therefore, we used AKT agonist (SC-79), AKT inhibitor (Triciribine, TCN) and PI3K inhibitor (LY294002, LY) to validate the mechanism by which PTL promotes Shh expression in C8-D1A. We found Shh expression was inhibited by SC-79 inhibited and increased by TCN and LY, suggesting that inhibition rather than activation of the PI3K/AKT pathway upregulates Shh in astrocytes. PTL was able to reverse the inhibitory effect of SC-79, and PI3K/AKT activators TCN, LY and PTL synergistically promoted Shh expression (Figure 6G). To verify the regulatory effect of PI3K/AKT pathway on Shh expression *in vivo*, radiation protection experiments in mice were carried out with TCN, SC79 and PTL. Interestingly, perhaps because of the complex conditions *in vivo*, IF assays showed that cerebellar Shh was upregulated after treatment with TCN alone without irradiation whereas PTL treatment alone had no significant effect on it. The TCN-treated mice in our experiments did not survive into adulthood. Consistent with the results of *in vitro* experiments, PTL or TCN increased the expression of Shh in the cerebellum after irradiation. Even *in vivo*, although the effect of PTL treatment was weaker than that of control group, PTL treatment could reverse the inhibition of SC79 (Figure 6H). These results suggest that PTL can not only protect astrocytes from radiation damage, but also promote the secretion of Shh by irradiated astrocytes by inhibiting the PI3K/AKT pathway.

Discussion

Generally, mammalian neurons do not regenerate after injury, which results in functional deficits. Although significant progress has been made in understanding the mechanisms of neuronal regenerative failure, there is no specific neuroregenerative therapy at present (Varadarajan et al., 2022). The ideal treatment for nerve injury should promote neuron regeneration while inhibiting cellular susceptibility to injury and cellular damage. PTL is a sesquiterpene lactone derived from the herb feverfew, which is traditionally used as medicine. As a covalently active compound, it exhibits anti-inflammatory, redox-regulatory, epigenetic and selective cytotoxicity against tumor stem and progenitor cells (Goodrich et al., 1997). Various published studies have focused on the antitumor activity of PTL. However, more recently, PTL has been shown to exhibit other bioactivities besides its classical function. At very low concentrations, PTL significantly accelerated nerve growth and regeneration by inhibiting microtubule detyrosine in damaged neurons (Diekmann and Fischer, 2016; Freund et al., 2019). In the present study, we utilized irradiation to induce injury to investigate the effect of PTL on cerebellum regeneration. It has been long known that reactive oxygen species (ROS) produced in the cells following irradiation are key mediators of oxidative damage to the cells. The Nrf2-Keap1 pathway is highly sensitive to ROS-dependent oxidative stress in cells. When Keap1 detects ROS, it releases Nrf2, which is then translocated from the cytoplasm to the nucleus. When activated, Nrf2 counteracts ROS production and cell survival (Bader et al., 2021). Previous studies suggest that PTL can increase the radiosensitivity of tumor cells and protect normal cells through its different effects on the KEAP1-NRF2 pathway (Xu et al., 2013). In this study, we first showed the protective effect of PTL on developing cerebellum with acute irradiation injury. γ-H2AX and cleaved caspase3 are proteins formed during irradiation-induced DNA damage and apoptosis, respectively. γ-H2AX and cleaved caspase3 staining showed reduced apoptosis and decreased damage to GNPs. Intracellular H_2O_2 levels can reasonably reflect general changes in intracellular ROS production during the conversion of various intracellular ROS to H_2O_2; therefore, selective measurement of H_2O_2 is essential for accurate assessment of oxidative stress (Calabria et al., 2020). Our results indicated that PTL can alleviate irradiation-induced oxidative stress by reducing H_2O_2 levels. Through *in vitro* cell experiment, we confirmed that PTL can provide radiation protection by reducing ROS, inhibiting KEAP1, promoting nuclear translocation of Nrf2 and reducing apoptosis. Previous reports on the relationship between PTL and ROS are still contradictory and incongruent. Some studies have indicated that PTL induces apoptosis *via* ROS generation and blockade of components of the intrinsically anti-apoptotic NF-κB pathway, including its downstream regulator IκB kinase. Other animal experiments have suggested that PTL displays anti-inflammatory effects by inhibiting ROS production *in vivo* (Freund et al., 2020; Wang et al., 2020). Data mostly from previous *in vitro* experiments using cultured cell lines with higher concentrations demonstrated that PTL increased ROS-induced apoptosis. In contrast, the results from normal cells as well as *in vivo* experiments mostly demonstrated its cytoprotective effect. We speculate that this apparent difference may be due to a more complex cellular environment *in vivo*, including the presence of regulatory networks in different types of cells. As early as 1955, it was reported that in irradiated cerebellum, glucose oxidative

conversion was somewhat disturbed and the effective glycolytic process was relatively poor (Golubtsova, 1956). It is known that, during aerobic oxidation, reactive oxygen species (ROS) are produced. Aerobic glycolysis can reduce the ROS produced by the respiratory chain of mitochondria during oxidative phosphorylation (OXPHOS), and reduce the oxidative stress burden of cells. (Pascale et al., 2020). PKM2 is a key regulator of aerobic glycolysis, and as an activator of PKM2, PTL may reduce ROS production to some extent by maintaining aerobic glycolysis in the proliferating GNPs. Our data are consistent with the results of previous studies on the reduction of normal cell irradiation sensitivity by PTL *via* the ROS/KEAP1/NRF2 pathway. Besides, some studies revealed that blocking the nuclear translocation of PKM2 and inhibiting the expression of apoptosis-regulated genes can alleviate AD neuronal lesions (Traxler et al., 2022), and PTL may also play an anti-apoptotic role by promoting the formation of PKM2 tetramers and inhibiting dimerization into the nucleus. Above all, PTL is potentially protective against irradiation-induced acute cerebellum injury.

In addition, the exploration of therapeutic strategies targeting neural regeneration may be more important, as increased regeneration is a more likely to attenuate permanent nerve deficits. Our previous study identified a novel population of progenitors in the developing cerebellum EGL that overexpressing Nestin. These NEPs are committed to the granule neuron lineage. In comparison with GNPs, NEPs express fewer genes associated with DNA repair, and exhibit increased genomic instability and tumorigenic potential (Li et al., 2013). In 2017, Wojcinski and colleagues revealed that Nestin-expressing cerebellar progenitor cells in the Purkinje cell layer form only interneurons and astroglia during normal development, expand their differentiation capacity, and originate as mature granule neurons after injury (Wojcinski et al., 2017). We suggest that the differences in location and time of the observations likely account for the seemingly conflicting results on NEPs lineage restriction. While destination of NEPs in inner-EGL was the main concern in our previous work, Wojcinski mainly focused on NEPs in the PCL. Indeed, the latter study also recognized that NEPs in PCL migrate to EGL to become committed to a neuronal lineage after being activated by injury. Together, these studies indicate that NEPs is a unique population of progenitors essential for regenerating GNPs after cerebellar injury. Therapeutic strategies that target activation of NEPs contribute to regeneration and functional recovery of the damaged developing cerebellum. By comparing the size and GNPs proliferation status of the cerebellum at different developmental stages, we confirmed that PTL pretreatment promoted the repopulation of the cerebellum after irradiation. Using transgenic Nestin-CFP mice, we found that a single PTL pretreatment alone could increase the NEPs in EGL and PCL of irradiated developing cerebellum. Also, in agreement with the results of Wojcinski. et al., we found that irradiation induced the appearance of SOX2+ cells in the ML and EGL, whereas PTL significantly increased SOX2+ cells in these regions. The SOX2 gene plays a critical role in regulating neural development, as well as in self-renewal of embryonic and neural stem cells (Jagga et al., 2021). Endogenous expression of Sox2 in neural progenitor cells has a role in promoting reprogramming (Wojcinski et al., 2017). These results suggest that PTL can facilitate the initial adaptive reprogramming of NEPs or promote the migration of NEP-derived cells to EGL after irradiation. Pax6 staining and lineage tracing analysis also verified that PTL contributes to the replenishment of damaged cerebellar granule neuronal cells. Although it has been suggested that NEPs in PCL can normally only differentiate into interneurons and glial cells, probably because of experimental methodological differences, we still found that a small amount of PAX6 positive NEPs exists in the normal cerebellum, which is consistent with the lineage tracing experiment. In contrast to single irradiation that reduces the expression of the interneuron marker PAX2 in NEPs, PTL pretreatment protects against the inhibitory effect of irradiation on interneurons derived from NEPs. This helps maintain the balance of excitatory and inhibitory neuronal cells in the microenvironment. PTL also exerts the same effect on glial cells to reverse the effects of irradiation and maintain homeostasis. These results suggest that PTL may have the ability not only to promote the reprogramming and differentiation of NEPs into granule neuron cells after irradiation, but also to expand NEPs cells and maintain their role of producing interneurons and glial cells. That is, although the cell lineage of NEPs is somewhat controversial, PTL mobilizes both NEPs located in the EGL and PCL, and may act on the most essential and basic level. Energy metabolism is a fundamental physiological activity in cells and individuals. Besides affecting the adaptive reprogramming capacity of brain progenitor cells, PTL may also regulate the energetic reprogramming of progenitor cells through its effects on PKM2. Katherine et al. (Tech et al., 2017) confirmed that PKM2 was highly expressed in developing cerebellar progenitor cells. GNPs with PKM2 deletion showed a decrease in lactic acid production and an increase in SHH-driven proliferation. The allosteric regulation of PKM2 activity is the basis of its effect on metabolic reprogramming. As a tetramer activator of PKM2, PTL may inhibit the glycolysis of PKM2 and regulate NEPs mobilization.

Previous studies have suggested that both NEPs in the PCL and a small number of scattered NEPs in the EGL are affected by Shh signaling after activation. Therefore, we believe that investigating Shh signaling could help elucidate PTL-promoted amplification of cerebellar NEPs after irradiation. Our *in vitro* experiments revealed that PTL itself neither promotes nor inhibits the response of GNPs to Shh, implying that it has no direct effect on Shh signaling. Shh was detected in cerebellar tissue at different time points, suggesting that although PTL is not an activator or inhibitor of Shh, it can promote Shh

production in the cellebellar microenvironment. DISP1 is indispensable in cholesterol bio-synthesis or secretion of cholesterol-modified Shh (Jamal et al., 2018). Yuan et al. (Yuan et al., 2020) found that PTL can affect the TCA cycle, amino acid metabolism, choline metabolism and lipid metabolism in thyroid cancer cells (TPC-1). Our data show that PTL increases DISP1 expression, contributing to the secretion of Shh. As the primary source of cerebellar Shh, Purkinje cell neurogenesis is synchronized with cerebellar development and begins at E10.5. Compared with prenatal irradiation, postnatal irradiation had slightly less effect on cerebellar Purkinje cells (Zhou et al., 2017). We found that PTL could upregulate Shh in irradiation-induced cerebellar Purkinje cells to some extent. Astrocyte is the major site of acute aerobic glycolysis in brain (Barros et al., 2021). Certain conditions induce astrocytes to produce Shh. It has been found that reactive astrocytes secrete Shh to promote the proliferation of astrocytes, microglia and NG2 positive progenitor cells (Pitter et al., 2014). Various CNS injury factors, including irradiation, can lead to an increase in reactive astrocytes (Liddelow and Barres, 2017). We found that PTL increases astrocyte number and Shh expression. Given that astrocytes are more sensitive to irradiation and PTL than Purkinje cells, we explored the possible mechanisms of PTL-induced astrocyte-associated Shh production. Regarding the relationship between astrocytes and Shh, studies have mostly focused on determining the important role of the Shh signaling pathway (Allahyari et al., 2019) and less on the regulation of Shh protein expression itself. The controllable temporospatial expression of Shh is the basis of its normal function. Multiple signaling pathways are associated with the regulation of Shh expression, and positive and negative transcriptional regulatory networks control its expression in restricted regions (Matsubara et al., 2017). Numerous tumor types are associated with dysregulation of the Shh signaling pathway, including constitutive overexpression of the Shh ligand (Tolani et al., 2018). Although the effects of PTL are mostly associated with its role as an NF-κB inhibitor, some studies have proposed that the overexpression of Shh in breast cancer is due to NF-κB activation (Cui et al., 2010), which apparently cannot explain the phenomenon that PTL promotes Shh expression. As previously described, PTL protects the peripheral nerve in an NF-κB-independent manner, and it mimics the beneficial effects of GSK3 mutations on axon regeneration in mice. In the exploration of the inhibition of Wnt/β-catenin signal by PTL, researchers found that PTL can reverse the inhibitory effect of GSK3 ion downstream genes (Zhu et al., 2018). GSK-3 is a key regulator of neuronal progenitor homeostasis. GFAP-cre-mediated deficiency of GSK-3 in astrocytes leads to enlarged brains and more and larger astrocytes (Jung et al., 2016). The PI3K/Akt is a major signaling pathway that regulates GSK3. Numerous studies have shown that the PI3K-Akt-GSK3β signal transduction pathway plays important roles in the neuroprotective process. Our results confirmed that PTL suppressed activation of PI3K/Akt signaling in astrocytes. The PI3K/Akt pathway has crosstalk with multiple cells signaling pathways. Activation of STAT3 is thought to upregulate Shh gene expression (Zagozewski et al., 2022), whereas PI3K/AKT inhibition promotes STAT3 activation. Recently, RARβ2, another promoter of Shh expression, was also found to be negatively regulated by PI3K/AKT (Huang et al., 2022). Through *in vitro* and *in vivo* experiments of PI3K/AKT inhibitor, we also confirmed that it can promote the expression of Shh in cells and irradiated cerebellar tissue. Surprisingly, a single administration of PTL *in vivo* did not promote cerebellar Shh expression. Our previous studies found that the homeostasis of NEPs is important. Under normal conditions, NEPs are quiescent *in vivo*, physiological concentrations of Shh do not trigger their mobilization, and abnormal proliferation of NEPs is associated with tumorigenesis. PTL functions only in the presence of abnormalities or injury, and is greatly likely to be attributable to changes in aerobic glycolysis, which facilitates the maintenance of homeostasis. Previous studies also confirmed that the Shh signaling pathway and PI3K/AKT synergize with each other to affect aerobic glycolysis of GNPs in the developing cerebellar EGL (Gershon et al., 2013; Tech et al., 2017). On the other hand, this suggests that the role of PTL on PI3K/AKT may be complex, especially since many studies have shown that PTL exert effects on various signaling pathways. Akt is a key kinase that promotes growth, metabolism, survival, and motility. Although further studies are needed to determine the specific molecular mechanisms by which PTL, PI3K/AKT and Shh regulate each other, these results are still encouraging.

Conclusion

Our work shows that PTL exerts a protective effect on irradiation-induced acute injury through the ROS/KEAP1/NRF2 pathway, and facilitates the expansion and reprogramming of NEPs in the damaged cerebellum, enhancing the regeneration of the damaged cerebellum by promoting Shh expression in irradiated cerebellar tissue. These findings suggest that therapeutic strategies based on PTL may provide new options for treatment after CNS injury, especially since PTL not only provides a protective effect on the damaged nerve cells themselves, but also facilitates nerve cell regeneration by affecting the microenvironment. Considering that PTL can also promote axonal regeneration of peripheral nerve cells and affect aerobic glycolysis, elucidating the metabolic mechanism of the effect of PTL on astrocyte Shh through PI3K/Akt inhibition, the effects of PTL on other cells (such as Purkinje cells, oligodendrocytes and microglia) and axonal regeneration of CNS neurons and the regulation of energy metabolism in NEPs warrant further study.

Summary

Given the multiple effects of PTL on the developing cerebellum after injury, which modulates both the injured cells and the microenvironment in which they reside, our findings provide a rationale for PTL as a potential CNS radioprotective agent.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

All authors contributed equally in the project design, execution and manuscript drafting. The manuscript was read and approved by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.1051103/full#supplementary-material

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Hyperglycemia-associated Alzheimer's-like symptoms and other behavioral effects attenuated by *Plumeria obtusa* L. Extract in alloxan-induced diabetic rats

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Diabetes mellitus is a chronic metabolic complaint with numerous short- and long-term complications that harm a person's physical and psychological health. *Plumeria obtusa* L. is a traditional medicine used in the treatment of diabetes to reduce complications related to behavior. *Plumeria* is a genus with antipsychotic activities. The objective of this study was to examine the effects of a methanolic extract of *Plumeria obtusa* L. in the attenuation of diabetes, on symptoms of Alzheimer disease, and on other associated behavioral aspects. A single dose of alloxan was administered to an experimental group of rats to induce development of diabetes (150 mg/kg, intraperitoneal) and the rats were then administered selected doses of methanolic extract of *Plumeria obtusa* L. (Po.Cr) or glibenclamide (0.6 mg/kg) for 45 consecutive days. Behavioral effects were evaluated using three validated assays of anxiety-related behavior: the open field test, the light and dark test, and the elevated plus maze. Anti-depressant effects of *Plumeria obtusa* L. were evaluated using the forced swim test (FST) and memory and learning were assessed using the Morris water maze (MWM) task. Po.Cr was also evaluated for phytochemicals using total phenolic content (TPC), total flavonoid content (TFC), and high-performance liquid chromatography assays, and antioxidant capability was assessed through assays of DPPH radical scavenging, total oxidation

Abbreviations: AAE/DW, the number of mg equivalents of ascorbic acid per gram of dry plant weight; DM, diabetes mellitus; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EPM, elevated plus maze; FST, forced swim test; HPLC, high performance liquid chromatography; MWM, Morris water maze; Po.Cr, crude methanolic extract of *Plumeria obtusa* L.; ROS, reactive oxygen species; TFC, total flavonoid content; TPC; total phenolic content.

capacity, and total reducing capacity. In the alloxan-induced model of diabetes, the administration of Po.Cr and glibenclamide for 45 days produced a marked decrease ($p < 0.001$) in hyperglycemia compared to control animals. Po.Cr treatment also resulted in improvement in indicators, such as body weight and lipid profile ($p < 0.05$), as well as restoration of normal levels of alanine transaminase (ALT) ($p < 0.001$), a biomarker of liver function. Diabetic rats presented more Alzheimer-like symptoms, with greater impairment of memory and learning, and increased anxiety and depression compared to non-diabetic normal rats, whereas treated diabetic rats showed significant improvements in memory and behavioral outcomes. These results demonstrate that Po.Cr reversed alloxan-induced hyperglycemia and ameliorated Alzheimer-related behavioral changes, which supports additional study and assessment of conventional use of the plant to treat diabetes and associated behavioral complications.

KEYWORDS

Alzheimer, anxiolytic, anti-depressant, learning, memory, anti-diabetic

1 Introduction

Diabetes mellitus (DM) is a heterogeneous metabolic complaint involving increased blood glucose (Talha et al., 2022), which is a result of inadequate insulin secretion, diminished insulin sensitivity, or both. DM is a polygenic condition with increased reactive oxygen species (ROS) and basal metabolic rate, along with deficiency in lipoproteins and free radical scavengers, and impairment of organs due to oxidative stress (Shah and Khan, 2014; Behl et al., 2022). Additionally, complications of DM include psychiatric complaints such as depression and anxiety, neurodegenerative impairments, and cognitive decline (Ceretta et al., 2012a; Reus et al., 2016). Numerous studies have shown an association between the pathophysiology of DM and psychiatric disorders due to alterations in glucose metabolism, formation of ketone bodies, oxidative stress, and negative effects on neuroplasticity (Hassan et al., 2022a, Ceretta et al., 2012a). The harmful consequences of DM are worsened by oxidative stress and inflammation, which contribute to the induction of DM and its complications, play a crucial role in diabetic tissue damage, and are a major contributors to diabetic neuropathy (Ceretta et al., 2012b). Oxidative stress arises from the imbalance between ROS and antioxidant defensive mechanisms (Hassan et al., 2022b). Hyperglycemia can elevate the production of ROS and lead to the damage of numerous cellular components, such as proteins, nucleic acids, amino acids, and lipids (Gupta et al., 2017). Decreased levels of circulating antioxidants associated with diabetes may be one of the risk factors for Alzheimer disease and depression (Abduljawad et al., 2022; Kabra et al., 2022). DM is also associated with alterations in neurochemicals and hormones that can be linked with anxiety and depression. The co-morbidity of diabetes with anxiety disorders has demonstrated greater diabetic complications, greater pain, increased depression, and decreased quality of life (Smith et al., 2013). Diabetes and its complications can be reversed or prevented by effective control of blood glucose levels. The utilization of medicinal plants based on ancient practices has had a resurgence (Sarwar et al., 2011; Mahnashi et al., 2022; Zou et al., 2022), and plant-based compounds should be part of an advanced treatment strategy. Present-day medications for diabetes are hampered by limited effectiveness and adverse effects that range in seriousness from negligible weakness to death from severe hypoglycemia, hepatic and kidney damage, or chronic toxicity. These adverse effects of established treatments have led to replacement with alternative medicines and herbal products, as they are safe and cost-effective. The effective use of medicinal plants to treat diabetes and related complications has been established in experimental animal models.

Plumeria obtusa L., a member of the family Apocynaceae, is commonly known as white frangipani, chafa, and gul cheen. In the traditional system of medicines, leaves of *Plumeria obtusa* L. have been frequently used to treat hyperglycemia (Ali et al., 2014; Dogra, 2016; Mulaudzi et al., 2019; Bihani et al., 2021). Furthermore, it has been traditionally applied in the treatment of skin diseases, fever, pain, inflammation (Zhang et al., 2022), arthritis, and gastrointestinal ailments (Zhang et al., 2021), bacterial, fungal, and viral (esp. herpes zoster) infections, as well as in cancer treatment (Wong et al., 2011; Devprakash et al., 2012; Asiimwe et al., 2013; Shah et al., 2015; Lotankar et al., 2016). Decoction of leaves is commonly employed to treat wounds and skin infections, cerebral pain, and asthma, and as a laxative, antitoxin, or diuretic (Ali et al., 2014; Shah et al., 2015). Roots are applied for the treatment of asthma, constipation, dysentery, leprosy, ulcers, skin and liver maladies, and tumors. Previous pharmacological studies revealed the presence of antifungals, antimicrobials, and antivirals (Ali et al., 2014), as well as gastro-protective (Singh et al., 2012), laxative, diuretic,

anti-tumor (Wong et al., 2011), and antioxidant activities in *Plumeria obtusa* L. (Dogra, 2016; Bihani et al., 2021). Some species of the genus *Plumeria* have been reported to have anxiolytic activities as well (Chatterjee et al., 2013). Phytochemical investigations of *P. obtusa* revealed the presence of tannins, triterpenoids, saponin, proteins, glycoside, flavonoids, essential oils, carbohydrates, and alkaloids (Singh et al., 2012). Furthermore, *Plumeria obtusa* L. (aerial parts) showed the presence of pentacyclic triterpenoids, including betulinic, oleanolic, and ursolic acids (Siddiqui et al., 1989; Devprakash et al., 2012; Alvarado et al., 2015). Another study reported that iridoids characterized as acetylplumieride coumarate and acetylplumieride-p-Z-coumarate are found in the plant, along with other constituents that include isoplumericin, plumieride, plumieride coumerate, and plumieride coumerate glucoside (Ali et al., 2014). Benzyl salicylate and benzyl benzoate are the essential oils found in *P. obtusa* (Devprakash et al., 2012).

Plumeria obtusa L. is a medicinally important plant with great potential and substantial traditional claims regarding its use to treat diabetes and associated Alzheimer-related and behavioral effects, but there remains a lack of sufficient experimental data to validate those claims. The purpose of this study was to examine the effect of the methanolic crude extract of *Plumeria obtusa* L. on the alloxan model of DM and associated Alzheimer-related and behavioral consequences, including cognitive impairment, anxiety, and depression.

2 Methods

2.1 Chemicals

Chemicals/reagents utilized in the experimental work were of analytical research grade. Methanol was procured from Duksan Pure Chemicals, Korea. Sodium chloride for normal saline was obtained from Otsuka, Pakistan, glucose from Merck, Germany, and alloxan and glibenclamide from Sigma-Aldrich, Germany.

2.2 Collection and pre-treatment of plant material

Plumeria obtusa L. (leaves) were collected from Multan in the spring of 2017. Sample "R.R. Stewart 565″" was preserved at Bahauddin Zakariya University (BZU), Institute of Pure and Applied Biology in Multan. Leaves were obtained, cleaned, dried, and ground into powder. A total of 1 kg of coarse powder was soaked in 80% v/v hydro-methanol for 7 days in an amber colored glass jar with occasional shaking. After filtering, the filtrate was dried in a rotary evaporator at 37°C and low pressure, and a viscous substance derived from the *Plumeria obtusa* (Po.Cr) leaves, with a yield of 12.6%, was produced. The extract was maintained at −20°C in an airtight, amber-colored vial for future experiments.

2.2.1 Dosage preparation

Po.Cr was dissolved in 1 ml of normal saline and 0.1 g/ml of Po.Cr was delivered orally for all experiments.

2.3 Experimental animals and their care

Male Sprague-Dawley rats weighing between 150 and 260 g were used and were kept at the Faculty of Pharmacy's animal house at BZU in Multan. Rats were kept in sawdust-lined polycarbonate cages with a 12 h light/dark cycle under regulated conditions. Rats were fed a high fat/carbohydrate-rich diet before induction of diabetes, and later fed regular rodent feed pellets containing 50% carbohydrates, 25% proteins, and 25% fats on a regular basis; the rats had free access to water.

2.4 Alloxan-induced experimental diabetes

On day 0 of the experiment, the selected rats were administered freshly prepared alloxan monohydrate (150 mg/kg/i.p.) in sterilized normal saline, after 12 h of fasting (Johar et al., 2018). Massive insulin discharge from the pancreas due to apoptosis of insulin producing beta cells generally leads to alloxan-induced hypoglycemia. Therefore, the rats were kept on 5% glucose for the following 24 h to prevent hypoglycemia.

2.5 Experimental design

The Po.Cr doses were selected based on preliminary experiments in our laboratory, in which rats were orally treated with four different doses: 100, 150, 250 and 500 mg/kg. For this study, rats were arbitrarily divided into five groups; details of grouping and dosing are given in Table 1. Drug and plant extract doses were administered to animals daily for 45 days *via* gavage feeding tube. After day 25, the animals were assessed using different behavioral tests, including the open field, light and dark, elevated-plus maze, forced swim test (FST), and Morris-water maze test. Body weight and blood glucose levels were assessed on alternate days using an electronic balance and glucometer, respectively.

On day 46, blood was taken from rats by cardiac puncture while under mild isoflurane (5% v/v) anesthesia (Kumar et al., 2017). Blood samples were immediately transferred to falcon tubes, kept at 15°C–25°C for an hour, and then centrifuged at 2,500 rpm for 15 min to obtain serum for biochemical analysis.

TABLE 1 Layout of animal groups and treatment of the alloxan-induced diabetic rat model.

Layout of animal groups and treatment				
Group I	Group II	Group III	Group IV	Group V
Normal control	Alloxan	Alloxan + Glibenclamide	Alloxan + Po.Cr extract, p.o.	Alloxan + Po.Cr extract, p.o.
1 ml/kg N.S.	150 mg/kg	150 mg/kg + 0.6 mg/kg	150 mg/kg + 300 mg/kg	150 mg/kg + 500 mg/kg
Administered once daily *via* oral gavage from day 1 to day 45	Single dose i.p.	Administered once daily *via* oral gavage from day 1 to day 45		

2.6 Behavioral tests

2.6.1 Behavioral test for learning and memory

2.6.1.1 Morris water maze test

A round water-filled swimming pool was utilized for this test, as previously described (Morris, 1984; Diegues et al., 2014). The apparatus comprised a large, dark water tank made of fiberglass, and was 150 cm in diameter, 50 cm in height, and full of water, at a temperature of 27 ± 1°C, to a depth of 30 cm. Non-toxic white dye was added to the water to make it opaque, and a platform of 29 cm in height and 10 cm × 10 cm in breadth was placed 1 cm below the surface of the water. The pool was separated into four equivalent quadrants, labeled northeast, southeast, southwest, and northwest. The platform was placed in the southwest quadrant and remained there throughout the experiment. On the higher border of the water pool, four indicators were set in the middle of the circumference of every quadrant. The position of the indicators was kept the same throughout the experiment. The apparatus was kept in the test room, with indirect light and a fixed video camera (Logitech, Webcam HD) on the ceiling to track the movement of the animals. The results were assessed using video capture and tracking via ANY-maze software. The animals were allowed to move freely and locate the platform only by means of distant signs placed in the experimental room. The time it took each rat to find and get on the platform was recorded. If the rat remained unable to find the platform within 90 s, it was put on the platform and left there for 30 s. The animal was then returned to its cage for 20 s prior to start of the next trial. The process was repeated by starting from another position in the pool according to the previous trial. Four consecutive trials were performed with each of the animals in similar order. The time to find the platform, i.e., escape latency, was measured during each trial.

On the fifth day, the platform was removed from the pool and a probe trial was performed. The animals were positioned in the pool opposite the prior platform-containing quadrant. The session lasted 90 s, during which the time spent in the targeted quadrant was noted.

2.6.2 Behavioral tests of anxiety

2.6.2.1 Open field test

The open field test (OFT) is a standard test used to assess the effects of test compounds on probing behavior and anxiety. The apparatus consisted of a square box with dimensions of 80 cm × 80 cm × 40 cm and made of white polyacrylic plastic. The apparatus was placed in the middle of the experiment room, which was properly illuminated and soundproof. At the beginning of the experiment, each rat was gently positioned in the center of the box and permitted to move freely for 5 min. The activity of each rat was recorded using a video camera and then analyzed *via* ANY-maze software version 5.3. The ANY-maze video tracking system facilitated analysis of behavioral experiments based on parameters such as total distance traveled and number of entries into the center zone or corner zone, including data on duration in the respective zones. Higher total number of entries and greater time spent in the central area are indicators of reduced anxiety (Turner and Burne, 2014).

2.6.2.2 Light and dark aversion test

The light and dark box (L/D) was also used to examine the anxiolytic effects (Turner and Burne, 2014) of Po.Cr. The apparatus was made up of two plastic boxes with dimensions of 40 cm × 25 cm × 20 cm; one white and the other black. The two boxes were connected *via* a small opening of 7 cm × 7 cm. The animal was allowed to move freely from one box to the other through the opening. The apparatus was set on a clapboard of transparent plastic-covered wood. The transparent white box was brightly illuminated with a 60 W bulb located above the box.

After an hour of pretreatment with extract, each animal was positioned in the center of the white box facing the open hole and permitted to explore the apparatus for 5 min. The apparatus was cleaned using 70% IPA after every trial. The activity of each rat was recorded using a video camera and behavior was assessed using parameters that included number of entries and total time spent in the light and dark boxes (Doukkali et al., 2015; Manikkoth et al., 2016). Decreased activity of animals in the light compartment of a light/dark box indicates anxiety-like behavior (Castillo-Gomez et al., 2015).

2.6.2.3 Elevated plus maze

For additional evaluation of anxiolytic effects of Po.Cr, the elevated plus maze (EPM), first developed by Lister in 1987, was employed (Adeyemi et al., 2010). This method was used with slight modification. The EPM apparatus was made of wood and the maze floor was made of black plexiglass. The maze consisted of two open arms of 110 cm in length and two opposite closed

arms of 110 cm in length, with a 35 cm high wall that formed a plus sign and a central square of 10 cm × 10 cm. The entire apparatus was raised from the ground by approximately 50 cm. The apparatus was brightly illuminated by the lights in the experiment room. After an hour of treatment with extract, the rats were positioned in the middle of the apparatus facing towards one of open arms and the experiment was performed for 5 min. The apparatus was cleaned after every trial using 70% IPA. All trials were recorded using a video camera and behavior was assessed using the following parameters: number of entries into the open and closed arms, and total time spent in the open and closed arms (Tang et al., 2015). Increased open arm entries and time spent in the open arm are indicators of reduced anxiety.

2.6.3 Behavioral test for depression

The forced swim test for evaluating the activity of anti-depressants was first used by Porsolt et al. (1977). In our study, the apparatus was made of a plexiglass cylinder (23 cm in diameter and 35 cm in height) that was filled with water (temperature 24°C–26°C). Each rat was placed in the water briefly as a test to ensure that it did not escape the container and that its feet did not touch the floor of the vessel (Tang et al., 2015). An hour after the preliminary test, the rat was subjected to FST and required to swim for 5 min. After completing the test, the rat was removed from the cylinder, dried with a towel, and placed under a heating fan for 15 min before being returned to its cage. After each trial, the water was removed from the cylinder and replaced with fresh water. The experiment was carried out in a brightly illuminated room. The activity of the animals was recorded using a video camera and then analyzed via ANY-maze software version 5.3. The following behavioral parameters were analyzed:

- Total time immobile (in seconds)
- Total time mobile (in seconds)

2.7 Phytochemical study

2.7.1 Evaluation of total phenolic content

Folin-Ciocalteu reagent analysis (Fatima et al., 2015) confirms the presence of phenolic compounds in tested substances. Each well of a 96-well plate included 20 µl of a 4 mg/ml solution of Po.Cr in DMSO plus 90 µl of Folin-Ciocalteu reagent. When the initial 5 min incubation period was complete, 90 µl of Na_2CO_3 was added to the reaction. The absorbance of each reaction mixture was measured at 630 nm in an ELX800 microplate reader (BioTek, United States), using gallic acid (GA) as the standard. The study was repeated three times, and the results are expressed as mg gallic acid equivalents per gram of sample in dry weight (GAE/g DW).

2.7.2 Evaluation of total flavonoid content

The flavonoid content was determined using a modified version of the aluminum chloride colorimetric technique (Fatima et al., 2015). Aluminum chloride solution (10%), potassium acetate (1.0 M), and distilled water (160 µl) were added to a plate containing 20 µl Po.Cr. After 30 min of incubation, absorbance of the reaction mixture was measured at 415 nm using a microplate reader. The flavonoid content was determined by repeating the experiment three times, and the results were expressed as mg of quercetin equivalents per gram of sample in dry weight (QE/g DW).

2.7.3 HPLC analysis

HPLC was performed in accordance with previously published methods (Fatima et al., 2015), with slight modification through use of a binary gradient pump from the Agilent ChemStation Rev series 260 and 1,200 attached to a diode array detector. Solvents used as the mobile phase were labeled solvent A and solvent B. Solvent A contained methanol (10): acetonitrile (5): water (85): acetic acid (1) and solvent B contained methanol (60): acetonitrile (40): acetic acid (1). The flow rate was maintained at 1 ml/min. Stock solutions of numerous standards were prepared in methanol and sequentially diluted to the final concentrations of 10, 20, 50, 100, and 200 µg/ml. The absorption of Po.Cr was recorded at various wavelengths, including 257 nm for rutin, 279 nm for gallic acid and catechin, 325 nm for caffeic acid and apigenin, and 368 nm for myricetin, quercetin, and kaempferol, and the analysis was performed three times. For the detection of compounds, retention time and absorption spectra were compared with known standards.

2.8 *In vitro* assessment of antioxidant markers

2.8.1 DPPH radical scavenging assay

The antioxidant capacity of Po.Cr was measured by its ability to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), with ascorbic acid acting as a reference standard (Fatima et al., 2015). The percent radical scavenging activity (RSA) and IC_{50} values were determined by spectrophotometric analysis. In 96-well plates, 180 µl of DPPH solution (9.2 mg/100 ml in methanol) were combined with four dilutions of Po.Cr (20 µl) to obtain concentrations of 200.0, 66.66, 22.22, and 7.406 µg/ml. The experiment was run, in triplicate, for 30 min at 37°C, and the absorbance was measured at 517 nm using a microplate reader. The percentage of resource savings due to scavenging was determined by:

$$\%RSA = Ab_c - Ab_s / Ab_c * 100$$

Ab_s = absorbance of sample (Po.Cr); Ab_c = absorbance of negative control.

2.8.2 Estimation of antioxidant potential

To determine the antioxidant potential of Po.Cr, the phosphomolybdenum assay was used. A 0.1 ml aliquot of Po.Cr (4 mg/ml of DMSO) was combined with 0.1 ml of ascorbic acid (4 mg/ml) in 1 ml of reagent containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. A blank solution was added to the same amount of solvent as done with the experimental sample. After 90 min in a boiling water bath at 95°C, the test tubes were cooled to room temperature. Using a PDA spectrophotometer (8354 Agilent Technologies, Germany), we compared the sample's absorbance to that of the blank at 695 nm. When describing the antioxidant activity, the unit of measure used was milligrams of ascorbic acid equivalents (AAE) per gram of dry weight (Fatima et al., 2015).

2.8.3 Total reducing power assessment

The reducing power of Po.Cr was evaluated using the potassium ferricyanide colorimetric assay, as previously published (Fatima et al., 2015). In short, a 200 µl aliquot of 4 mg/ml Po.Cr in DMSO was dissolved in 400 µl of 0.2 mol/L phosphate buffer and 1% potassium ferricyanide. The reaction mixture was incubated at 50°C for 20 min. The mixture was then centrifuged at 3,000 rpm in a solution of trichloroacetic acid (400 µl). A 100 µl aliquot of 0.1% $FeCl_3$ and 500 µl of distilled water were added to the top layer. The absorbance at 700 nm was noted; an increase in absorbance of the reaction mixture indicated increased reducing power. The blank consisted of the above-mentioned reaction mixture plus 200 µl DMSO instead of the extract. The reducing power was articulated as mg AAE/g DW, and the assay was run in triplicate.

2.9 Statistical analysis

The behavioral test data were analyzed using two-way ANOVA and subsequent multiple Dunnett's tests in GraphPad Prism (version 8.0.1), while the remaining experimental data were analyzed using one-way ANOVA and a subsequent Dunnett's test. The results are reported as mean ± standard deviation; $p < 0.05$ indicates a statistically significant difference between groups.

3 Results

3.1 Po.Cr exhibits *in vivo* antidiabetic activity

The blood glucose level remained higher in alloxan-induced diabetic rats compared to normal rats during the 45 days of the study ($p < 0.001$) (Figure 1A). Furthermore, rats treated with glibenclamide (Group III) and Po.Cr at the selected doses had significantly reduced blood glucose levels during the study compared to untreated rats ($p < 0.001$). The study revealed that Po.Cr had significant anti-diabetic activity in treated diabetic rats compared to untreated diabetic rats.

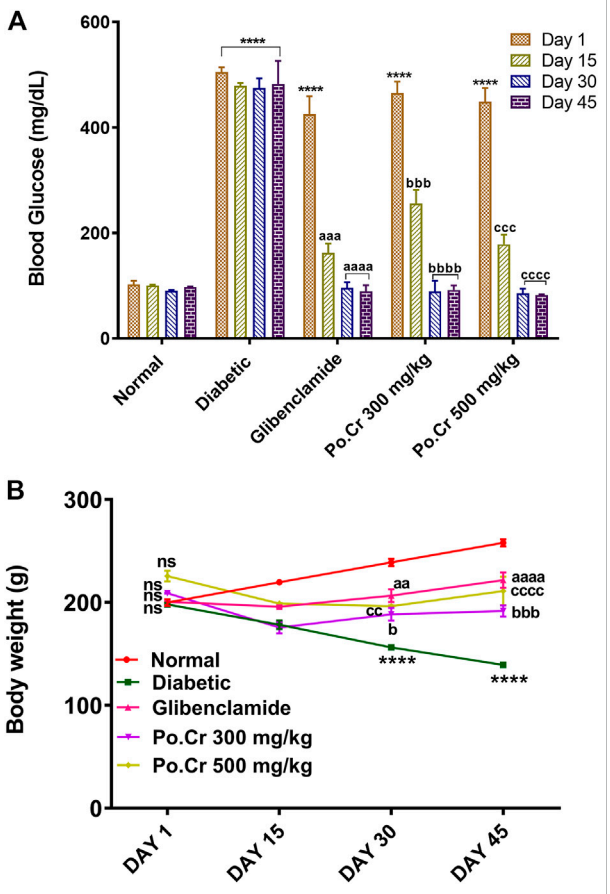

FIGURE 1
Graphical representation of the effects of methanolic extract of *Plumeria obtusa* L. on **(A)** blood glucose levels (mg/dl) and **(B)** body weight of rats. Group II (diabetic control) was compared to Group I (normal control), whereas all the treated groups (Groups III [glibenclamide] to V [Po.Cr]) were compared with Group II. ANOVA (two-way) and the multiple comparison Dunnett's test were applied, and the data values are mean ± SEM. *$p < 0.05$ shows comparison of the diabetic control (Group II) to the normal control (Group I), whereas $^a p < 0.05$, $^b p < 0.05$, and $^c p < 0.05$ indicate comparison of glibenclamide (Group III); Po.Cr, 300 mg/kg (Group IV); and Po.Cr, 500 mg/kg (Group V) *versus* the diabetic control group, respectively.

During the experimental period (45 days), untreated diabetic rats showed prominent weight loss, from 199.66 ± 7.19 to 139.67 ± 3.41 g, compared with normal healthy rats that showed weight gain, from 199.33 ± 5.73 to 258.6 ± 3.25 g. However, treatment with 300 or 500 mg/kg of Po.Cr protected diabetic rats from the significant weight loss observed in untreated diabetic rats (Figure 1B).

TABLE 2 The levels of TC, TG, LDL, HDL, and ALT in blood serum ($n = 8$). Group II (diabetic control) was compared to Group I (normal control), whereas all the treated groups (Groups III [standard treatment] to V [Po.Cr]) were compared with Group II (diabetic control). ANOVA (two-way) and the multiple comparison Dunnett's test were applied, and the data values are mean ± SEM. *$p < 0.05$ and $^{a}p < 0.05$ indicate comparison of the diabetic control (Group II) to the normal control (Group I) and glibenclamide (Group III), respectively. Whereas $^{b}p < 0.05$ and $^{c}p<0.05$ indicate comparison of Po.Cr, 300 mg/kg (Group IV); and Po.Cr, 500 mg/kg (Group V) *versus* the diabetic control group, respectively.

Groups	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	ALT (U/L)
Normal	90.45 ± 1.73	77.83 ± 1.39	22.16 ± 0.87	43.16 ± 1.01	28.83 ± 2.40
Diabetic	254.5 ± 1.67****	197.16 ± 1.09****	92.54 ± 1.79****	19.34 ± 1.75****	92.33 ± 3.34****
Glibenclamide 0.6 mg/kg	116.5 ± 3.74^{aaaa}	124.23 ± 2.25^{aaaa}	39.65 ± 0.73^{aaaa}	40.3 ± 0.76^{aaaa}	38.83 ± 1.83^{aaaa}
Po.Cr 300 mg/kg	163.8 ± 1.88^{bbb}	149.5 ± 1.87^{bbb}	56.6 ± 2.04^{bbb}	29.5 ± 0.51^{bbb}	50.66 ± 1.86^{bb}
Po.Cr 500 mg/kg	127.33 ± 1.31^{cccc}	136.3 ± 1.79^{cccc}	42.21 ± 1.42^{cccc}	37.65 ± 0.88^{cccc}	45.16 ± 2.17^{cccc}

**** $p < 0.001$ and aaaa $p < 0.001$ show respective comparisons of diabetic control (Group II) to normal control (Group I) and glibenclamide (Group III). While, bbb $p < 0.005$ and cccc $p < 0.001$ show comparison of Po.Cr; 300 mg/kg (Group IV) and Po.Cr; 500 mg/kg (Group V) versus diabetic control group respectively.

3.2 Po.Cr improves metabolic fitness of diabetic rats

Apart from alterations in glucose metabolism, DM is often associated with alterations in cholesterol metabolism and hepatic dysfunction (Aleissa et al., 2020). After administration of alloxan (150 mg/kg) to induce diabetes, the rats showed a noticeable increase in levels of total cholesterol (TC), triglycerides (TG), LDL cholesterol, and the liver function biomarker ALT, along with a reduction in HDL cholesterol levels in comparison to untreated controls (Group I). As expected, the standard diabetes drug glibenclamide (0.6 mg/kg) significantly reduced TC, TG, and LDL levels, reduced liver serum markers ($p < 0.001$), and increased HDL levels when compared to the diabetic group that did not receive glibenclamide ($p < 0.05$) (Table 2). Po.Cr significantly reduced harmful cholesterol biomarkers, including TC, TG, and LDL and led to an increase in ALT and HDL levels. Our results in Table 2 demonstrate that Po.Cr treatment can significantly improve the metabolic profile of diabetic rats.

3.3 Po.Cr decreases Alzheimer-like complications of diabetes and improves behavioral outcomes in diabetic rats

3.3.1 Behavioral test for learning and memory

The Morris water maze test was performed to explore the effects of Po.Cr on learning and memory in diabetic rats. Our results indicated that escape latency and distance traveled by normal control rats to reach the hidden platform was reduced, whereas the number of entries into the platform zone (SW zone) increased as they were trained over 4 days of testing. In contrast, diabetic rats exhibited thigmotaxic behavior and reduced capacity for task execution as escape latency and distance traveled to reach the platform increased, along with decreased numbers of entries into the SW zone compared to the control group (Figures 2A–D). The results also indicated that treatment of diabetic rats with Po.Cr (300 or 500 mg/kg) led to a marked increase in performance ($p < 0.001$) as demonstrated by more rapid location of the platform in comparison to diabetic rats that did not receive Po.Cr. The probe day results showed that time spent in the platform zone (Figure 2A) and the number of entries into the platform zone (Figure 2B) were decreased in diabetic rats, and the total distance traveled to reach the platform was increased in comparison to the control group. Rats treated with Po.Cr at either dose, however, presented a significant ($p < 0.001$) increase in number of entries and time spent in the target quadrant and a significant ($p < 0.001$) reduction in distance traveled to reach the target quadrant in comparison to diabetic rats that did not receive Po.Cr. Overall, these data suggest that *Plumeria obtusa* L. can attenuate the learning and cognitive deficits observed in diabetic rats (Figures 2A–D).

3.3.2 Behavioral tests for anxiety

3.3.2.1 Open field test

On day 25 of the experiment, animals were subjected to OFT an hour after administration of Po.Cr at one of the two doses and treatment with diazepam as a standard single dose. A significant difference was detected between groups in the number of entries into the center square (Figure 3A) and time spent in the center square (Figure 3B). There was a higher mean number of entries and greater time spent in corner squares in the diabetic group (Group II) compared to the normal (non-diabetic) group and the glibenclamide and Po.Cr treated groups (Figures 3C, D). After treatment with Po.Cr at either dose, there was a significant increase ($p < 0.001$) in total distance traveled (Figure 3E), number of entries, and time spent in the central zone of the open field apparatus, and a decrease in the number of entries and time spent in the corner zones in comparison to the diabetic control group (Group II) ($p < 0.001$). Outcomes were comparable to that of the standard and shown in Figures 3A–E).

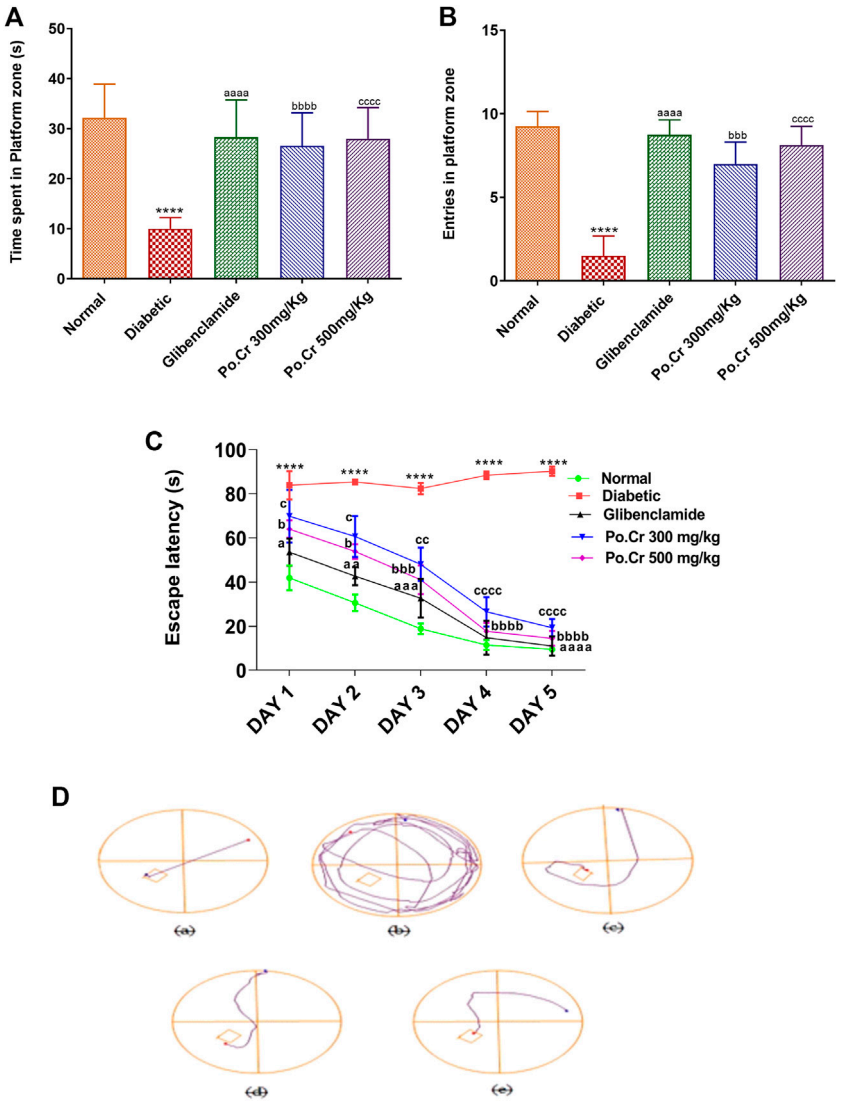

FIGURE 2
Representation of the effects of crude methanolic extract of *Plumeria obtusa* on the performance of diabetic rats in the Morris water maze test. **(A)** Time spent in platform (SW) zone, **(B)** entries into platform (SW) zone, **(C)** escape latency, and **(D)** swim paths taken by rats to the hidden platform in the SW zone. Group II (diabetic control) was compared to Group I (normal control), whereas all the treated groups (Groups III [glibenclamide] to V [Po.Cr]) were compared with Group II (diabetic control). ANOVA (two-way) and the multiple comparison Dunnett's test were applied, and the data values are mean ± SEM. *$p < 0.05$ indicates comparison of diabetic control (Group II) to normal control (Group I), whereas $^{a}p < 0.05$, $^{b}p < 0.05$, and $^{c}p < 0.05$ indicate comparison of glibenclamide (Group III); Po.Cr, 300 mg/kg (Group IV); and Po.Cr, 500 mg/kg (Group V) *versus* the diabetic control group, respectively.

3.3.2.2 Elevated plus maze

Experimental animals treated with Po.Cr at either dose were exposed to the field of elevated-plus maze. Results showed that alloxan-induced diabetic rats demonstrated anxiety-like behavior, including significantly decreased number of entries and time spent in open arms of the apparatus and increased number of entries and time spent in closed arms compared to normal control rats. Moreover, the results indicated that rats treated with Po.Cr at either dose were less anxious, as they made fewer entries and spent shorter periods of time in closed arms of the maze ($p < 0.01$–$p<0.001$) compared to diabetic control rats (Figures 4A–D).

3.3.2.3 Light and dark aversion test

Experimental animals of each group were subjected to the L/D aversion test to further explore anxiety-like behavior. The results revealed that diabetic rats are more anxious compared to normal rats, as demonstrated by reduction in time spent and

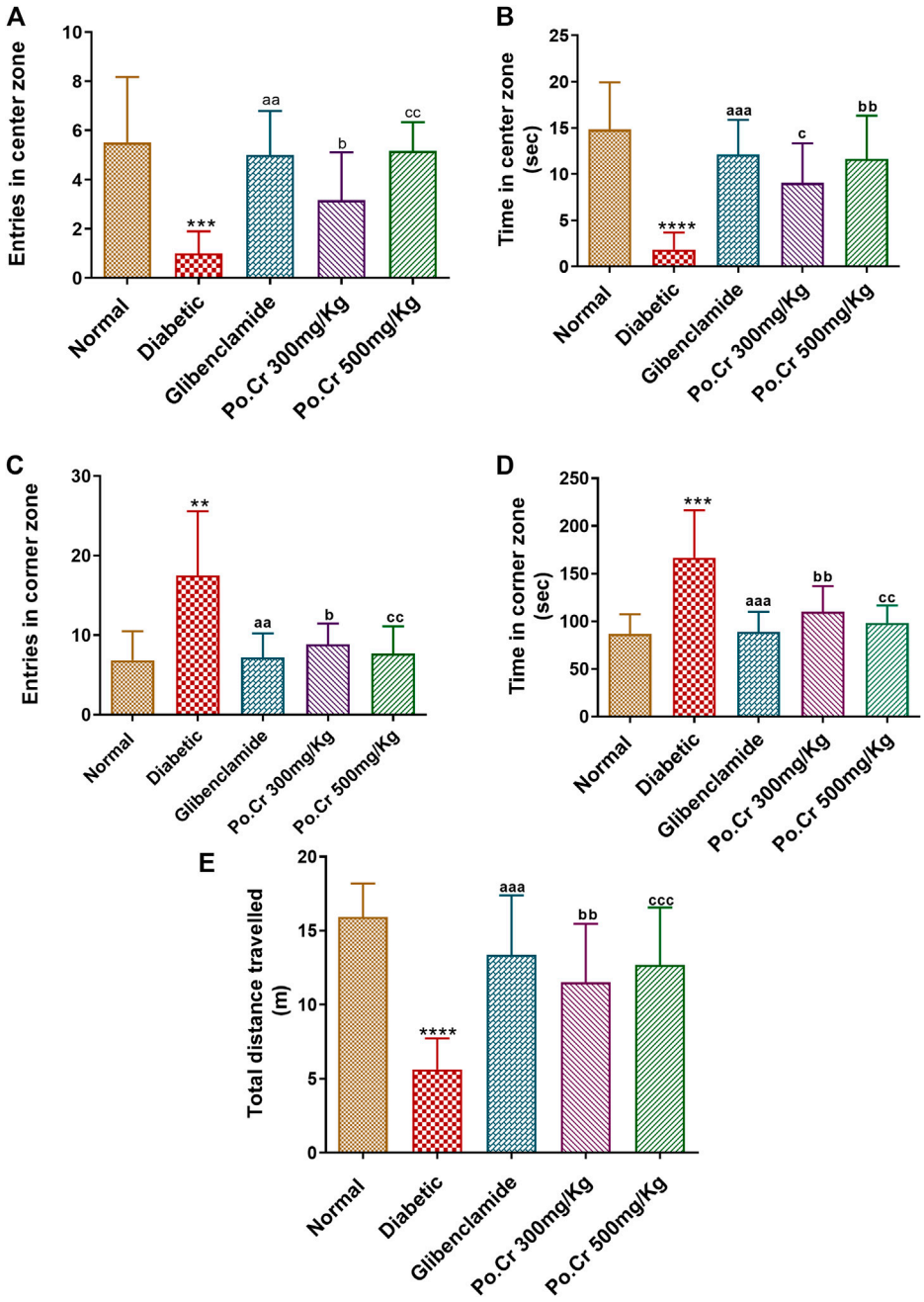

FIGURE 3
Anxiolytic capacity of aqueous extract of *Plumeria obtusa* at doses of 300 and 500 mg/kg assessed using the open field test. **(A)** Entries into center zone. **(B)** Time spent in center zone. **(C)** Entries into corner zone. **(D)** Time spent in corner zone. **(E)** Total distance travelled. Group II (diabetic control) was compared to Group I (normal control), whereas all the treated groups (Groups III [glibenclamide] to V [Po.Cr]) were compared with Group II (diabetic control). ANOVA (two-way) and the multiple comparison Dunnett's test were applied, and the data values are mean ± SEM. *$p < 0.05$ indicates comparison of the diabetic control (Group II) with the normal control (Group I), whereas $^{a}p < 0.05$, $^{b}p < 0.05$, and $^{c}p < 0.05$ indicate comparison of glibenclamide (Group III); Po.Cr, 300 mg/kg (Group IV); and Po.Cr, 500 mg/kg (Group V) *versus* the diabetic control group, respectively.

number of entries into the light chamber and by increased time spent and number of entries into the dark chamber of the L/D box. Furthermore, Po.Cr at either dose decreased anxiety in diabetic rats as demonstrated by significantly increased time spent and number of entries in the light chamber and decreased time spent and number of entries in the dark chamber compared to diabetic rats without Po.Cr treatment ($p < 0.001$), as shown in Figure 5.

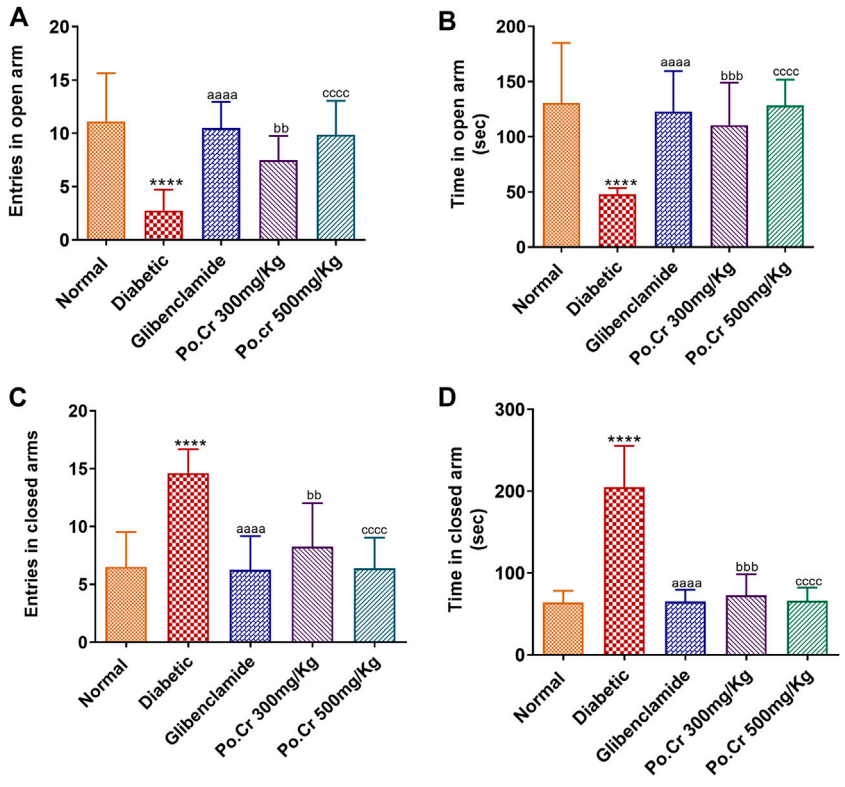

FIGURE 4
Anxiolytic effects of Po.Cr in the elevated plus maze (EPM) test of alloxan-induced diabetic rats. **(A)** Entries into open arm. **(B)** Time spent in open arm. **(C)** Entries into closed arm. **(D)** Time spent in closed arm. Group II (diabetic control) was compared to Group I (normal control), whereas all the treated groups (Groups III [glibenclamide] to V [Po.Cr]) were compared with Group II (diabetic control). ANOVA (two-way) and the multiple comparison Dunnett's test were applied, and the data values are mean ± SEM. $*p < 0.05$ indicates comparison of diabetic control (Group II) to normal control (Group I), whereas $^{a}p < 0.05$, $^{b}p < 0.05$, and $^{c}p < 0.05$ indicates comparison of glibenclamide (Group III); Po.Cr, 300 mg/kg (Group IV); and Po.Cr, 500 mg/kg (Group V) *versus* the diabetic control group, respectively.

3.3.3 Behavioral test for depression

Animals of all experimental groups were forced to swim to allow investigation of the anti-depressant effects of Po.Cr and of fluoxetine as standard treatment. The results indicated that diabetic rats showed increased duration of immobility and a decreased mobility period compared to normal control rats. Treatment with Po.Cr at either dose significantly reduced the immobility period and increased the mobility time compared to diabetic control rats ($p < 0.001$) (Figure 6).

3.4 Po.Cr demonstrates strong antioxidant potential

In DM, there is disturbance of the redox equilibrium and more and more free radicals are generated (Matough et al., 2012). Neurodegenerative effects of ROS are often found to be responsible for Alzheimer disease symptoms. Several experiments were performed to study the impact of Po.Cr on free radical scavenging. Phytochemical assays were carried out to identify active constituents of Po.Cr that have antioxidant potential. The results indicated the TPC and TFC in Po.Cr were 53.11 ± 1.90 gallic acid equivalents (GAE)/mg extract and 38.19 ± 0.98 quercetin equivalents (QE)/mg extract, respectively. HPLC-DAD analysis demonstrated the presence of syringic acid, coumaric acid, emodin, gentisic acid, and caffeic acid in the aqueous-methanolic extract of *Plumeria obtusa*, as shown in Table 3; Figures 7A, B. Po.Cr exhibited excellent antioxidant and free radical scavenging properties (Table 4). Overall, these results indicate the presence of several antioxidant compounds in the methanolic extract of Po.Cr (HPLC analysis) and the antioxidant and anti-diabetic potential of this traditional medicine *via* the improvement of the metabolic profile and neuropsychiatric symptoms in diabetic rats.

4 Discussion

The prevalence of metabolic and neurodegenerative complications is increasing with time in the developed

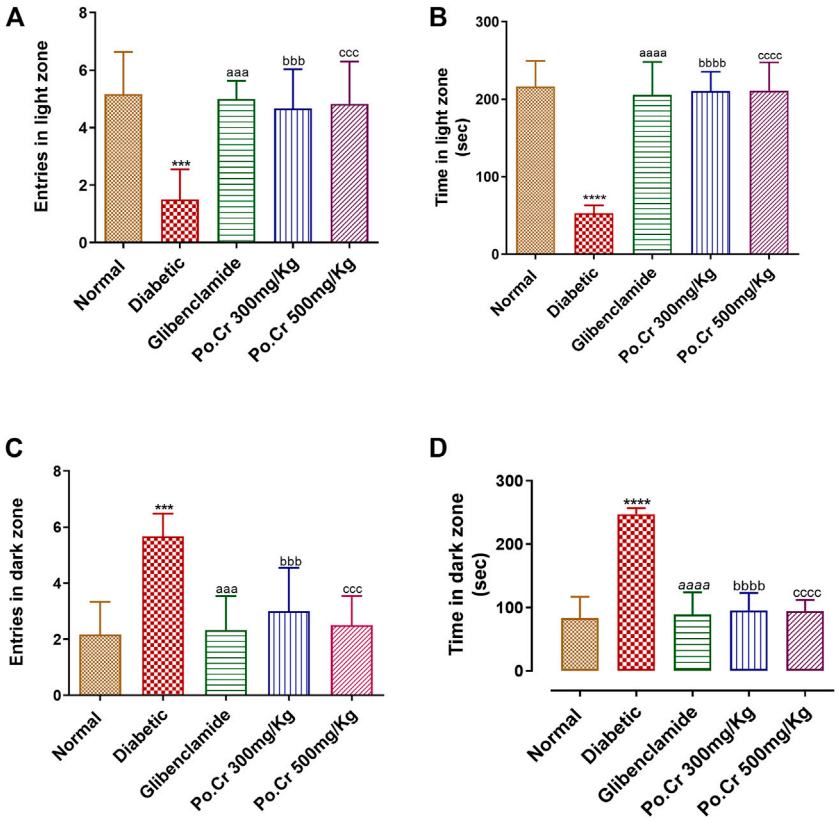

FIGURE 5
Anxiolytic effects of Po.Cr on alloxan-induced diabetic rats in the light and dark (L/D) test. **(A)** Entries into the light zone. **(B)** Time spent in the light zone. **(C)** Entries into the dark zone. **(D)** Time spent in the dark zone. Group II (diabetic control) was compared to Group I (normal control), whereas all the treated groups (Groups III [glibenclamide] to V [Po.Cr]) were compared with Group II (diabetic control). ANOVA (two-way) and the multiple comparison Dunnett's test were applied, and the data values are mean ± SEM. *$p < 0.05$ indicates comparison of the diabetic control (Group II) to the normal control (Group I), whereas $^{a}p < 0.05$, $^{b}p < 0.05$, and $^{c}p < 0.05$ indicates comparison of glibenclamide (Group III); Po.Cr, 300 mg/kg (Group IV); and Po.Cr, 500 mg/kg (Group V) *versus* the diabetic control group, respectively.

world. Diabetes is the most common metabolic disorder affecting the population worldwide and is associated with numerous microvascular and macrovascular complications. Both type-1 and type-2 DM are found to have close association with cognitive dysfunction. Early cognitive deficits in learning and memory and in mental flexibility and speed might be associated with diabetes as depicted in Figure 8 (Sims-Robinson et al., 2010). Various available anti-diabetic medicines were found to exert limited control over the glycemic index and associated cognitive complications, which stimulated researchers to search for novel therapeutics to address this critical health challenge (Mechchate et al., 2021). Natural resources have gained attention among researchers worldwide for use in the development of novel therapeutics due to their attractive safety profile and economic benefits. Our study revealed that crude methanolic extract of *Plumeria obtusa* L. (Po.Cr) significantly attenuates diabetes and associated Alzheimer-like symptoms in an alloxan-induced diabetic rat model. Phytochemical analysis using high-performance liquid chromatography confirmed the presence of flavonoids, phenols, and phenolic acids, including syringic acid, coumaric acid, ferulic acid, caffeic acid, and gentisic acid in *Plumeria obtusa* L extract. Furthermore, antioxidant and free radical scavenging activity of Po.Cr was confirmed, which may be due to the presence of flavonoids, phenols, and phenolic acids.

Single dose alloxan monohydrate resulted in increased blood glucose levels and decreased body weight in rats for about 45 days. These parameters presented effective establishment of diabetes in rats, as similar findings have been reported previously (Yin et al., 2018). Long-term administration of Po.Cr significantly controlled blood glucose level and improved weight compared to diabetic rats that did not receive Po.Cr. The flavonoids in Po.Cr (Table 3) might exert a hypoglycemic effect through stimulation of insulin secretion, as demonstrated in previous studies (Baharvand-Ahmadi et al., 2016). Flavonoids and phenolic compounds are found to have several benefits against many disorders, including diabetes (Sarian et al., 2017; Memariani et al., 2021), by targeting different pathways and

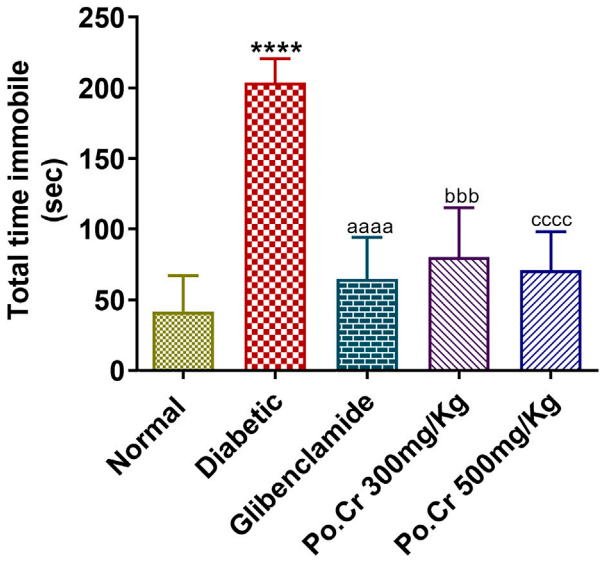

FIGURE 6
Anti-depressant effects of Po.Cr in the forced swim test (FST) of alloxan-induced diabetic rats, showing total time immobile (in sec) for the different groups. Group II (diabetic control) was compared to Group I (normal control), whereas all the treated groups (Groups III [glibenclamide] to V [Po.Cr]) were compared with Group II (diabetic control). ANOVA (two-way) and the multiple comparison Dunnett's test were applied, and the data values are mean ± SEM. *$p < 0.05$ indicates comparison of the diabetic control (Group II) to the normal control (Group I), while $^{a}p < 0.05$, $^{b}p < 0.05$, and $^{c}p < 0.05$ indicates comparison of glibenclamide (Group III); Po.Cr, 300 mg/kg (Group IV); and Po.Cr, 500 mg/kg (Group V) *versus* the diabetic control group, respectively.

TABLE 3 Compounds identified in methanolic extract of *Plumeria obtusa* L. using HPLC-DAD.

Compound	Groups	Quantity
Syringic acid	Phenols	1.14 (μg/mg DW)
Coumaric acid	Hydroxycinnamic acid	0.21 (μg/mg DW)
Emodin	Trihydroxyanthraquinone	0.77 (μg/mg DW)
Gentisic acid	Dihydroxybenzoic acid	1.25 (μg/mg DW)
Caffeic acid	Hydroxycinnamic acid	0.33 (μg/mg DW)
Ferulic acid	Hydroxycinnamic acid	0.62 (μg/mg DW)

The calculated IC_{50} values of different antioxidant activities are given in Table 4.

affecting β-cell proliferation, as well as insulin signaling and secretion (Graf et al., 2005).

The diabetes-like metabolic disorders are associated with dyslipidemia due to elevated ROS and related oxidative stress (Samarghandian et al., 2013). An accumulation of triglycerides and LDL, and reduced HDL levels were found in alloxan-induced diabetic female rats (Júnior et al., 2017), which might be due to reduced utilization of glucose and additional disposal of fats from adipose tissues (Draganescu et al., 2021). Our study showed that long-term administration of Po.Cr reduces the hyperlipidemia associated with diabetes, which suggests the presence of phenols and flavonoids that might enhance insulin release from pancreatic β-cells, as well as decrease LDL oxidation (Fuhrman and Aviram, 2001; Hossain et al., 2011). Furthermore, phenols attenuate oxidative stress and inflammatory mediators (including *Nf*-KB), and reduce the production of eicosanoid derivatives by inhibiting the arachidonic cascade (Feldman et al., 2021; Aleem et al., 2022). Furthermore, previous experiments suggested that alloxan-induced diabetes affects multiple organ systems, including the liver (Lucchesi et al., 2015). Hepato-cellular injury was indicated by increased levels of ALT enzymes in this study, which might have been due to toxic effects of alloxan and/or the diabetic state of the rats (Aleissa et al., 2020). Administration of Po.Cr reduced the ALT levels, which may have been mediated by flavonoids in the extract. Flavonoids have been shown to reduce inflammation and oxidative stress in hepatic cells and modulate pathways of insulin signaling and liver gluconeogenesis (Yin et al., 2018; Kang et al., 2020), and are potential contributors to the hepatoprotective effects observed in Po.Cr-treated diabetic rats.

Both types of diabetes result in increased production of ROS (Matough et al., 2012), which is a contributing factor in diabetic neuropathy. Alloxan induces diabetes through intracellular generation of ROS, with subsequent increases in cytosolic calcium level and thus oxidative pressure through reduction of endogenous anti-oxidation mechanisms (Ceretta et al., 2012b) following the suppression of insulin release and synthesis (Rohilla and Ali, 2012). Some of the anti-diabetic potential of Po.Cr in the alloxan-induced diabetic rat model may be due to the antioxidant potential of phenols and flavonoids contained in Po.Cr extract that combat the oxidative stress, mediated by alloxan, that affects pancreatic β-cells.

The metabolic signaling *via* glucose and insulin are important phenomenon for healthy activity of brain (Sims-Robinson et al., 2010). Therefore, diabetes has been associated with cognitive deficit and psychiatric comorbidities (Raffield et al., 2016). Dementia and cognitive impairment are common complications of DM, and elderly patients with DM are at higher risk of developing Alzheimer disease due to serious neuronal damage (Jiang et al., 2012; Behl et al., 2021). The prospective mechanisms for this incorporate direct impacts of hypo or hyperglycemia and hypo or hyperinsulinemia and indirect impacts include increased intracellular calcium levels, mitochondrial dysfunction, oxidative stress, and neurochemical changes that cause cerebrovascular modification (Sims-Robinson et al., 2010; Li et al., 2019; Xu et al., 2021; Song and Wu, 2022). The Morris water maze (MWM) test is one of the most widely used models for the assessment of memory and learning. The results from the MWM test in our study indicate improved memory in Po.Cr-treated diabetic rats compared to untreated diabetic rats. Phenols

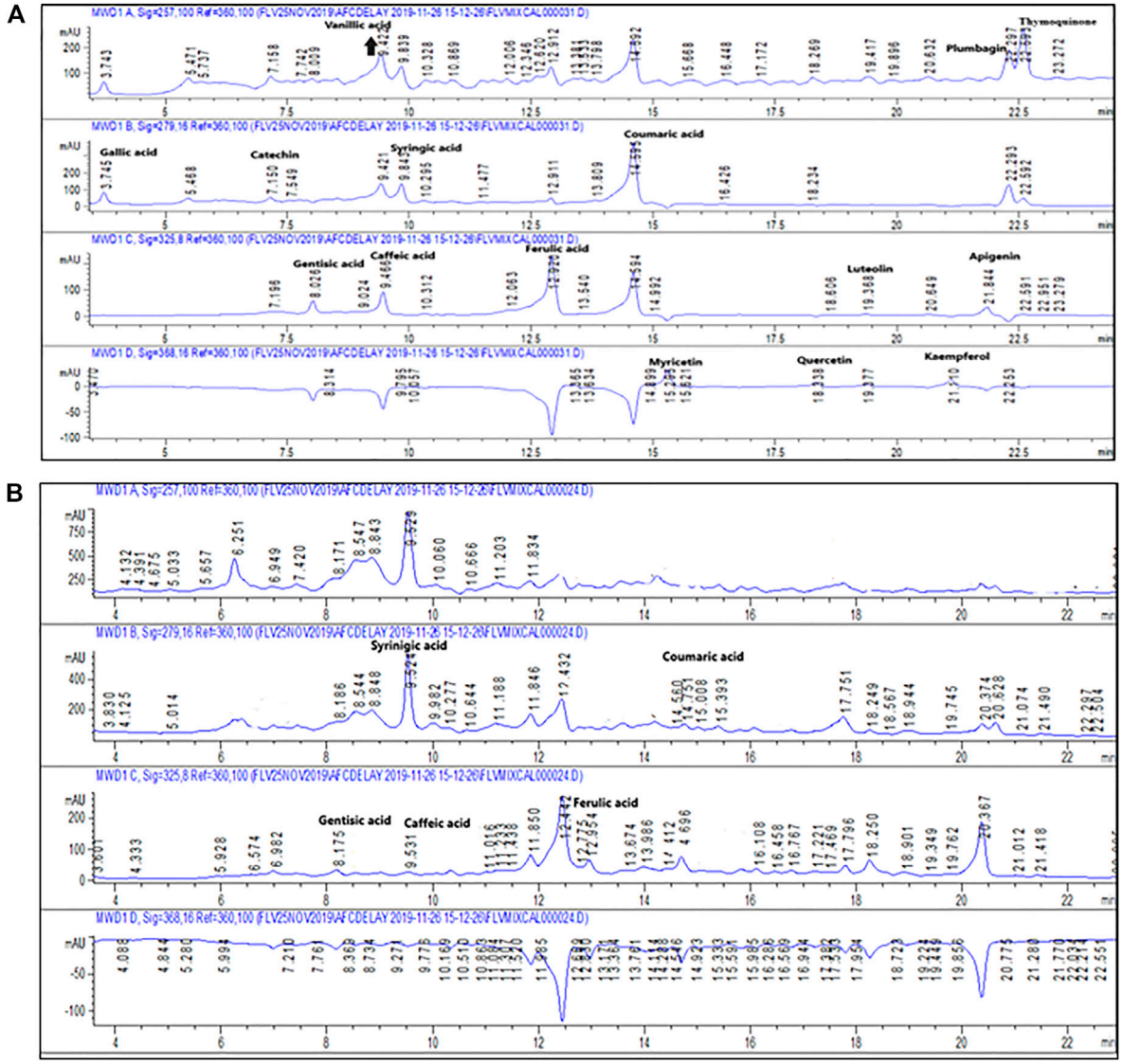

FIGURE 7
(A) HPLC chromatogram of the standard sample. **(B)** HPLC chromatogram of methanolic extract of *Plumeria obtusa* L.

TABLE 4 Assessment of antioxidant markers.

Antioxidant marker	IC_{50}	Unit
DPPH radical-scavenging activity	16.56 ± 1.43	μg/ml
Total antioxidant capacity	226.21 ± 1.57	Ascorbic acid equivalents (AAE)/mg extract
Total reducing power	390.33 ± 1.35	Ascorbic acid equivalents (AAE)/mg extract

and flavonoids have been reported to have neuroprotective effects by controlling neuroinflammation, reducing oxidative stress and neuronal dysfunction, and improving neuronal differentiation in the hippocampus (Vauzour, 2012; Hussain et al., 2018). Thus, the antioxidant capacity of Po.Cr might be due to presence of phenols and flavonoids that regulate the levels of antioxidant enzymes and attenuate neuronal damage in rat brains (Dogra, 2016; Singh et al., 2020; Ul Hassan et al., 2021).

Several previous studies have documented an association between diabetes and psychiatric disorders, such as anxiety

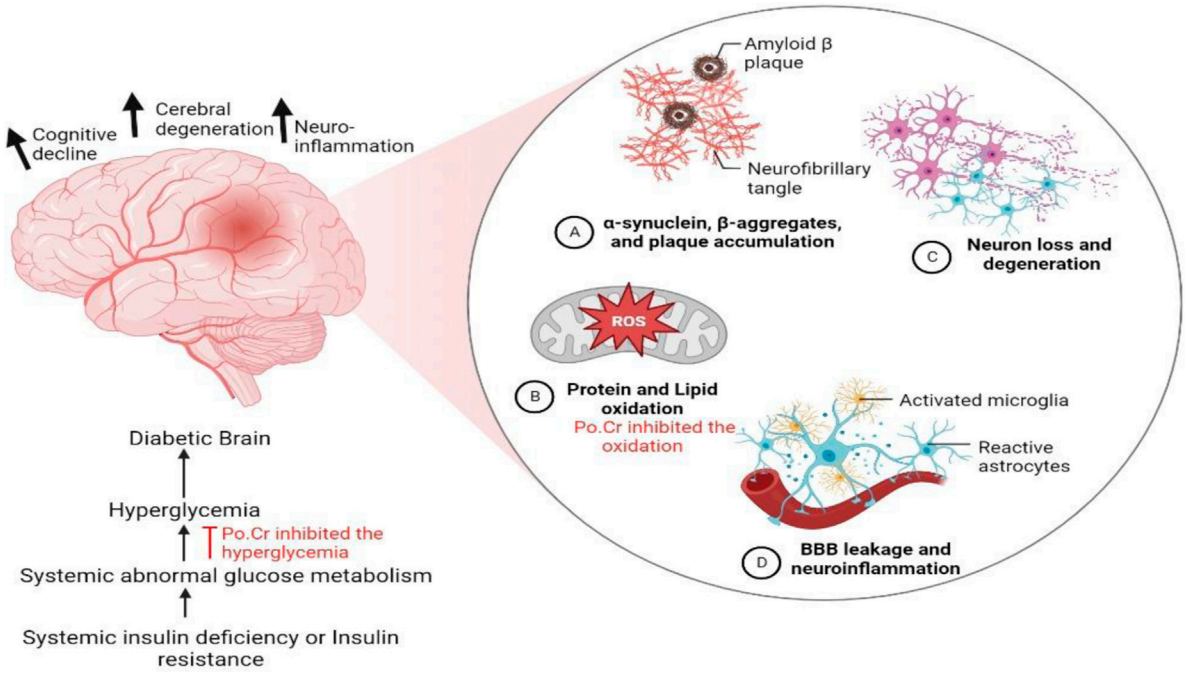

FIGURE 8
Illustration explaining the possible mechanism of development of cognitive impairment and dysfunction associated with diabetes mellitus. The long-term administration of crude extract of *Plumeria obtusa* L. not only reduces blood glucose level but also reduces oxidative stress and therefore prevents diabetes-associated neuroinflammation and cognitive dysfunction.

and depression, that is potentially due to a disturbance in levels and functions of some neurotransmitters, including those that are serotonergic, GABAergic, dopaminergic, or noradrenergic, caused by increased glucose level (Shpakov et al., 2011). Patients with DM are 14.3 times more likely to develop comorbid depression and expresses poor glycemic control and poor adherence to diet and medicine (Andreoulakis et al., 2012). Reagan (2012) reported that similar psychiatric problems were observed in diabetic animal models. In our study, exaggerated symptoms of anxiety-like behavior were noted in diabetic rats as they stayed longer in hidden and darker areas during the experiment and treatment with Po.Cr at either dose significantly attenuated the anxiety compared to untreated diabetic rats. The anxiolytic effects of Po.Cr might be due to the presence of flavonoids, phenols, and terpenoids in Po.Cr extract. It has been indicated in a previous report that flavonoids and phenols found in natural medicinal plants are known to augment $GABA_A$ receptor neurotransmission in the brain and have additional antioxidant properties (Singh et al., 2012; Komaki et al., 2016; Muhasaparur Ganesan et al., 2021). Likewise, the Po.Cr treatment of diabetic rats attenuated depression-like symptoms, resulting in increased mobility in FST compared to untreated diabetic rats. Phenols and polyphenols may attenuate depression by regulating monoamine neurotransmitters in the brain (Li et al., 2020) and the antioxidant potential of flavonoids and phenols may alleviate depressive behavior by protecting the brain from oxidative stress and neuronal damage.

The results of our study suggest that long-term administration of crude methanolic extract of *Plumeria obtusa* L. not only attenuates hyperglycemia in alloxan-induced diabetic rats, but also improves associated metabolic disorders, Alzheimer-like symptoms, and psychiatric disorders, potentially due to the presence of phytochemical constituents with strong antioxidant capacity.

5 Conclusion

The findings of our study revealed the presence of flavonoids and phenolic compounds in crude methanolic extract of *Plumeria obtusa* L. Po.Cr attenuates diabetes, and controls body weight, liver function enzyme levels, and lipid profile parameters in an alloxan-induced diabetic rat model. Moreover, Po.Cr improved diabetes-associated cognitive impairment and psychiatric disorders in diabetic rats, which may be due to its antioxidant capacity and prevention of neuronal damage resulting from oxidative stress. These data demonstrate the importance of further study of the potential of Po.Cr in providing protection against the development of Alzheimer disease in patients with diabetes. This study provides

scientific evidence that supports the traditional uses of this plant, yet further investigation is required to clarify the mechanisms responsible for the beneficial effects of *Plumeria obtusa* L. in the treatment of diabetes.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Ethical committee, Faculty of Pharmacy, Bahauddin Zakariya University, Multan.

Author contributions

Conceptualization; AA and SN. Methodology; AA, SN, and II. Resources; AA and II. Data Analysis; AA, SN, II, JS, and ZZ. Writing-original draft; AA, SN, II, AA, JS, and ZZ. Review and editing; SAHS, MAF, IA, and MIS. Revisions; MAF, IA, JS, AA. Supervision; AA. All authors have contributed to the article and approved the submitted version. All authors have read and agreed to the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ursolic acid and rosmarinic acid ameliorate alterations in hippocampal neurogenesis and social memory induced by amyloid beta in mouse model of Alzheimer's disease

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Alzheimer's disease (AD) is a multifaceted neurodegenerative disorder characterized by substantial neuronal damage which manifests in the form of deficits in memory and cognition. In spite of the debilitating nature of Alzheimer's disease (AD), a dearth of treatment strategies calls for the need to develop therapeutic agents that stimulate neurogenesis and alleviate the associated cognitive deficits. The present study investigates the therapeutic potential of two major phytochemicals, rosmarinic acid (RA) and ursolic acid (UA) in an amyloid beta$_{1-42}$ (Aβ_{1-42})-induced model of AD. UA, a natural pentacyclic triterpenoid and RA, a phenolic ester are major bioactive constituents of *Rosmarinus officinalis*, which is a medicinal herb belonging to family Lamiaceae and exhibiting significant biological properties including neuroprotection. Donepezil, a second generation cholinesterase inhibitor approved for the treatment of mild, moderate and severe Alzheimer's disease (AD) is used as control. Out of eight groups of male BALB/c mice, stereotaxic surgery was performed on four groups (n = 6 each) to introduce Aβ_{1-42} in the hippocampus followed by treatment with vehicle (phosphate-buffered saline (PBS)), donepezil, UA or RA. The other four groups were given vehicle, donepezil, UA and RA only. Behavior analysis for social interaction was performed which constitutes the social affiliation and the social novelty preference test. Presence of Aβ plaques and expression of neurogenesis markers i.e., doublecortin (DCX) and Ki-67 were also assessed. Results revealed the neuroprotective effect of UA and RA observed through substantial reduction in Aβ plaques as compared to the Aβ_{1-42}- and donepezil-treated groups. The neuronal density was also restored as evident *via* DCX and Ki-67 immunoreactivity in Aβ_{1-42} + RA and Aβ_{1-42}+UA-treated groups in comparison to Aβ_{1-42}-treated and Aβ_{1-42}+donepezil-treated groups. The social affiliation was reestablished in the Aβ_{1-42} administered groups treated with UA and RA. Molecular docking studies further validated the comparable binding of UA and RA with Ki-67 and DCX to that of donepezil. Our findings suggest that UA and RA are potential neuroprotective compounds that reverses the histological hallmarks of AD and ameliorate impaired social memory and hippocampal neurogenesis.

KEYWORDS

Alzheimer's disease, neurodegeneration, adult hippocampal neurogenesis, rosmarinic acid, ursolic acid, DCX, Ki-67

Introduction

Alzheimer's disease (AD) is the most common form of dementia accounting for more than 80% of the cases diagnosed. With a prevalence that continues to grow as the world population ages, it has emerged as a leading health problem. The debilitating disorder affects nearly 50 million people worldwide (Crous-Bou et al., 2017). It is characterized by the formation of neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein and amyloid beta (Aβ) plaques which manifests as deficits in cognition and memory (Lopez et al., 2019). Aβ is a major contributing factor in neurotoxicity and neural function and the deposition of amyloid plaques in the hippocampus, cerebral cortex and amygdala can lead to stimulation of astrocytes and microglia, axonal and dendritic damage and synaptic loss which manifest as cognitive impairments (Armstrong, 2009; Chen et al., 2017). The plaque formation constitutes the primary pathological process associated with AD while NFT formation and the subsequent neurodegeneration are downstream processes (Lane et al., 2018).

Adult hippocampal neurogenesis, is a unique phenomenon hosted by the hippocampus, which confers significant levels of plasticity to the hippocampal circuitry improving pattern separation and spatial memory (Anacker et al., 2018). AD leads to a sharp decline in adult hippocampal neurogenesis in comparison to neurologically healthy subjects (Moreno-Jiménez et al., 2019). Impaired neurogenesis is thereby considered as a relevant mechanism that leads to cognitive deficit associated with AD.

Among the various markers of neurogenesis, Doublecortin (DCX) is a brain-specific protein associated with the microtubules which regulates neuronal migration through the polymerization and stabilization of microtubules in migrating neuroblasts (Sadeghi et al., 2018). DCX is vital for the proper initiation and maintenance of differentiation as well as migration during neurogenesis. Neural cells with reduced expression of DCX exhibit impaired migration, differentiation and neurite formation (Shahsavani et al., 2018). Ki-67 another widely acclaimed marker of cell proliferation and neurogenesis is expressed in dividing cells during mitosis except the G0 phase (Sun and Kaufman, 2018).

Currently, only two classes of drugs have been approved for the treatment of AD. Cholinesterase enzyme inhibitors and *N*-methyl d-aspartate (NMDA) antagonists function mainly by treating the symptoms of AD and do not possess preventive or curative effects (Breijyeh and Karaman, 2020). Although a huge amount of research on AD has been directed towards the development of disease-modifying therapy in the last decade, however there is still a dearth of therapeutic agents which will alter the course of disease rather than providing symptomatic treatment alone. Lack of disease modifying drugs even after decades of studies indicates the challenges associated with the development of therapeutic agents with curative potential against AD (Salomone et al., 2012).

In the recent times, natural compounds have garnered significant interest due to their pharmacologically significant activities. These natural products, including herbs and spices, possess various phytochemicals which serve as potential sources of natural antioxidants and neuroprotectants and are devoid of the potentially life-threatening side effects characteristic of the existing approved drugs (Twilley et al., 2020; Karthika et al., 2022). Rosmarinic acid (RA), a phenolic ester is abundantly present in the herbs belonging to the family *Labiatae* and exhibits antioxidant, antimutagenic, antiapoptotic and several other pharmacological activities (Amoah et al., 2016). It also plays a beneficial role against AD through the suppression of Aβ aggregation (Hase et al., 2019). Ursolic acid (UA), a natural pentacyclic triterpenoid also exerts health benefits against inflammation, oxidative stress and fibrosis (Wang X. T. et al., 2018; Xu et al., 2018). Also, RA and UA substantially improve the deficits in cognition as well synaptic dysregulation and the associated neurodegeneration in AD model of $A\beta_{1-42}$ -induced neurotoxicity suggesting their therapeutic significance against AD (Mirza et al., 2021). Furthermore, an *in silico* study also presents the therapeutic potential of these compounds based on drug-likeness, pharmacokinetic properties and binding affinity with AD-associated proteins (Mirza et al., 2022).

In the current investigation we evaluated the neuroprotective effects of RA and UA against impaired neurogenesis and social memory deficits produced by $A\beta_{1-42}$. The effects were assessed in comparison to donepezil, commonly prescribed for AD (Eskandary et al., 2019). It is routinely used as a standard drug in studies investigating the therapeutic potential of different agents against AD (Adlimoghaddam et al., 2018). Moreover computer-aided molecular docking assessment also elucidated the interaction of RA and UA with the markers of neurogenesis. The data obtained for the current study may provide further insights into molecular mechanisms and clinical intervention options for AD and its associated consequences.

Materials and methods

Experimental animals

Male BALB/c mice were maintained in the animal facility of Atta-ur-Rahman School of Applied Biosciences (ASAB) National

University of Sciences and Technology (NUST). Mice were raised under 12 h natural light-dark cycles and had free access to food and water *ad libitum*. All animal experiments were conducted in conformity with the standards of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institutes of Health, United States. The internal review board of ASAB, NUST approved the study design (IRB # 64).

Animal model induction

BALB/c mice (male, age 10–12 weeks; 35–45 g) were used for experimentation. A previously described method was used to establish the animal model (Mirza et al., 2021). The mice were administered 50 mg/ml ketamine (80 µL) and 5 mg/ml Diazepam (60 µL) per 40 g of animal weight prior to microinjection of $A\beta_{1-42}$ (ab120301, Abcam, Cambridge, MA, United States; dilution 1 µg per µL) into the hippocampus (CA1 region) from bregma: anteroposterior, 2.3 mm; mediolateral, 1.8 mm; dorsoventral, 2.0 mm, using a stereotaxic apparatus using a Hamilton syringe. Similarly, the control mice were administered phosphate-buffered saline (PBS). The mice were kept in individual cages until further use.

Animal grouping

The male BALB/c mice were segregated into eight groups (n = 6 each). The groups 1–4 were pre-treated with $A\beta_{1-42}$. The groups 2, 3 & 4 received donepezil (15 mg/kg) (Ahmed et al., 2017), RA (16 mg/kg; ab141450, Abcam, United Kingdom) (Farr et al., 2016), or UA (40 mg/kg; ab141113, Abcam, United Kingdom) (Liang et al., 2016), respectively for 15 days post $A\beta_{1-42}$ administration. Group 5 received vehicle (PBS) while groups 6, 7, 8 received the similar dose of donepezil, RA and UA, respectively for similar duration. All the groups were orally administered with normal feed and water. The purity of UA and RA was >99% and >95% respectively while donepezil was taken as a positive control. After the end of treatment period the mice were subjected to behavioral tests and subsequently brain tissue harvesting.

Social interaction behavior

The procedure was performed as reported previously (Rizwan et al., 2016). The apparatus comprised of a glass box having dimensions 40 × 40 × 40 cm with two similar cages placed inside the box. The mice placed in the cages were characterized as mouse A and B while the treated mice were referred to as test subject. Mice A and B were never encountered before with the test subject and were of the same background in terms of age, weight and gender.

Session I: Social affiliation test

After habituating the test subjects (5 min) inside the box, they were introduced back into the box with a cage having mouse A on one side and an empty cage on the other side. The test subject was then left in the box for 10 min and allowed to move in both the cages. The discrimination index (DI) or the interaction time or with the empty cage and mouse A was evaluated and the difference between the time spent interacting with the mouse A or empty cage and the total time was scored.

Session II: Social novelty preference test

The mouse B was released in the empty cage while the mouse A remained unaltered. The test subject was again allowed an exploration time of 10 min. DI or interaction time was measured as the difference between the time spent interacting with mouse A or mouse B and the total time spent with mouse B.

Histology and immunohistochemistry

Histology was performed according to the protocol used by Amber et al., 2020. The tissue was dewaxed and rehydrated in ethanol and double distilled water (ddH_2O). Congo red stain (1%) was applied to the tissue for 25 min after which it was thoroughly rinsed with ddH_2O, followed by counter Haematoxylin and eosin staining. The sections were rinsed again with ddH_2O, dried and incubated for 20–25 min before clearing in xylene solution.

Immunostaining was performed in accordance with the protocol previously described by Mirza et al., 2021. Hippocampal tissue sections (5 µm) were deparaffinized after antigen retrieval. H_2O_2 (1%) was applied to quench the peroxidase activity and blocked with BSA (5%) in PBS for 1 h. After an overnight incubation with primary antibodies at 4°C the sections were incubated for 1 h with a secondary antibody followed by washing thrice with TBST and visualised with 0.025% 3, 3′ diaminobenzidine (DAB Kit, ab50185, Abcam, MA, United States). Ki-67 (Leica Biosystems, PA0230) and DCX (Abcam, ab207175) diluted in the block solution 1:200 and 1:100 respectively, were used as primary antibodies while anti-rabbit IgG-HRP conjugated (ab97051, Abcam, MA, United States) diluted in block solution 1: 100 was used as secondary antibody. The images were visualized using B-150, OPTIKA microscope (Italy) at 4× and 10× resolution. The images were captured using Optika Vision Lite 2.1 image analysis software. Quantitative analysis was performed for cell count in an area of 10,000 µm^2 from three randomly selected areas and the average values were calculated and plotted.

Molecular docking

PatchDock server was used for docking using cluster RMSD at default value of 4.0 to identify the interaction among RA and UA with target proteins DCX and Ki-67. The interaction was compared with donepezil. Patchdock algorithm produces potential complexes based on the criteria of shape complementarity (Schneidman-Duhovny et al., 2005). The 3D structures of the target proteins Ki-67 and DCX were acquired from RCSB Protein data bank (PDB) (https://www.rcsb.org/). The PDB IDs of Ki-67 and DCX were 5J28 and 2BQQ, respectively. The 3D structures of RA, UA and donepezil were taken from PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The automated docking models generated were further assessed using FireDock (Mashiach et al., 2008) and visualized through BIOVIA Discovery Studio (Systèmes, 2016).

Statistical analysis

Data are presented as mean ± SEM and statistical significance was set at 95% confidence level and a *p*-value <.05. Behavioural and biochemical data was analysed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons as *post hoc test* using GraphPad Prism 5.0.

Results

Rosmarinic and ursolic acid improve social affiliation and social novelty preference in $A\beta_{1-42}$ treated mice

To evaluate the effects of RA and on sociability in mice, social interaction test was performed. $A\beta_{1-42}$ treated group (2.798 ± 0.7145; $p < 0.0001$) exhibited a significant reduction in interaction with mouse A, a conspecific placed in one of the cages, in comparison to the control (22.66 ± 1.726; $p < 0.0001$) and other experimental groups exhibiting a significant decrease in social affiliation. The $A\beta_{1-42}$ + RA (20.25 ± 3.779; $p < 0.001$) and $A\beta_{1-42}$+ UA-treated groups (23.74 ± 3.434; $p = 0.0003$) performed significantly better than the diseased group and comparable to the $A\beta_{1-42}$+donepezil-treated group (22.91 ± 5.629; $p = 0.0051$). $A\beta_{1-42}$+ UA-treated group exhibited improved social affiliation in comparison to all of the other groups (Figure 1).

Interactions with the empty cage also show least amount of interaction by the $A\beta_{1-42}$ -treated group. The decrease social affiliation pattern observed during session I for $A\beta_{1-42}$ -treated group is inferred from the same length of time spent with the mouse A (2.798 ± 0.7145; $p < 0.0001$) as well as empty cage (2.276 ± 0.7505; $p = .0664$). Conversely, the control and experimental groups interacted more with the mouse A in comparison to the empty cage (Figure 1).

The $A\beta_{1-42}$-treated group (0.5600 ± 0.01703; $p < 0.0001$) exhibited significantly low DI in comparison to the control (0.8117 ± 0.02400; $p < 0.0001$) and the treated groups. A significant improvement in DI was observed in $A\beta_{1-42}$ + RA (0.8300 ± 0.02887; $p < 0.0001$) and $A\beta_{1-42+}$ UA-treated (0.8220 ± 0.03541; $p = .0002$) groups depicting restoration of social affiliation (Figure 1).

In the session II, the mice were assessed for novelty preference and social memory. The mice treated with $A\beta_{1-42}$ showed diminished levels (6.572 ± 2.116; $p = .0144$) of interaction with an unfamiliar mouse B in comparison to the control group (21.38 ± 4.261). The treated groups did not show preference with any mouse cage.

The DI for session II revealed a significantly low value for the $A\beta_{1-42}$ -treated group (0.3820 ± 0.02107; $p < 0.0001$) relative to the control (0.7880 ± 0.05054; $p < 0.0001$) and the other experimental groups, thus indicating alterations in social memory. A significant improvement in DI was evident in $A\beta_{1-42}$ + RA (0.7300 ± 0.06258 $p = .0007$) and $A\beta_{1-42}$+ UA-treated (0.6867 ± 0.03783 $p < 0.0001$) groups in comparison to the control. The DI in RA- and UA-treated groups was comparable to that of the standard drug donepezil (0.6850 ± 0.01893; $p < 0.0001$) demonstrating their protective effects against social memory deficit in AD (Figure 2).

Improvement in neurogenesis by RA and UA

A considerable reduction in neuronal proliferation was significantly apparent in the group treated with $A\beta_{1-42}$ relative to the control mice. An improvement in the density of Ki-67-positive neurons was observed with treatment with RA (22.87 ± 0.4667; p= .0111) and UA (25.33 ± 0.8819; p = 0.0027) post $A\beta_{1-42}$ administration as compared to the mice treated $A\beta_{1-42}$ only (19.50 ± 0.6455). It was found that RA and UA have greater improvement activity than donepezil (21.30 ± 1.825; p = 0.34), evident through the restoration of the $A\beta_{1-42}$ deteriorated neurons. No obvious neuronal loss was encountered by the control mice (27.65 ± 1.525), donepezil, RA and UA in $A\beta_{1-42}$ untreated groups (Figure 3).

A notable decrease in the hippocampal proliferating cells of group treated with $A\beta_{1-42}$ (14.30 ± 0.4813; p = 0.0003) relative to control (29.33 ± 1.856) indicated the intensity of neurotoxic effects of Aβ. RA (24.53 ± 1.619; p= 0.0009) and UA (28.13 ± 2.551; p= 0.0015) treatment post $A\beta_{1-42}$ administration showed improved immunoreactivity with DCX with increase DCX positive cells relative to control and donepezil-treated group (16.07 ± 0.7881; p= .0984) (Figure 4).

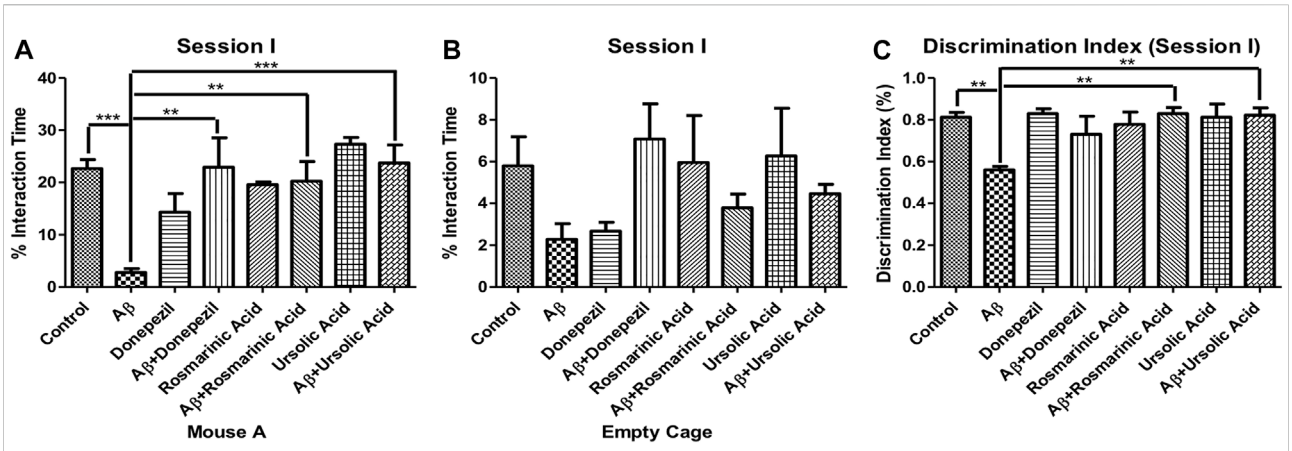

FIGURE 1
Effect of RA and UA on Social Affiliation behavior. **(A)** Interaction time with the Mouse A. **(B)** Interaction time with the empty cage. **(C)** Discrimination index during session (I). Significance was analyzed by one-way ANOVA followed by Bonferroni comparison test (mean ± SEM) using Graphpad Prism. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

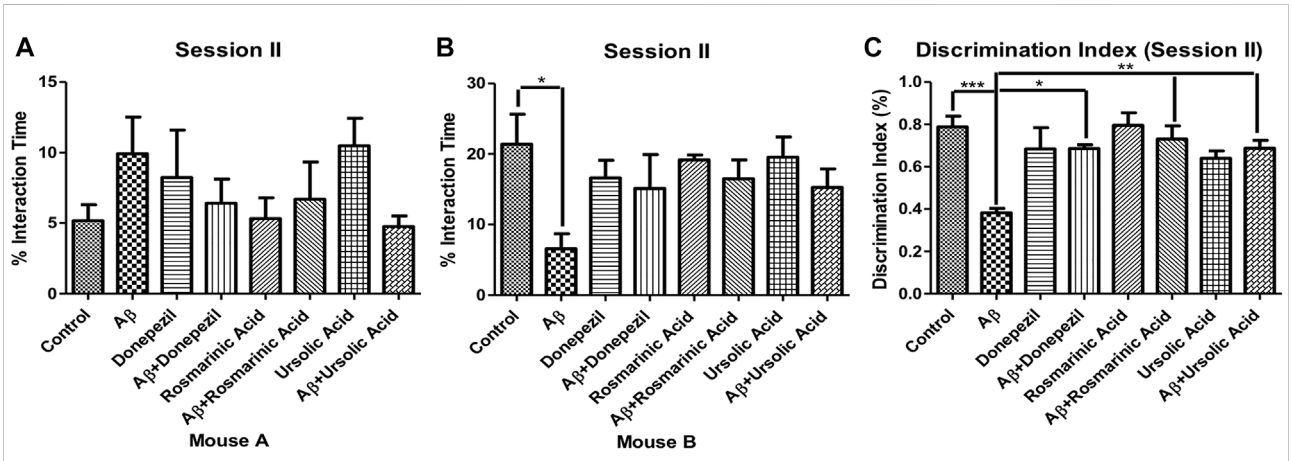

FIGURE 2
Effect of RA and UA on social novelty preference behavior. **(A)** Interaction time with the Mouse A. **(B)** Interaction time with the Mouse B. **(C)** Discrimination index during session II. Significance was analyzed by one-way ANOVA followed by Bonferroni comparison test (mean ± SEM) using Graphpad Prism. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

RA and UA treatment reduces the accumulated amyloid beta burden

The presence of congophilic amyloid plaques in the mice administered with $A\beta_{1-42}$ was substantially decreased with the treatment of RA and UA indicating a reversal of the plaque formation. RA and UA treatment significantly rescued the cellular density and morphology in $A\beta_{1-42}$ -treated groups and comparatively have greater neuronal restoration than donepezil. The control littermates showed a normal neuronal pattern (Figure 5).

Molecular docking studies of Ki-67 and DCX with RA, UA and donepezil

Molecular docking studies were used to predict the receptor-ligand interaction geometrics of RA, UA and donepezil with the neurogenesis markers Ki-67 and DCX. All the compounds successfully docked against the target proteins. Lowest atomic contact energy (ACE) is depictive of higher binding affinity, therefore, the ligand molecules that had the lowest ACE were considered better ligands to Ki-67 and DCX. The docking scores of the complexes are shown in Table 1. The docking results were

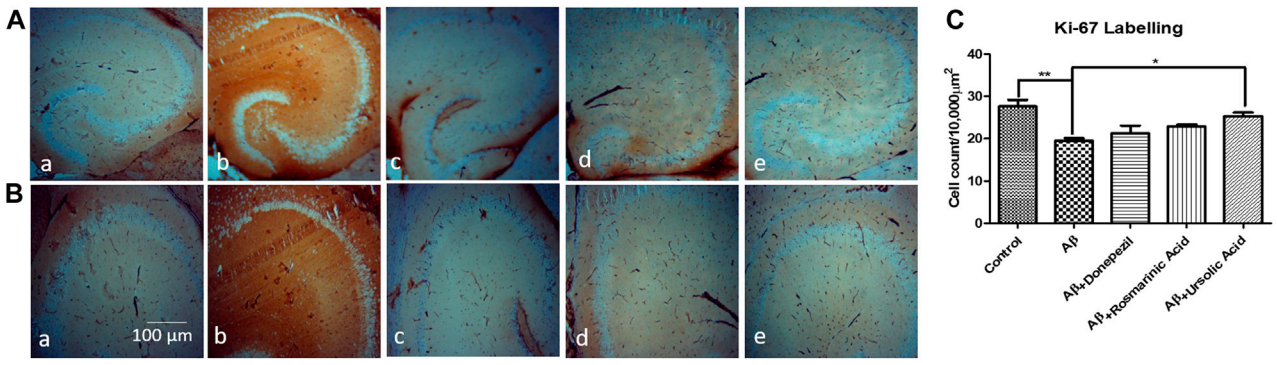

FIGURE 3
Neuron specific Ki-67 Labelling of mice hippocampal tissue: (a) Control (b) $A\beta_{1-42}$ -treated group (c) $A\beta_{1-42}$ + donepezil-treated group (d) $A\beta_{1-42}$ +RA-treated group (e) $A\beta_{1-42}$ + UA-treated groups at magnification **(A)** 4X **(B)** 10X. **(C)** Graph showing cell count/10000 μm^2 of Ki-67 labelled cells in mice hippocampus in experimental groups respectively. Significance was analyzed by One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) using Graphpad Prism. *$p < 0.05$ **$p < 0.01$.

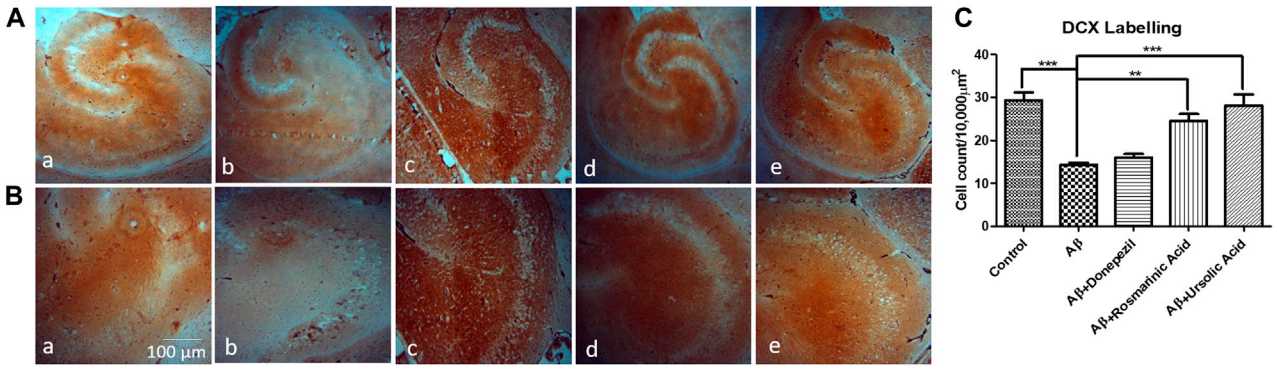

FIGURE 4
Neuron-specific DCX Labeling of mice hippocampal tissue: (a) Control (b) $A\beta_{1-42}$ -treated group (c) $A\beta_{1-42}$ + donepezil-treated group (d) $A\beta_{1-42}$ + RA-treated group (e) $A\beta_{1-42}$ +UA-treated groups at magnification **(A)** 4X **(B)** 10X **(C)** Graph showing cell count/10000 μm^2 of DCX labelled cells in mice hippocampus in experimental groups respectively. Significance was analyzed by One-way ANOVA followed by Bonferroni comparison test (mean ± SEM) using GraphPad Prism. **$p < 0.01$, ***$p < 0.001$.

further refined using FireDock and the results obtained are stated in Table 2.

UA exhibits ACE comparable to donepezil in binding interactions with Ki-67 and DCX

Binding interaction of UA, RA, and donepezil with Ki-67 revealed that amongst the other compounds, UA with an ACE of −290.71 had better affinity than RA (−203.58) which was comparable to that of donepezil (−293.37). Interactions with DCX also demonstrated a comparable binding energy of UA (−154.2) and donepezil (−157.92). These binding energy values suggest an interaction of UA with Ki-67 and DCX validating the present *in vivo* results (Table 1).

UA shows global energy comparable to donepezil in binding interactions with Ki-67 and DCX

Global energy depicts the binding energy of the complex while the ACE is based on its contribution to global energy. Low values of ACE correspond to more stable complexes. UA

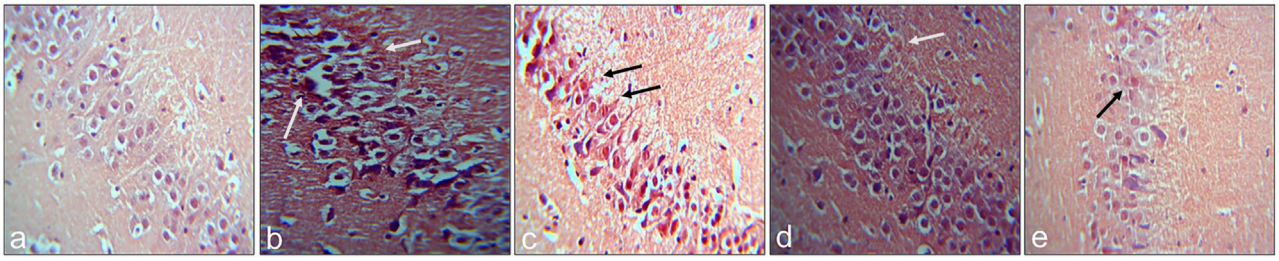

FIGURE 5
Congo red-stained sections of hippocampus for visualization of amyloid plaques: **(A)** Control **(B)** $A\beta_{1-42}$ -treated group **(C)** $A\beta_{1-42}$ + donepezil-treated group **(D)** $A\beta_{1-42}$ + RA-treated group **(E)** $A\beta_{1-42}$ + UA-treated group. Original Magnification 40X.

TABLE 1 Protein docking scores evaluated by Patchdock.

Receptor	Ligand	Geometric shape complementarity score	Approximate interface area size of the complex (Å^2)	Atomic contact energy (Kcal/mol)
Ki-67	Rosmarinic Acid	3652	420.90	−203.58
Ki-67	Ursolic Acid	3796	525.10	−290.71
Ki-67	Donepezil	4426	524.00	−293.37
DCX	Rosmarinic Acid	4000	467.20	−84.83
DCX	Ursolic Acid	4172	493.00	−154.20
DCX	Donepezil	4546	569.40	−157.92

TABLE 2 Protein docking scores refined by Firedock.

Receptor	Ligand	Global energy (kcal/mol)	Attractive vdw	Repulsive vdw	Atomic contact energy (Kcal/mol)
Ki-67	Rosmarinic Acid	−27.62	−15.86	4.81	−8.74
Ki-67	Ursolic Acid	−32.58	−14.64	7.10	−10.96
Ki-67	Donepezil	−32.99	−14.38	3.84	−10.92
DCX	Rosmarinic Acid	−25.10	−19.45	3.45	−2.38
DCX	Ursolic Acid	−31.01	−18.90	8.53	−10.06
DCX	Donepezil	−39.49	−21.83	9.16	−11.84

Vdw, Van der waal's forces.

exhibited an ACE of −10.96 in its interaction with Ki-67 which is comparable to that of donepezil (−10.92). UA (−10.06) also showed a comparable ACE to that of donepezil (−11.84) in its interaction with DCX. Attractive and repulsive vdw (Van der waal forces) are a quantification of the input of van der waal's forces to the global binding energy. The values are tabulated in Table 2 and the interacting residues are shown in Figure 6.

Discussion

Our study elucidated the potential effects of the bioactive compounds of *R. officinalis*, RA and UA on $A\beta_{1-42}$ -induced neurotoxicity in comparison to donepezil. The data revealed the restoration capability of RA and UA of altered social memory in AD mouse models. Social engagement with the surrounding environment is associated with an improved

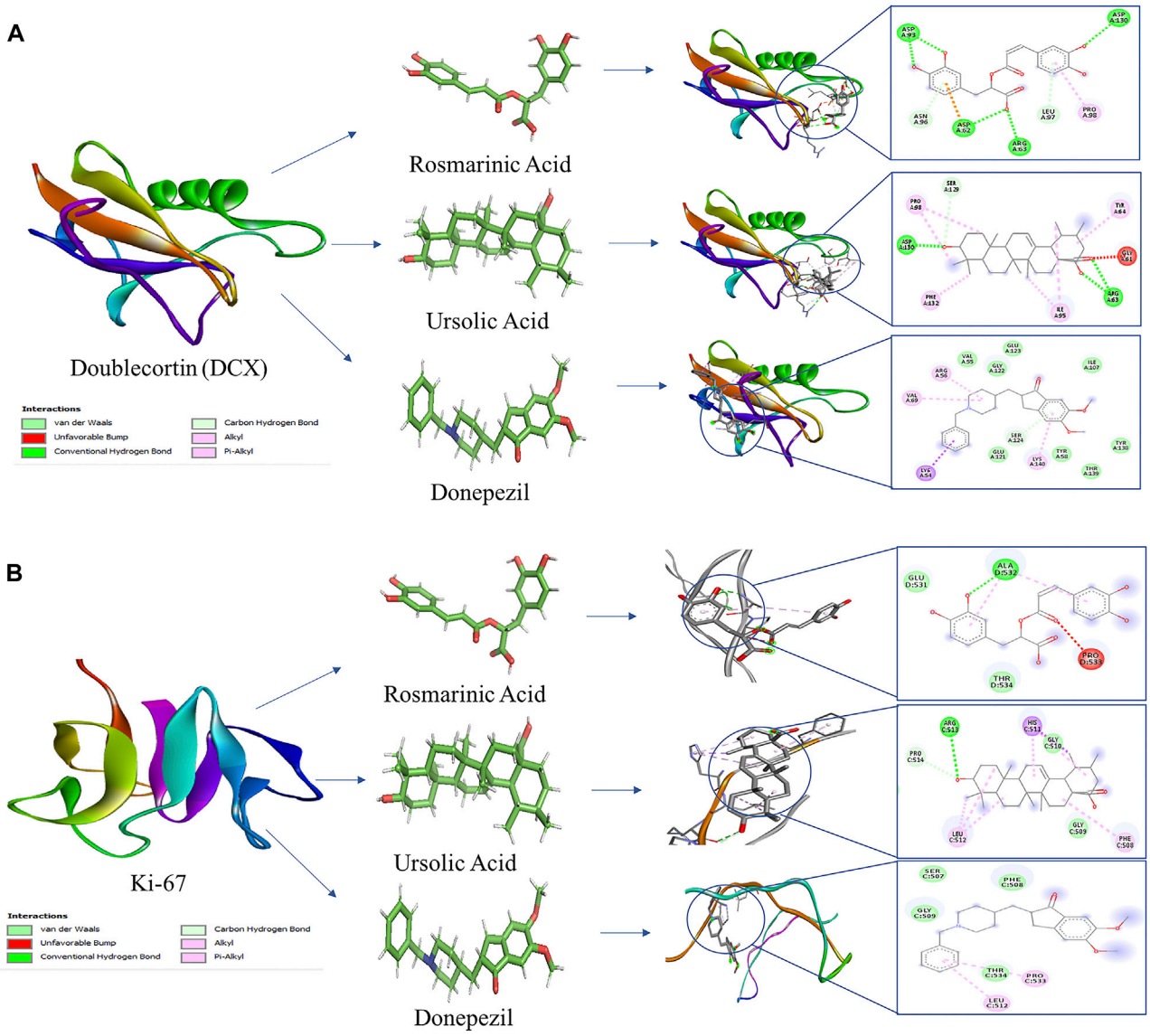

FIGURE 6
Molecular docking interaction models of RA, UA, and donepezil with DCX and Ki-67. 2D structures of the compounds are shown by line and stick models with the surrounding amino acids of **(A)** DCX and **(B)** Ki-67. The interactions are denoted by the following colors: hydrogen bonding interactions (green), alkyl bonds (pink) and bumps (red).

angiogenesis, synaptogenesis, and neurogenesis which are crucial in delaying the progression of AD (Fratiglioni et al., 2004).

Notably, RA and UA are previously known to exert anxiolytic and antidepressant effects in various models of neurotoxicity (Colla et al., 2015; Ramos-Hryb et al., 2019; Lataliza et al., 2021). The present study showed that RA and UA treatment post $A\beta_{1-42}$ administration exhibited significantly greater sociability levels relative to the diseased group. RA and UA treatment post $A\beta_{1-42}$ administration also displayed significant improvement in social novelty preference in mice. The social interaction behavior of mice with RA and UA treatment was comparable to that of the standard drug donepezil which is indicative of their comparable protective effects to restore social memory. These data suggest the potential of RA and UA in alleviating the behavioral deficits in social affiliation and novelty preference induced by $A\beta_{1-42}$.

Altered social behavior is demonstrated by a decrease in sociability, avoidance of novel social stimuli and exacerbation

of aggression associated with amyloid pathology (Kosel et al., 2021). Impaired social interaction memory also results due to mitochondrial damage contributed to increased oxidative stress and deficits in hippocampus and medial pre-frontal cortex activity (Misrani et al., 2021). According to Okada et al., the removal of cholinergic cell groups in the basal forebrain cause an impairment of social behavior which was significantly reinstated by cholinesterase inhibitors, suggesting the critical role of cholinergic dysfunction in sociability deficits corelated with psychological and behavioral symptoms of dementia in AD (Okada et al., 2021). Interestingly, modulation of matrix metallopeptidase 9 (MMP9) caused an improvement in sociability and social recognition memory, along with a reduction in anxiety in AD mouse model, supporting the notion that targeting MMP9 could serve as a therapeutic strategy in restoring the neurobehavioral damage in AD (Ringland et al., 2021).

Although the effect of RA and UA on social memory has not been reported previously but their effect on the restoration of spatial memory and object recognition memory has been documented. A mechanistic study on RA describes its role in the inhibition of cognitive decline through the suppression of tau phosphorylation (Yamamoto et al., 2021) while UA ameliorates oxidative stress and inflammation to improve cognitive deficits in an Aβ-induced mouse model (Liang et al., 2016). UA also exerts radioprotective effects improving radiation induced deficits in memory and learning in BALB/c mice (Tang et al., 2017) whereas also exhibit a potent anti-dementia effect observed in olfactory bulbectomized mice (Nguyen et al., 2022). Improvement of spatial memory and amelioration of $A\beta_{25-35}$ accumulation by UA has also been demonstrated (Li et al., 2020).

We further studied the effects of RA and UA on altered hippocampal neurogenesis induced by $A\beta_{1-42}$. Several studies indicate the relevance of $A\beta_{1-42}$ induction in the impairment of adult hippocampal neurogenesis eliciting neurodegeneration associated loss of memory and cognition. The intra-cerebrovascular injection of $A\beta_{1-42}$ in male kunming mice caused mitochondrial damage, inflammation, loss of memory (Qi et al., 2019) and altered adult hippocampal neurogenesis observed in balb/c mice (Amber et al., 2020). In addition, the interneuronal accumulation of phosphorylated tau protein is also crucial for AD progression and impairs adult hippocampal neurogenesis through the suppression of GABAergic transmission (Zheng et al., 2020). Contrarily, promotion of neurogenesis reconstructs the degenerated neural circuits in AD hindering the associated cognitive decline (Choi et al., 2018). $A\beta_{1-42}$ -induced neurotoxicity causes significant deterioration of Ki-67 and DCX expression levels in the hippocampal tissue (Amber et al., 2020). Our results indicated a reduction in the neuronal proliferation induced by $A\beta_{1-42}$ evident through a significant decline in the immunoreactivity of DCX and Ki-67 positive cells in the mice treated with $A\beta_{1-42}$, however it was considerably restored upon treatment with UA and RA.

The results of behavioral and immunohistochemical analysis indicated the neurogenic potential of UA and RA. Based on these results, *in silico* analysis was further conducted to determine the binding interactions of the compounds with the neurogenesis markers, Ki-67 and DCX in comparison to donepezil. Molecular docking analysis showed comparable binding energy values of UA to that of donepezil. UA had an ACE value comparable to that of donepezil in its interaction with Ki-67 and DCX. Previously, we reported the effect of UA and RA in normalizing the mRNA expression levels of neurogenesis markers, Ki-67, DCX and NeuN. Interestingly, UA exhibit significant restoration of the expression levels of these markers in comparison to RA and donepezil (Mirza et al., 2021). It also enhanced neurogenesis and repressed inflammation in temporal lobe epilepsy and cerebral ischemia models (Wang Y. et al., 2018; Liu et al., 2022). Its role in neurite outgrowth and neuronal survival mediated by nerve growth factor has also been reported (Theis et al., 2018). These results reiterate the neurogenic potential of UA through interaction with Ki-67 and DCX indicating its therapeutic potential against neurodegeneration associated with AD.

The reduction in the plaque formation by RA and UA post $A\beta_{1-42}$ administration also suggests their role in the suppression of AD progression. RA has been previously found to be effective against copper (II)-induced neurotoxicity through the formation of an original ternary association between Aβ and Cu (II) (Kola et al., 2020). Also, the prevention of fibrillization and assembly of β sheets in tau protein suggest its therapeutic potential against AD (Cornejo et al., 2017). Interestingly, RA also reduces the formation of Aβ and ameliorated tissue structure in an AD-like dementia model induced by scopolamine (Deveci et al., 2021). It also exerts anti-apoptotic effect that results in the alleviation of inflammation and oxidative stress associated neurodegeneration as observed in a model for Parkinson's disease (Lv et al., 2020). Consistently UA also hindered the deposition of Aβ and lowered the levels of its oligomers and monomers in an Aβ-induced *Caenorhabditis elegans* transgenic model (Wang et al., 2022). These results are indicative of the potential of UA and RA in the reduction of Aβ plaques which constitute a hallmark feature of AD.

Conclusion

This study suggests the pro-neurogenic potential and neuroprotective effects of UA and RA on neurotoxicity

induced by $A\beta_{1-42}$ that represents pathological hallmarks of AD. Our findings revealed that UA and RA can rescue the AD like alterations characterized by accumulated amyloid plaques, impaired social memory and neurogenesis induced by Aβ, thereby reiterating their potential as promising therapeutic agents against AD.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Internal Review Board, Atta ur Rahman School of Applied Biosciences-NUST.

Author contributions

SZ, substantial contribution to conception and design of the study and finalization of the manuscript; FM, all experimental work, data analysis, interpretation, and drafting the article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A mechanistic review of pharmacological activities of homeopathic medicine licorice against neural diseases

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The use of medicinal plants has grown in popularity in recent decades because, as natural ingredients, they have fewer adverse effects and are more effective than synthetic alternatives. As a small perennial herb, *Glycyrrhiza glabra* L. (Licorice) has been investigated for its therapeutic efficacy against neural disorders mainly ischemic stroke as well as the neurodegenerative diseases such as dementia and Alzheimer's disease, and Parkinson's disease which has been attributed to its HMGB inhibitory function, reactive oxygen scavenging and anti-inflammatory activity. The objective of current review is to review the evidence for the pharmacological effects of licorice and its vital active components on neurological disorders and the underlying signaling networks. We reviewed Papers published from 2000.1.1 up to 2 January 2023 in web of science, Google Scholar and PubMed data bases using key words including "Licorice," "*Glycyrrhiza glabra* L.," "Glycyrrhizic acid," "brain," "neurodegenerative disease," "Alzheimer's," and "Parkinson" were used to search in title/abstracts. Licorice extract and/or its active components can be used safely in therapeutic doses for optimizing the management of a multiple neurodegenerative disorders, and hampering the extent of neural tissue injury and neurologic deficits subsequent to cerebrovascular accidents.

KEYWORDS

licorice, Parkinson, Alzheimer's disease, herbal medicine, brain

1. Introduction

Licorice root is commonly used in the preparation of commercial products for the food industry, tobacco flavoring, and herbal medicine (Fu et al., 2013). Since ancient eras, licorice has been utilized as a medicinal plant for a variety of human diseases, including infections, neural disorders, peptic ulcers, and asthma (Ayeka et al., 2016). Recent

investigations have shown many more medicinal properties. Flavonoids [isoliquiritigenin (ISL), liquiritigenin, LQapioside, and liquiritin (LQ)], isoflavonoids [Dehydroglyasperin C (DGC)], and triterpenes [glycyrrhizic acid (GA) and glycyrrhetinic acid monoglucuronide (GM)] are the active components of licorice root. The sugary flavor of licorice is due to GA (Jiao et al., 2013; Ton et al., 2013; Hosseinzadeh and Nassiri-Asl, 2015; Han et al., 2017). Flavonoids derived from licorice have antimicrobial, anti-inflammatory, antioxidant, and antispasmodic attributes (Guo et al., 2016). Besides, DGC has recently been shown to have anti-cancer properties (Shi et al., 2015). Licorice and its constituents have been shown to mediate several signaling pathways involved in acute and chronic neurodegeneration. Ischemic stroke, which causes a burst of infarctions in the center of a hypoperfusion zone, is an acute neurotoxic process. Neurodegenerative diseases like Parkinson's and Alzheimer's are examples of chronic neurotoxicity (AD) (Gaur et al., 2014; Abduljawad et al., 2022; Hassan et al., 2022a). Recent studies showed that plant based active ingredients are effective in neurodegenerative disease (Wei et al., 2021; Hassan et al., 2022b; Mahnashi et al., 2022). Both active components and the entire extract of licorice have been shown to have neuroprotective properties (Hopkins, 2008; Dai et al., 2013; Huang et al., 2016). The licorice root contains several active ingredients with biological functions. Using High-performance liquid chromatography techniques, multiple chemical compounds, including flavonoids and triterpene saponins, have been identified (Hopkins, 2007; Zhu et al., 2018; Heidari et al., 2021). Other minor components identified include DGC, glycerol, glycerin, licoflavone, and glycycoumarin (Gao et al., 2016).

Acetylcholinesterase, nitric oxide synthase, cholinesterase, monoamine oxidase A (MAOA), monoamine oxidase B (MAOB), and are among the afferent nervous system targets that licorice influences. Both MAOA and MAOB belong to the monoamine oxidase (MAO) family and play a crucial role in maintaining mental health by catalyzing the oxidative deamination of neurotransmitters and xenobiotic amines (Ramsay and Tipton, 2017). The proper regulation of MAO activity is required for the effective treatment of neurodegenerative diseases. MAO-B inhibition is a well-known treatment strategy for Alzheimer's disease and Parkinson's disease (Dezsi and Vecsei, 2017). Various constituents of licorice including licocoumarone, licopyranocoumarin and glycyrrhisoflavone inhibit MAO activity (Hatano et al., 1991; Ramalingam et al., 2018). Most of the inhibitory mechanism of licorice is dependent to the presence of glicoricone and structure of MAO (Hatano et al., 1991). Furthermore, licorice can mediate the function of acetylcholinesterase, a key enzyme in the hydrolysis of acetylcholine (Coloviæ et al., 2013). Licorice contains 52 compounds that have been shown to inhibit acetylcholinesterase activity (Chen et al., 2019). The current review concentrated on the available evidence regarding the pharmacologic effects of active compounds of licorice on neural disorders and the underlying signaling pathways (**Figure 1**).

2. Method of searching

Papers had published from 2000.1.1 up to 2 January 2023 in web of science, Google scholar and pubmed were investigated. 65 papers from web of science database, 73 from Google scholar and 318 in pubmed were find with including criteria (key words) "Licorice." "*Glycyrrhiza glabra* L.," "glycyrrhizic acid," "brain," "neurodegenerative disease," "Alzheimer's," and "Parkinson" in title/abstracts. Paper without the keywords, review articles, abstracts of congress, and non-English papers were excluded from this review.

3. Licorice in ischemic brain stroke

Ischemic stroke is one of the important causes of death worldwide, causing irreversible brain tissue damage. Current ischemic stroke mainstay therapy includes blood supply recovery, however, blood supply reestablishment is not obtained during the golden time due to the patient's late arrival or contraindications related to the use of endovascular and thrombolytic agents (Roaldsen et al., 2021). Various agents have been proposed to reduce ischemia-related neural tissue injury by inhibiting inflammatory and neurotoxic pathways (DeLong et al., 2022). Licorice-derived glabridin has substantially modulated the middle cerebral artery occlusion (MCAO)- induced cerebral injuries in rats and also in staurosporine-treated cultured rat cortical neurons. The results indicated that glabridin escalated the levels of endogenous antioxidants and prevents cellular apoptosis (Yu et al., 2008). It has been shown that post-treatment of the ischemic stroke mice with 125 mg/kg *Glycyrrhizae Radix et Rhizoma* was effective in cerebral infarction and inflammatory response by regulating the activation of microglia and astrocytes (**Figure 2**; Choi et al., 2022).

In animal models, licorice effectively blocked neuroexcitatory damage cascades (Wei et al., 2021). It has also significantly reduced lactate dehydrogenase release in hypoxia-induced cultured gerbil hippocampus (Hwang et al., 2006). *In vivo*, licorice treatment has increased superoxide dismutase activity in a carotid artery occlusion model (Sathyamoorthy et al., 2020). In another study, intravenous administration of GA after ischemia induction significantly reduced infarction size, microglia activation, and the production of pro-inflammatory cytokines (Kim et al., 2012). GA in combination with candesartan have significantly ameliorated the expression of toll like receptor (TLR) (TLR-2 and TLR-4) and subsequent downstream inflammatory markers (Barakat et al., 2014). The activity of HMGB is linked to post-ischemia inflammation of neural tissue (Ramalingam et al., 2018). GA, a known HMGB inhibitor, has reduced the inflammatory response in mice with MCAO (Lim et al., 2018). The mechanism of this inhibition has been attributed to the HMGB1-TLR4-IL-17A signaling pathway (Zhang et al., 2014). In another study GA has exerted protective effects on ischemia-reperfusion injury in rat brains through the prohibition of oxidative stress, inflammation, and apoptotic injury by inhibiting the cytokine activity of HMGB (Gong et al., 2014). Also, the HMGB1 inhibitory role of GA has been shown to be connected with ferroptosis and the related signaling network. Ferroptosis is a caspase-independent type of cell death triggered by lipid peroxidation and could be caused as a result of glutathione peroxidase impairment (Wang et al., 2018). GL can prevent neuronal ferroptosis, suppress oxidative stress, diminish mitochondrial injury, and decrease neuro-inflammation in HIBD *via* the HMGB1/GPX4 pathway

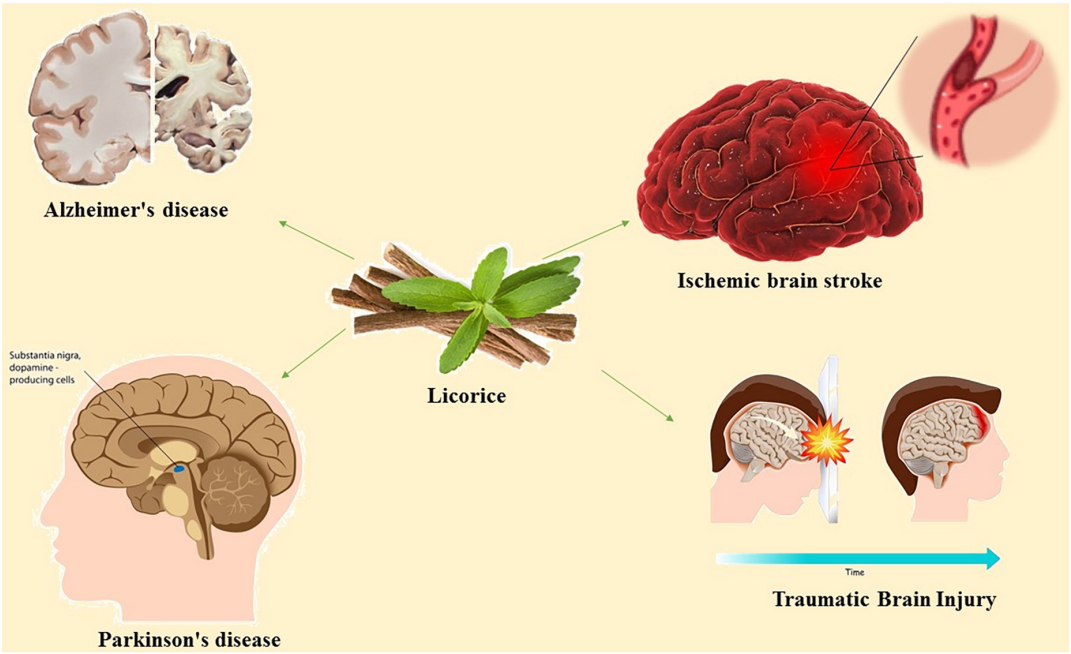

FIGURE 1
Schematic illustration of the effects of licorice on neural disorders.

(Zhu et al., 2022). Furthermore, GA treatment can significantly reduce CD68+ macrophage infiltration, indicating a role in T-cell-mediated cytotoxicity (Xiong et al., 2016). ISL derived from licorice has been shown to reduce the expression of apoptotic factors and the formation of reactive oxygen species (ROS) in neural tissues (Hwang and Chun, 2012). The first clinical trial of licorice extract in dried powder capsules found that it effectively improved neurologic function after the onset of ischemic stroke symptoms (Ravanfar et al., 2016). In this study 450 and 900 mg licorice extract was orally prescribed for 7 days and National institute of Health stroke scale (NIHSS) and Modified Rankin Scale (MRS) scores were evaluated prior to licorice intake and 90 days after treatment.

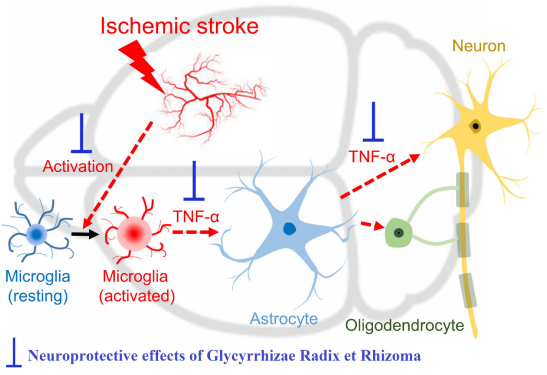

FIGURE 2
Mechanism of the neuroprotective effect of *Glycyrrhizae Radix et Rhizoma* in the alleviation of inflammation in ischemia/reperfusion-induced brain damage (Choi et al., 2022).

4. Licorice in Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative condition defined by the gradual death of brain cells through many signaling pathways, including glutamate, PI3K/Akt, extracellular signal-regulated kinase (ERK), HMGB, and Necrotic factor kappa B (NFkB) (Kao et al., 2009; Srinivasan and Lahiri, 2015; Miculas et al., 2022). Studies showed that GA blocked the activity of NF-kB as a key element of neurodegenerative disease pathogenesis (Wang et al., 2011). GA has been shown to inhibit the activity of NFkB, which is essential in the pathogenesis of neurodegenerative diseases (Hwang et al., 2006). Following activation, NFkB sends several downstream signals that terminate in inflammation (Shih et al., 2015). Glutamate has been shown to induce apoptosis in cultured hippocampal cells, which has been confirmed by microscopic analysis of the morphological properties of apoptosis. At the same time, GA treatment may impair apoptotic machinery function in a concentration-dependent manner. In this study, GA significantly reduced glutamate-mediated NMDA receptor signaling and prevented the activation of NFkB as a downstream signal in the mentioned pathway (Cherng et al., 2006). Another study found that GA and GM inhibited NFkB and other inflammatory pathways in an LPS-induced inflammation model (Wang et al., 2011). Furthermore, GA and GM have been shown to lower Bcl-2 levels and increase PI3K signaling activity, resulting in the inhibition of cytotoxic mechanisms. Also, DGC administration has been shown to reduce the inflammatory response to LPS and NFkB activity in microglial cells (Kim et al., 2013). ERK signaling is another important pathway involved in the potential neuroprotective effects of GA. GA has been shown to increase ERK activity in neural cultures (Wang et al., 2014). Licorice-derived ISL inhibits glutamate-related neurotoxicity by decreasing stress

mediators such as ROS, membrane lipid peroxidation, calcium influx, decreasing apoptosis signaling markers, and increasing cell survival factors. In addition, by suppressing ROS formation and blocking the release of apoptotic factors (Bcl2, Bax, and AIF) from mitochondria into the cytosol, ISL has attenuated glutamate-induced mitochondrial injury and further hippocampal neural loss (Peng et al., 2015). It has been reported, licorice reduced microglial cell activation and inflammation in LPS-induced neurotoxicity by inhibiting activator protein 1 (AP1) and NFkB. This suppression can prevent neurotoxic processes in inflammatory-related neural disorders such as Alzheimer's (Zhu et al., 2022). Indeed, ILS has been shown to significantly reduce amyloid peptide (2535) [A(2535)]-induced neurotoxicity by modulating downstream signaling mediators (Ahn et al., 2010; Lee et al., 2012). Interestingly, licorice has been shown to improve cognitive markers of Alzheimer's disease *in vivo*. Licorice has been shown to have anticholinesterase activity in reversing scopolamine and diazepam-induced amnesia. Anticholinesterase agents are well-known medications used in the treatment of Alzheimer's disease (Dhingra et al., 2004). Three consecutive recipients of licorice-derived glabridin were able to effectively reduce cholinesterase activity comparable to standard medication (Cui et al., 2008). Glabridin decreases MDA levels in rat brains while raising superoxide dismutase and glutathione levels (Yu et al., 2008). A research demonstrated that feeding hypoxic rats with *G. glabra* restored low levels of brain neurotransmitters such as glutamate and dopamine and decreased AChE activity.

Another study tested the neuroprotective properties of an aqueous root extract of *G. glabra* in Wistar albino rats. The dosages of 150 and 225 mg/kg showed a considerable neuroprotective effect. The neuroprotective action is linked to the presence of the active isoflavone "Glabridin" in *G. glabra* (Hasanein, 2011). Furthermore, when used for 30 days, higher concentrations reversed diabetes-induced memory and learning dysfunction *in vivo* (Hasanein, 2011).

Recent research has found that HMGB1 plays a pathogenic role in memory impairment, primarily *via* the TLR4 and RAGE signaling pathways (Rong et al., 2021; Miculas et al., 2022). Furthermore, HMGB1 neutralization has been shown to reduce cognitive dysfunction and post-TBI cognitive impairment (Hei et al., 2018; Okuma et al., 2019). TLR4 and NF-B phosphorylation, followed by activation of the NLRP3 inflammasome, is one proposed mechanism by which HMGB1 affects cognition (Costello et al., 2011). Previous research has found that NLRP3 contributes to the worsening of cognitive dysfunction (Li et al., 2017). In LPS-treated animal models, GA has been shown to slow memory decline (Song et al., 2013). GA protects by lowering the expression of inflammatory markers such as TNF- and IL-1, as well as the protein expression of COX-2 and iNOS (Song et al., 2013). In addition, by inhibiting HMGB1/NF-B signaling-mediated neuroinflammation, GA treatment improved spatial memory in isoflurane-exposed animals (Wang et al., 2016). By preventing brain inflammation and AD-like pathology through HMGB1 neutralization, GA has been found to protect mice from surgery-induced cognitive impairments (short swimming latency and distance in the MWM test) (Kong et al., 2017). GA also can significantly decrease inflammatory markers, NF-B, and hippocampal A levels (Kong et al., 2017). GA has been shown to reduce cell death in AD experimental models by inhibiting HMGB1 (Jang et al., 2013).

5. Licorice in Parkinson's disease

Another significant neurodegenerative disorder is Parkinson's disease, which is characterized by neural loss and gliosis in the substantia nigra. In Hwang and Chun (2012), the first study using licorice to treat Parkinson's disease was conducted, in which 6hydroxydopamine (6OHDA)-induced neurotoxicity was used to mimic PD-like dysfunction in dopaminergic neurons *in vivo*. It was discovered that ISL, by mediating intracellular signals, could significantly reduce ROS formation and inhibit the release of apoptotic factors. ISL and liquiritigenin have been shown to significantly reduce synuclein fibril deposition (the pathologic hallmark of Parkinson's disease) in neural tissues. Furthermore, ISL has the potential to disaggregate previously formed deposits (Liao et al., 2016).

The pathophysiology of Parkinson's disease is linked to several signaling axes that are involved in cell survival, protein aggregation, inflammation, oxidative stress, apoptosis, mitochondrial damage, and autophagy (Angelopoulou et al., 2019; Paudel et al., 2020). The aggregation of -synuclein-containing Lewy bodies causes cognitive and motor dysfunction (Angelopoulou et al., 2018; Kirkeby and Barker, 2019). Furthermore, HMGB1 signaling appears to be tightly linked with inflammatory response and degeneration in Parkinson's disease, as increased levels of HMGB1 have been detected in PD patients (Yang et al., 2018; Baran et al., 2019). Therefore, HMGB1 targeting has great potential as a treatment for PD (Song et al., 2013; Wang et al., 2016). Lower levels of HMGB1 and RAGE in the midbrains of MPTP-treated rats were associated with this protective effect (Kong et al., 2017). Increasing antioxidant protein levels and lowering MDA and carbonyl production, another research found that GA and 18-glycyrrhetinic acid (a metabolite of GA) prevented cell death in differentiated PC12 cells treated with MPTP and 1-methyl-4-phenylpyridinium (MPP+) (Kim and Lee, 2008). Furthermore, the combination of GA and 18-glycyrrhetinic acid has been shown to improve caspase 3 activity GA and 18-glycyrrhetinic acid was found to inhibit mitochondrial permeability transition in MPP+-induced neurotoxicity (Yim et al., 2007). GA has also been shown to have neuroprotective effects in the rotenone-induced Parkinson's disease model by increasing intracellular glutathione levels, decreasing MDA, increasing cellular antioxidant capacity, and decreasing pro-inflammatory cytokine release (Ojha et al., 2016).

Rotenone induces Parkinson's disease-associated cell cycle re-entry-mediated G2/M arrest, mitochondria-related oxidative stress, and triggering of the caspase-3 apoptotic pathway through MEK-ERK-1/2 hyperactivation (Karthikkeyan et al., 2021). Glycyrrhiza glabra L, when used in combination with other therapies, has been shown to decrease cellular ROS and improve mitochondrial health (Karthikkeyan et al., 2021). By downregulating the MEK-ERK-1/2 axis, it stops the cell cycle from restarting after a mitotic catastrophe and stops caspase activation. Findings suggest that *G. glabra* L protects cells against neurotoxic stress (**Figure 3**; Karthikkeyan et al., 2021).

These results indicate that licorice and its compounds may possess neuroprotective capabilities against Parkinson's disease. However, further study is required to determine the therapeutic effectiveness and safety of different formulations.

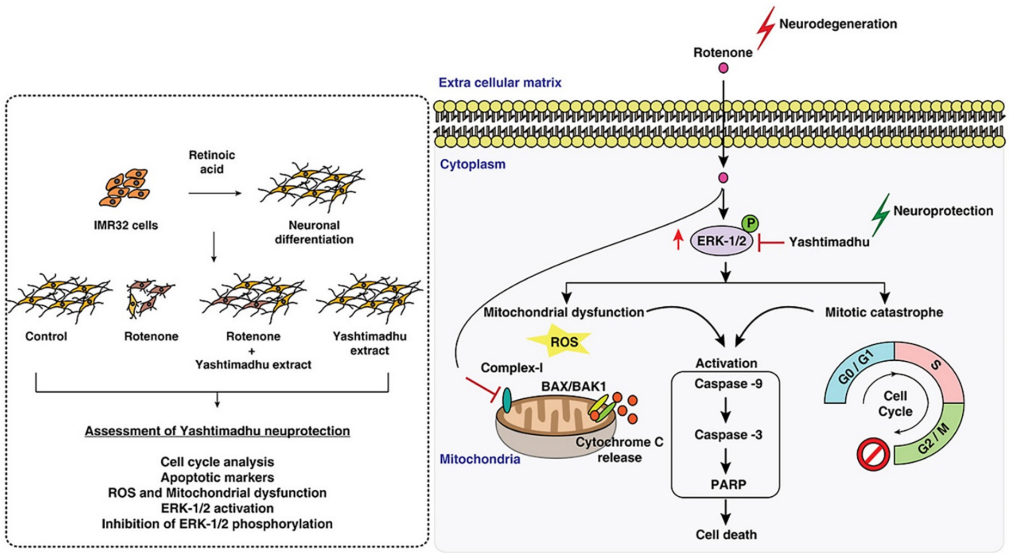

FIGURE 3
Mechanism of licorice's neuroprotective action against rotenone-induced toxicity. Reproduction authorized. Elsevier[r] 2021 Elsevier B.V. (Karthikkeyan et al., 2021).

6. Licorice in traumatic brain injury

Traumatic Brain Injury (TBI) is a brain injury caused by an external mechanical force, such as a blow to the head (Webster et al., 2017). There are two types of traumatic brain injury depending on the underlying mechanism: closed and penetrating. The severity of a traumatic brain injury is measured by structural damage and the Glasgow coma scale (GCS) (Maas et al., 2008). TBI encompasses both primary and secondary damage. The primary damage consists of an external force disrupting the blood-brain barrier (BBB), which is followed by an increase in inflammatory markers, ROS formation, excitotoxicity, and neural cell death (Woodcock and Morganti-Kossmann, 2013; Parker et al., 2017). Intracranial pressure (ICP), seizures, infection, and hematomas are all caused by the inflammatory response. The secondary injury occurs next, which can be reversed to improve (Parker et al., 2017). Untreated brain injuries can cause behavioral and cognitive disruption, seizures, chronic encephalopathy, and Alzheimer's disease (Barman et al., 2016; Hay et al., 2016). Despite recent advances in basic and clinical research, treatment options with favorable outcomes following TBI remain limited (Hasanein, 2011). Though, numerous strategies have been proposed for TBI treatment, with inflammation targeting by pharmaceutical agents being a particularly prominent option (Kochanek et al., 2015). Necrotic neurons release HMGB1 during TBI *via* an N-methyl D-aspartate receptor subtype 2B (NR2B)-mediated mechanism (Richard et al., 2017). When HMGB1 is produced, it binds to TLR2, TLR4, and RAGE, initiating the HMGB1/TLR4/RAGE/NF-B cascade, which leads to the release of inflammatory cytokines and further aggregation of the secondary damage (Yang et al., 2005; Gu et al., 2014). GA has been shown to have promising results in animal models of TBI. For example, in the fluid percussion injury (FPI)-induced model of TBI, GA could, in a concentration-dependent manner, block the translocation of HMGB1 from the nucleus to the cytosol and thus protect BBB permeability (Cai et al., 2016). Furthermore, GA has been shown to improve cognitive function and locomotor activity (Parker et al., 2017). Another pre-clinical study found that GA treatment improved walking balance while decreasing brain edema and apoptosis (Gu et al., 2014). GA significantly lowered cytoplasmic expression of HMGB1 and the number of TLR4 and RAGE positive cells. GA's neuroprotective benefits were ascribed mostly to its anti-inflammatory action through HMGB1 inhibition (**Figure 3**).

The pre-treatment of C57Bl/6 mice with GA before the imitation of TBI had a significant impact on the reduction of HMGB1 levels in the brain. However, administering GA 1 h after TBI did not produce the same results, whereas chronic use of GA may improve memory and spatial learning. GA administration in TBI-induced animals may also mediate the polarization of microglia associated with secondary injury (Gao et al., 2018). In a focal contusion animal model, GA has been shown to reduce neurological function recovery, lesion volume, and HMGB1 expression. Notably, GA inhibited post-TBI M1 phenotype activation, increased M2 phenotype activation, and reduced TBI consequences, most likely by blocking an M1-like pro-inflammatory phenotype in microglia and, in part, inhibiting HMGB1 (Gao et al., 2018). These findings suggest that targeting HMGB1 to mediate microglia polarization could be a promising therapeutic option for TBI.

Glycyrrhizic acid treatment has also been shown to suppress apoptosis, reduce axonal damage, inhibit the release of pro-inflammatory cytokines, and improve cognitive impairments in patients with diffuse axonal injury (Pang et al., 2016). As a result, GA treatment may be an effective therapy for various brain injuries. However, the precise underlying mechanisms of neuroprotection must be determined (**Figure 4**).

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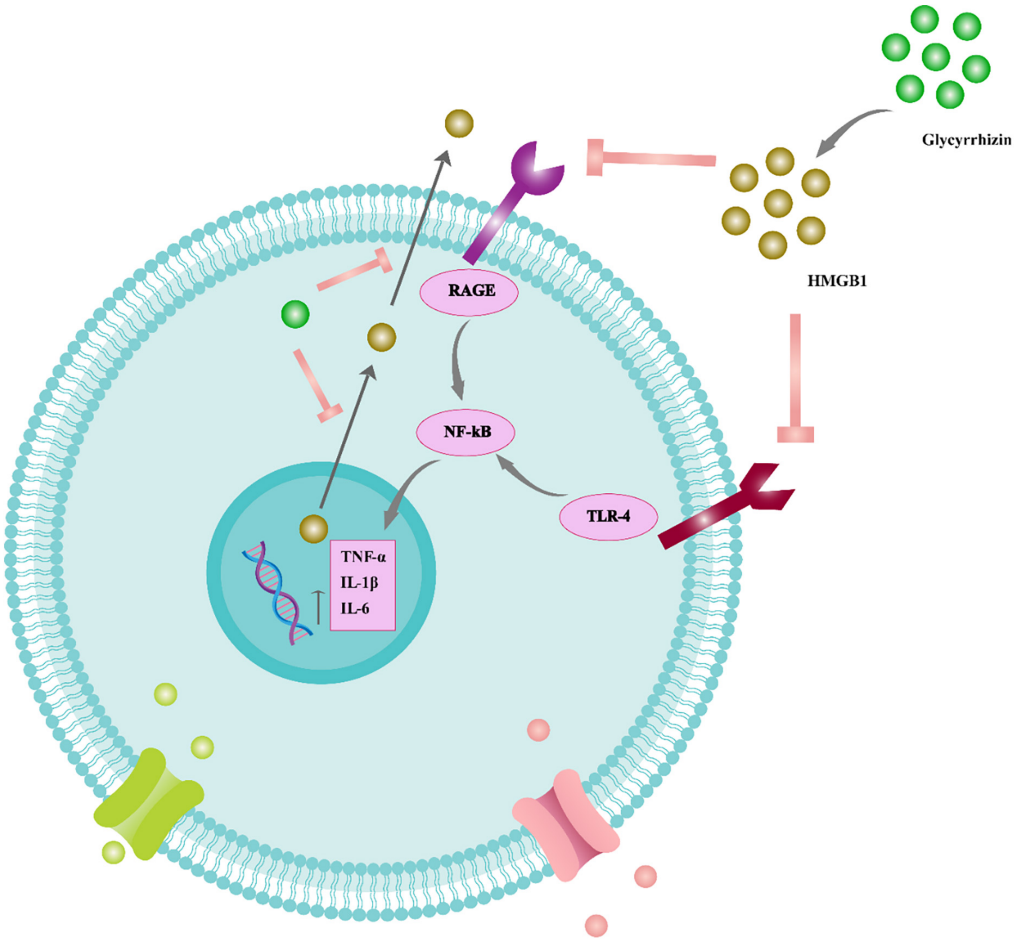

FIGURE 4
The function of glycyrrhizin in the inflammatory cascade of the neural system. Glycyrrhizin binds to HMGB1 and prevents HMGB1/RAGE and HMGB1/TLR4 interaction. Therefore, NF-B signaling is suppressed and pro-inflammatory cytokines such as TNF-, IL-6, and IL-1 are reduced. Furthermore, glycyrrhizin prevents nuclear translocation of HMGB1 to the cytoplasm and consequent extracellular release, thus also reducing HMGB1's extracellular pro-inflammatory actions. Tumor necrosis factor (TNF), receptor for advanced glycation end products (RAGE), interleukin (IL), nuclear factor light chain enhancer of activated B cells (NF-B), toll-like receptor 4 (TLR4).

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7. Possible toxicity of licorice

Some worries have been expressed concerning prolonged high-dosage ingestion of licorice and its compounds, notwithstanding the apparent therapeutic potential. A large amount of licorice may have adverse consequences, and GA and GM are to blame. Renal 11-hydroxysteroid dehydrogenase2, an enzyme involved in the breakdown of cortisol, is inhibited by GA. Forbidden foods may trigger hypermineralocorticoid states, which in turn can lead to moderate hypertension because of elevated potassium and sodium/water retention excretion. Safe human dosing ranges for GA are between 0.015 and 0.229 mg/kg body weight per day (Isbrucker and Burdock, 2006; Asl and Hosseinzadeh, 2008).

It should be noted that different licorice preparations contain varying levels of GA and glycyrrhizin. As a result, the precise concentration of the manufactured preparations should be measured to adjust the dosage within safe limits. The toxicological test results show that licorice has no carcinogenic and/or teratogenic effect *in vivo*. In addition, therapeutic doses of licorice are considered safe for humans based on toxicological assay

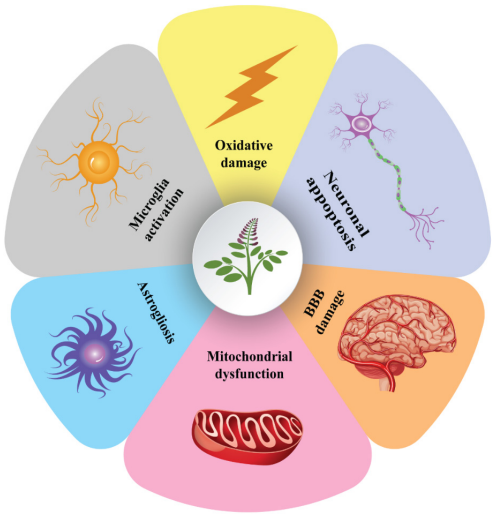

FIGURE 5
The cellular processes behind the neuroprotective properties of glycyrrhizin. Glycyrrhizin may enhance the integrity of the blood-brain barrier by preventing astrogliosis, neuronal apoptosis, microglia activation, oxidative-induced cellular damage, and mitochondrial dysfunction. These methods enable glycyrrhizin to boost anti-excitotoxicity (for epilepsy treatment), decreasing axonal damage and brain edema (TBI improvement), cognitive and motor function improving (AD and PD treatment), and reducing demyelination (MS treatment). MS, multiple sclerosis; TBI, traumatic brain injury; PD, Parkinson; AD, Alzheimer's disease.

recommendations and without developmental or reproductive harm (Cosmetic Ingredient Review Expert Panel, 2007). Doses of 900 mg whole extract three times per day for 1 week did not affect human blood pressure or electrolyte hemostasis (Ravanfar et al., 2016). For an additional 8 weeks, healthy volunteers were given 0, 1, 2, and 4 mg/kg/day doses of GA. A six-gr daily licorice intake for a 60 kg individual was found to have no known side effects (van Gelderen et al., 2000).

8. Future perspective

Currently the treatment of certain neural disorders is not possible. For instance, medications for ischemic stroke should be prescribed within a short duration after the ischemic attack. On the other hand, current therapeutic option for specific molecular targeting of neurodegenerative disorders are few and costly. Recent studies have been devoted on enlightening novel pharmacologic specifications of the well-known herbal remedy, licorice extract, and its active constituents such as GA, GL, ISL, and glabridin. The newly discovered neuroprotective effects of licorice has provided a new shift in paradigm of neural disease treatment plausible for both acute and chronic brain damages. The active substances of licorice can effectively inhibit cytotoxic pathways in brain. Whole licorice extract and/or purified ingredient can hamper the volume of infarction after ischemic injuries *in vivo*. HMGB has been revealed to be one of the major cellular pathways in the neuroprotective effects of licorice. Combining separated phytochemical elements from licorice and their biological significance in battling multiple neurological disorders and their secondary metabolites may lead to the creation of potential pharmacological formulations.

9. Conclusion

To summarize the present review, licorice extracts and flavonoids have been employed to reduce neuro-inflammatory processes after acute ischemia injury to brain cells, TBI, and neurodegenerative diseases. Licorice is safe for human intake at therapeutic doses that have been researched. These results can lead to the discovery and manufacture of novel medications for neurodegenerative illnesses and acute brain tissue injury. However, further ***in vivo*** and clinical studies are needed to extrapolate their action method into other neuro-therapeutic actions.

Author contributions

PZ: data curation, formal analysis, investigation, and writing—original draft. TZ-G and SM: methodology, visualization, and formal analysis. EA: formal analysis, methodology, and visualization. VT: methodology, visualization, formal analysis, data curation, and writing—review and editing. DS and GR: methodology, formal analysis, data curation, writing—original draft, and writing—review and editing. RK: resources, writing—original draft, and writing—review and editing. AE: conceptualization, study design, supervision, manuscript revision, and final approval of the version to be declaration of competing interest. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Natural coumarins from *Murraya paniculata* as mixed-type inhibitors of cholinesterases: *In vitro* and *in silico* investigations

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Currently, acetylcholinesterase (AChE) inhibiting drugs in clinical use, such as tacrine, donepezil, rivastigmine, and galanthamine, are associated with serious side effects and short half-lives. In recent years, numerous phytochemicals have been identified as inhibitors of cholinesterases with potential applications in the management of Alzheimer's disease (AD). In this study three natural coumarins, 2′-O-ethylmurrangatin (**1**), murranganone (**2**), and paniculatin (**3**) isolated previously by our group from the leaves of Murraya paniculata, were tested against the two cholinesterases (ChE) enzymes, AChE and butyrylcholinesterase (BChE) using *in vitro* assay. Molecular docking was performed to highlight the structural properties that contribute to the molecular recognition pattern in the inhibition of ChE and the structural differences resulting in the selectivity of these compounds toward AChE. Classical enzyme inhibition kinetics data suggested that compounds **2** and **3** were potent inhibitors of AChE and BChE, while **1** was found inactive against both enzymes. The findings from molecular docking studies revealed the competitive and non-competitive inhibition mechanisms of compounds **2** and **3** against both enzymes. Molecular docking and simulations have revealed that hydrogen bonding, mediated by ketone and hydroxyl functionalities in various positions, significantly contributes to the binding of the inhibitor to the receptor. According to MD simulation studies, the stability of the ligand-AChE complex for the most active compound (**3**) is found to be comparable to that of the widely used drug Tacrine. In addition, to evaluate the drug-likeness of compounds, *in silico* ADME evaluation was performed, and the compounds presented good ADME profiles. Data suggested that the coumarin nucleus having diverse side chains at the C-8 position can serve as a potential inhibitor of cholinesterases and can act as a lead to develop a new semisynthetic drug for the treatment of AD.

KEYWORDS

acetylcholinesterase, butyrylcholinesterase, natural coumarins, inhibition kinetics, molecular docking, MD simulation, ligand-enzyme interactions

1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that leads to a gradual loss of neuronal structure and function, causing cognitive decline and dementia (Kabir et al., 2021; Onoja et al., 2021; Khalid et al., 2022). The neurotransmitter Acetylcholine (ACh), which is essential for learning and memory processes, was the first neurotransmitter to be implicated in Alzheimer's disease (Kaur et al., 2022; Hussain et al., 2023). The ChE family of enzymes consists of AChE, which hydrolyzes ACh in the cholinergic synapses, and BChE, which preferably hydrolyzes butyrylcholine (BCh), having an elusive function in the central nervous system (CNS). Both enzymes are considered potential targets for discovering mechanism-based inhibitors for the treatment of AD and related neurodegenerative disorders (Rampa et al., 2013; Karthika et al., 2022). The role of BChE in the normal, aging, and diseased brain is still unknown; however, it was found that the BChE existed in significantly higher quantities in Alzheimer's plaques than in the plaques of age-related non-demented brains (Darvesh et al., 2012). Moreover, the inclusion of cymserine, a highly potent selective BChE inhibitor in the clinical trials for AD treatment, proved that BChE inhibition could be an essential tool for the treatment of AD and related dementias (Kamal et al., 2008). The structures of AChE and BChE are very close to each other. Overall, cholinesterases have α/β hydrolase protein fold, and the active sites of both cholinesterases are buried near the bottom of a deep gorge which consists of esteratic and π-cation subsites (Hotelier et al., 2004).

In AChE, the gorge is lined by 14 conserved aromatic amino acids, whereas in BChE, Leu286 and Val288 are positioned by replacing structurally equivalent AChE residues, Phe288 and Phe290, respectively, facilitating BChE to accommodate bulkier and non-polar ligands. At the opening of the gorge, an anionic site termed as peripheral anionic site (PAS) is present, which makes the diffusion of the substrate possible towards the active site (Fang et al., 2011). In contrast to AChE, BChE lacks Trp279 resulting in weaker binding of bisquaternary ligands at the PAS. The ChE is known to be inhibited by a wide variety of synthetic and natural compounds (Houghton et al., 2006; Murray et al., 2013), and previously, several natural ChE inhibitors isolated from various medicinal plants were reported by our group (Parveen et al., 2001; Atta-ur-Rahman et al., 2002; Atta-Ur-Rahman et al., 2003; Choudhary et al., 2006). The *in vitro* and *in silico* studies aimed to perform inhibition kinetics, Comparative Molecular Field Analysis (CoMFA), Comparative Molecular Similarity Indices (CoMSIA), ligand docking, and molecular dynamics (MD) simulation studies have also been conducted on these inhibitors (Wellenzohn et al., 2003; Wheeler, 2003; Zaheer et al., 2003).

Coumarins are one of the most important moieties that are present in a number of natural and synthetic drugs acting on various targets. Recently, coumarins have been explored for their potential as an important pharmacophore in the development of AChE inhibitors, and promising results were obtained (Abu-Aisheh et al., 2019; de Souza et al., 2016; Ekstrom et al., 2022; Sonu et al., 2019; Yusufzai et al., 2018). Previously, we reported the isolation and characterization of three 7-methoxy-8-substituted coumarins, namely, 2′-*O*-ethylmurrangatin (**1**), murranganone (**2**), and paniculatin (**3**) from *Murraya paniculata* (Orange Jasmine) (Figure 1) (Choudhary et al., 2002). *Murraya paniculata* has multiple traditional medicinal uses, including treatment for diarrhea, abdominal pain, and headaches, as well as anticonvulsant and antibacterial properties, and has been found to have antinociceptive, anti-inflammatory, antidiabetic, antimalarial, and antioxidant activities (Dosoky et al., 2016).

In this study, the efficacy and druggability of isolated coumarin derivatives were investigated against the two ChE enzymes using *in vitro* enzyme inhibition assay, molecular docking, MD simulation, and ADME profile evaluation studies.

2 Materials and methods

2.1 Materials

The three coumarins utilized in this study, i.e., 2′-O-ethylmurrangatin (**1**), murranganone (**2**), and paniculatin (**3**) were isolated from M. paniculata (Orange Jasmine) collected from Karachi, Pakistan as previously reported (Choudhary et al., 2002).

All the chemicals and solvents used in this study were purchased from Sigma Aldrich (Steinheim, Germany). The enzyme inhibition studies were performed on 96-well microtiter plates using a SpectraMax microplate spectrophotometer (Molecular Devices, CA, United States). AChE, BChE, acetylthiocholine, butyrylthiocholine, 5,5-dithiobis-2-nitro benzoic acid (DTNB), and Eserine (standard inhibitor) were also purchased from Sigma Aldrich (Steinheim, Germany). The enzyme kinetic study was performed using the EZ-Fit enzyme kinetics program (Perrella Scientific Inc., Amherst, United States). The software used for molecular docking was Molecular Operating Environment (MOE) version 2018 (Chemical Computing Group, Montreal, Canada). MD simulation study was performed using GROMACS

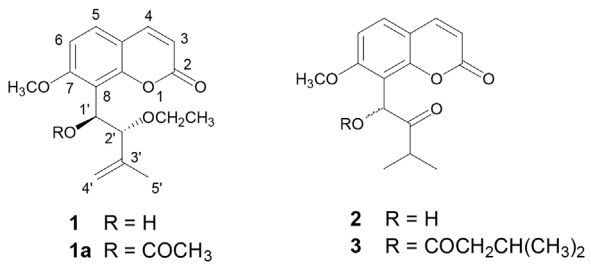

FIGURE 1
Strcutures of isolated coumarins from *Murraya paniculata* (Orange Jasmine) 2′-*O*-ethylmurrangatin (**1**), murranganone (**2**), and paniculatin (**3**).

version 2018 (University of Groningen, Netherlands), and ADME prediction was made using SwissADME (Swiss Institute of Bioinformatics, Lausanne, Switzerland).

2.2 *In vitro* enzyme inhibition assay

The AChE and BChE enzymes inhibition activity was measured *in vitro* by a modified spectrophotometric method developed by Ellman et al. (1961) with slight modifications. The test compounds were prepared fresh immediately before each experiment. Test compounds were added to 150 µl 0.1 mM phosphate buffer (pH 8.0), 200 µl methanol, and 20 µl of the test enzymes and were incubated at 25°C for 15 min. A sample with an equal amount of solvent (ethanol) in place of the test compounds served as a negative control. 10 µl of DTNB reagent was added to the samples, and the reaction was initiated by adding 10 µl acetylthiocholine (ATCh) or butyrylthiocholine (BTCh) for the estimation of AChE and BChE, respectively. All the experiments were conducted in triplicate. The hydrolysis of ATCh and BTCh was measured by assessing the formation of a yellow anion of 5-thio-2-nitrobenzoate at wavelength 412 nm due to the reaction of DTNB with thiocholine. The rate of enzyme inhibition was calculated as the change in optical density per minute (OD/min). The rate of enzymatic reaction was calculated using the extinction coefficient of 5-thio-2-nitrobenzoate by the following equation:

$$Rate\ (moles/L/\min) = \frac{change\ in\ absorbance}{\varepsilon_{TNB}}$$

where ε_{TNB} = molar extinction coefficient of 5-thio-2-nitrobenzoate = 13.6×10^3 at 412 nm (Ellman, 1958).

2.3 IC_{50} estimation

The IC_{50} values were determined spectrophotometrically by measuring the concentrations of test compounds that inhibited the hydrolysis of substrates ATCh or BTCh, by 50%. The enzyme inhibition activities of serially diluted concentrations of test compounds were measured, and the IC_{50} values were calculated using EZ-Fit Enzyme Kinetics Program.

2.4 Enzyme kinetics and measurement of the dissociation constant

The dissociation constant (*Ki*) values, which signified the dissociation of the enzyme-inhibitor complex into free enzyme and inhibitor, were also determined. The *Ki* values were calculated by the interpretation of the Dixon plot (Cheng and Prusoff, 1973; Fukushima et al., 2002), the Lineweaver-Burk plot (Fjellstedt and Schlesselman, 1977), and its secondary re-plots by using initial velocities. These velocities were obtained over a range of substrate concentrations between 0.1 and 0.4 mM for ATCh and 0.05 and 0.2 mM for BTCh. The assay conditions for measurement of the residual activities of all inhibitors were identical to the aforementioned spectrophotometric assay procedure, except that the fixed concentrations of inhibiting compounds were used in the assay medium. Assays were conducted in triplicate at each concentration of the inhibitor.

2.5 Determination of inhibition pattern

The inhibition patterns of tested coumarins were determined by the graphical views of Dixon plots, Lineweaver-Burk plots, and their secondary re-plots. Graphs were plotted using the GraFit 7.0 curve fitting program (Erithacus Software Ltd., West Sussex, United Kingdom) (Yamada et al., 2017). The linear regression analysis values, correlation coefficient, slope, intercept, and standard errors were obtained using the same software. Two different methods were applied to monitor the effects of the test sample on both Michaelis constant (K_m) and maximum velocity ($V_{\max}$) values. This was done first by plotting the reciprocal of the reaction rate against the reciprocal of the substrate concentration as the Lineweaver-Burk plot (Frère et al., 1983); and secondly by the Dixon plot in which the reciprocal of rates of reaction were plotted against the concentrations of the inhibitor (Yao et al., 2012). The secondary re-plots of the Lineweaver-Burk were also constructed in two ways; firstly, the reciprocal of apparent V_{max} ($1/V_{maxapp}$) values were determined at each intersection point for every inhibitor concentration line on the *y*-axis of the Lineweaver-Burk plot and then re-plotted against different concentrations of the respective inhibitor. Secondly, in the non-competitive and linear mixed-type inhibitions, the slope of each line of inhibitor concentration on the Lineweaver-Burk plot was plotted against inhibitor concentrations. The secondary re-plot of the Dixon plot was constructed as the slope of each line of substrate concentration in the original Dixon plot against the reciprocals of substrate concentrations.

2.6 Molecular docking studies

Molecular docking studies were performed to get insights into the binding pattern of the natural coumarins against AChE and BChE enzymes. All the tested coumarins were sketched in the MOE program (Vilar et al., 2008) using a builder module and subjected to structure correction and protonation followed by energy minimization using an MMFF94 force field (Sulimov et al., 2017). Crystal structure of Tacrine in complex with AChE and BChE were retrieved from the Protein Data Bank (PDB ID: 1ACJ and 4BDS, respectively) (Harel et al., 1993; Nachon et al., 2013). All the water molecules were removed except the conserved water molecules, and hydrogen atoms were added for both proteins.

Crystal structures of both enzymes were minimized using an Amber99 force field (Wang and Wolf, 2004) prior to docking. Active aromatic gorge served as grid spacing during docking, and 30 poses were generated for all three coumarins. Triangular Matcher was used as placement method with London dG and GBVI/WSA dG as scoring and re-scoring functions, respectively. The binding patterns of coumarins in the active aromatic gorge of AChE and BChE were analyzed using Chimera.

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TABLE 1 The kinetics data for cholinesterase enzyme inhibition by the tested coumarins (**1**–**3**).

Compound	AChE			BChE		
	IC_{50}[a] (μM)	K_i[b] (μM)	Type of inhibition	IC_{50} (μM)	K_i[b] (μM)	Type of inhibition
2′-*O*-Ethylmurrangatin (**1**)	No activity upto 1 mM	ND[c]	ND	842.8 ± 13.0	ND	ND
Murranganone (**2**)	79.1 ± 2.02	50 ± 0.58	NC[d]	74.3 ± 0.75	22 ± 0.44	LM[e]
Paniculatin (**3**)	31.6 ± 0.03	43 ± 0.65	LM	>100	22.0 ± 0.60	LM
Tacrine	0.021 ± 0.002	0.23 ± 0.02	MT[f]	0.051 ± 0.005	0.025 ± 0.003	MT
Galanthamine	0.45 ± 0.02	0.19 ± 0.01	MT	39.1 ± 0.032	32 ± 0.33	NC

[a]K_i and IC_{50} values are expressed as (mean ± SEM), where SEM, is the standard mean error of 3 experiments.
[b]K_i is the mean of four values calculated from Lineweaver-Burk plot, its secondary re-plots, and Dixon plot.
[c]ND, Not determined.
[d]NC, non-competitive.
[e]LM, linear mixed.
[f]MT, mixed type.

2.7 Molecular dynamics (MD) simulation studies

The MD simulation studies were performed for AChE in complex with paniculatin **(3)** and the standard drug tacrine using GROMACS (Berendsen et al., 2005). Automated Topology Builder (ATB) was used to generate the topologies of test compounds, and the topology for the receptor was processed using GROMOS96 54a7 force field (Schmid et al., 2011). The cubic box was generated, the protein was placed at a distance of 1.0 nm from the box edge, and the systems were solvated using the SPC water model with periodic boundary conditions. Sodium ions were added to neutralize the systems. For energy minimization, the steepest descent algorithm was used, and the minimized systems were equilibrated in NVT and NPT ensemble for 100 ps by using Berendsen barostat and thermostat algorithms. For constraining the bond length and calculation of electrostatic long-range interactions, LINCS algorithm, and PME (particle mesh ewald) method were applied, respectively. The working conditions were set at 298.1 K temperature, 1 atm pressure, and 2.0 fs time per step. Finally, the MD production of 100 ns was run for the equilibrated systems. Root mean square deviation (RMSD) was analyzed to determine the stability of protein-ligand complexes.

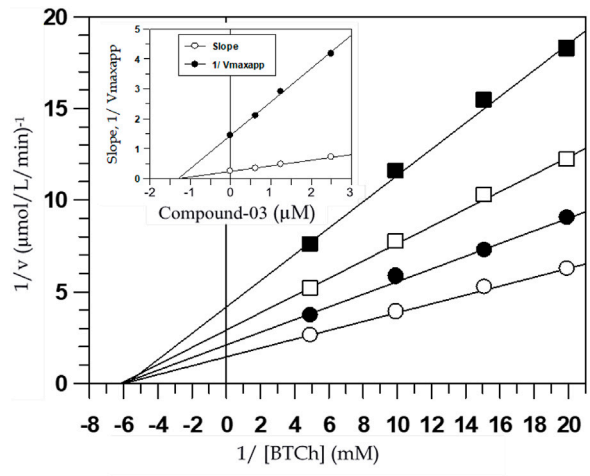

FIGURE 2
Lineweaver-Burk plot of reciprocals of the initial velocities versus reciprocals of BTCh in the absence (○) and presence of 1.25 μM (●), 2.5 μM (□) and 5 μM (■) of compound-03 against BChE. Inside frame shows the secondary replots of the Lineweaver-Burk plot: 1/V_{maxapp} or Slope versus various concentrations of inhibitor, the correlation coefficient for all the lines of all the graphs was >0.99, each point in all the graphs represents the mean of three experiments.

2.8 ADME analysis

The ADME profiles of test compounds were predicted using a web-based server SwissADME (DeLano, 2002; Muhammad et al., 2021). The SMILES notations of all the three compounds were generated and subjected to the webserver for the prediction of physiochemical and pharmacokinetic properties. BOILED-Egg predictive model was applied to predict and estimate the probability of gastrointestinal absorption and permeation to brain.

3 Results and discussion

3.1 Cholinesterase inhibition activity

Compounds (**1**–**3**) were subjected to *in vitro* inhibitory activity assessment against AChE and BChE enzymes using the standard method. Experimentally, IC_{50} values (Table 1) showed paniculatin (**3**) was the most active against AChE (IC_{50} = 31.6) followed by murranganone (**2**) (IC_{50} = 79.1), while that 2′-O-ethylmurrangatin (**1**) was not active at 1 mM conc. Murranganone (**2**) was the most active compound against BChE (IC_{50} = 74.3 μM).

Classical inhibition kinetics was used to investigate the mode of inhibition and to determine the *Ki* values. Results showed that paniculatin (**3**) was more potent against AChE, while murranganone (**2**) was more potent against BChE and both compounds did not show selectivity towards any of the two enzymes (Table 1). Paniculatin (**3**) was observed to be a mixed-type inhibitor of both enzymes (AChE and BChE), whereas murranganone (**2**) exhibited a pure non-competitive inhibition of AChE and mixed-

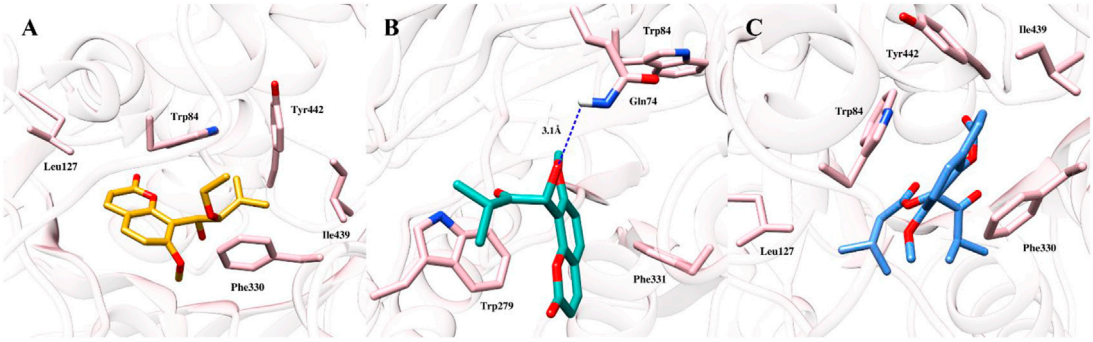

FIGURE 3
Docked pose of all the coumarin derivatives with AChE. **(A)** 2′-*O*-ethylmurrangatin (**1**) resided in the active aromatic gorge; **(B)** murranganone (**2**) fitted well in the peripheral anionic site of AChE and acted as non-competitive inhibitor; **(C)** paniculatin (**3**) resided in the anionic subsite of AChE.

type inhibition of BChE. The compound 2′-*O*-ethylmurrangatin (**1**) did not show any inhibition against AChE and a very weak activity against BChE, in concentrations up to 1 mM.

The inhibition patterns of tested compounds were determined using Lineweaver-Burk plots. Plots of compounds (**2**) and (**3**) for AChE inhibition were observed to be linear and the lines intersected at a point different from x- and *y*-axis. The K_i values were determined from the secondary plots of slopes of Lineweaver-Burk plots drawn against the concentration of inhibitors and were found to be 50 ± 0.58 and 43 ± 0.65 μM for compounds (**2**) and (**3**), respectively for AChE inhibition (Figure 2). Similarly, plots of BChE inhibition by both the compounds were also found to be linear and not intersecting at x- or *y*-axis. The Ki values calculated from the secondary re-plots were found to be 22 ± 0.44 and 22.0 ± 0.60 μM for compounds (**2**) and (**3**), respectively. These results showed that both compounds (**2**) and (**3**) acted as mixed-type inhibitors of AChE and BChE enzymes.

Mixed-type inhibitors decrease both V_{max} values and the affinity of the substrate towards the enzyme. Figure 2 shows the graphical representation of steady-state kinetic analysis of AChE and BChE inhibition by compounds (**2**) and (**3**). Mixed-type inhibition showed by naturally occurring coumarins under study was found to be a combination of partially competitive and pure non-competitive inhibitions (Rahman et al., 2006). The pure non-competitive inhibition of AChE by compound (**2**) was envisaged from the ability of the compound to decrease the V_{max} value without affecting the affinity of the enzyme for the substrate (K_m values). The purity of the non-competitive inhibition was further confirmed by the linearity obtained in the secondary re-plots of Lineweaver-Burk plots. This indicated that the compound (**2**) could fit in the substrate-binding loci of these enzymes and bind to the enzyme-substrate complex at other allosteric site such as the peripheral site.

The primary coumarin nucleus did not seem to have much effect on the inhibitory activity as the compounds (**1–3**) were structurally similar in having the coumarin nucleus but diverse at the side chain at the C-8 position, which might have accounted for their activity. Therefore, the structural basis of inhibitors binding at the active site gorge of both cholinesterases was important to investigate. To test the selectivity of these compounds for cholinesterases, all the three natural coumarins (**1–3**) were screened for their inhibition activity against several other enzymes such as urease, acid phosphatase, β-glucuronidase, and α–glucosidase. All the compounds were found inactive at concentrations up to 1 mM against all aforementioned enzymes, which indicated their selectivity towards cholinesterase enzymes.

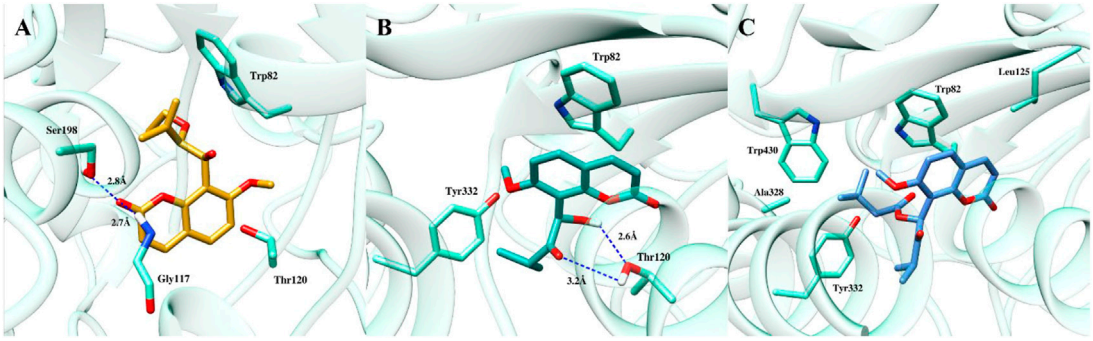

FIGURE 4
Docked pose of all the coumarin derivatives with BChE. **(A)** 2′-*O*-ethylmurrangatin (**1**), **(B)** murranganone (**2**) and **(C)** paniculatin (**3**) resided in the active site of BChE.

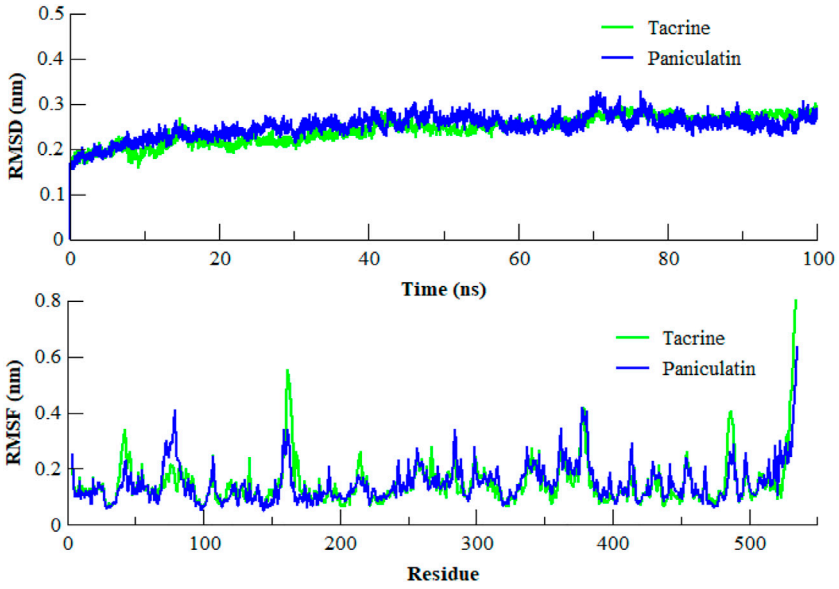

FIGURE 5
RMSD and RMSF of the backbone atoms (CA, N, and C) of AChE in complex with Paniculatin (**3**) and Tacrine with respect to the initial structure as a function of time. RMSD of Tacrine shown in green color while Paniculatin (**3**) shown in blue color.

TABLE 2 Predicted ADME profile of tested coumarin derivatives (1–3).

Ligands	*Mol Weight (g/mol)*	*HBA*	*HBD*	*TPSA (Å²)*	*iLOGP*	*WLOGP*	*GI absorption*	*BBB*	*P-gp substrate*	*Lipinski violations*
1	304.34	5	1	68.90	3.00	2.49	High	Yes	No	0
2	276.28	5	1	76.74	1.97	1.74	High	No	No	0
3	360.40	6	0	82.81	3.31	3.33	High	No	No	0

3.2 Molecular docking studies

To gain further insights into the binding modes and plausible mechanism of cholinesterase inhibition, molecular docking studies of all three compounds were carried out using crystal structure of AChE and BChE. The docking results revealed that all three compounds gained access to the deep aromatic gorge of AChE and BChE. The docking poses of compound (**1**) at the binding site of AChE suggested that the compound was unable to accommodate between Trp84 and Phe330 properly and showed the binding affinity of −3.28 kcal/mol. This might be attributed to the presence of unsaturated methylene (-enyl) group in the side

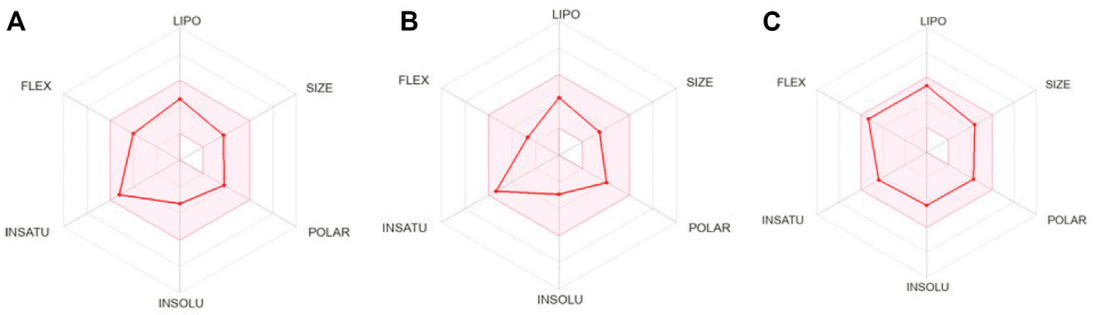

FIGURE 6
Bioavailability radar of **(A)** 2′-*O*-ethylmurrangatin (**1**), **(B)** murranganone (**2**) and **(C)** paniculatin (**3**).

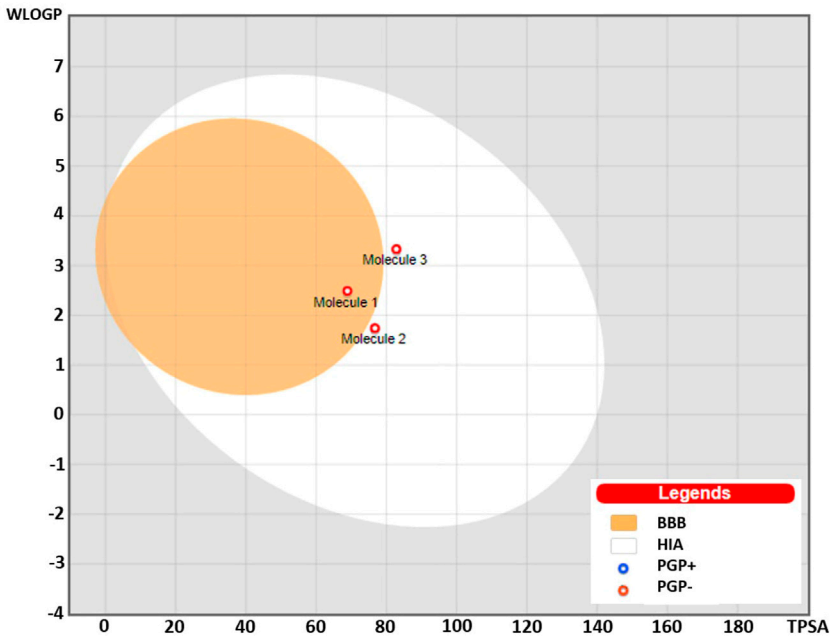

FIGURE 7
BOILED-Egg predictive model showing placement of compounds (**1**), (**2**), and (**3**). Compound (**1**) was placed in the yolk suggesting probability for permeability to the brain, while compounds (**2**) and (**3**) were placed in the white region predicting high gastrointestinal absorption. The red dots predicted to be not effluated from the central nervous system (CNS) by the P-glycoprotein.

chain at the C-3′ position as well as the absence of carbonyl group at C-2′ position which decreased the binding and made the compound inactive against AChE enzyme (Figure 3A). However, the ethoxy group at C-2′ position of compound (**1**) was observed to have hydrophobic interaction with Tyr442 residue.

Most of the docked poses of compound (**2**) showed that the compound bound to the peripheral anionic site at the aromatic gorge of AChE by interacting with Trp279 and Phe331 (Figure 3B) residues with the coumarin ring stacked between them. Similarly, isopropyl group present in the side chain at the carbonyl group-containing C-2′ position mediated alkyl-alkyl interaction with Trp279 which helped the compound to firmly bind to the AChE with the binding affinity of −4.07 kcal/mol. In addition, hydroxyl group at C-1′ position participated in the formation of a hydrogen bond with Gln74 which further improved the binding. The obtained results suggested that the compound (**2**) resided into the peripheral anionic site with good stability and showed non-competitive binding. Compound (**3**) was observed to be stacked in the binding site of AChE between the indole and phenyl rings of Trp84, Phe330 and Tyr442 amino acids with the highest binding affinity of −4.5 kcal/mol. Results revealed that the compound (**3**) bound to the anionic substrate binding site of AChE and would act as a competitive inhibitor (Figure 3C) of the enzyme. Isopropyl group in the side chain at carbonyl group-containing C-2′ position mediated π-alkyl interaction with Phe330. In addition, butyric acid ester group present in the compound (**3**) at C-1′ position mediated alkyl-alkyl interaction with Trp84 and Leu127 amino acid residues further increased the binding.

At the binding site of BChE enzyme, compound (**1**) resided into the aromatic gorge by establishing hydrophobic and hydrogen bond interactions with moderate binding affinity of −6.47 kcal/mol (Figure 4A). Carbonyl group at C-2 position mediated hydrogen bonding with Gly117 and Ser198. Similarly, methylene group in the side chain of C-8 bent towards the Trp82, while the coumarin ring was observed to be bent towards the Thr120. The docked pose of compound (**2**) at the binding site of BChE demonstrated two hydrogen bonds with the highest binding affinity of −7.92 kcal/mol (Figure 4B). Hydroxyl group at C-1′ and ketonic group at C-2′ mediated hydrogen bonds with Thr120, whereas the coumarin ring stacked against Trp82 residue. Furthermore, isopropyl group present in the side chain of ketonic group at C-2′ position mediated hydrophobic interaction with Tyr332. The potency of compound (**2**) against the enzyme BChE can be attributed, in part, to the two hydrogen bonds with the Thr120 and π–π stacking with Trp82 residue. The third coumarin paniculatin (**3**) presented hydrophobic interactions only at the binding site of BChE with good binding affinity of −7.01 kcal/mol (Figure 4C). The ester group at C-1′ position also mediated hydrophobic interactions with Ala328, Tyr332, and Trp430, whereas the coumarin ring interacted with Trp82 by mediating hydrophobic interaction.

The docking studies revealed that 2′-*O*-ethylmurrangatin (**1**) which has ethoxy group at C-2′, unlike murranganone (**2**) and paniculatin (**3**) having ketonic group at C-2′, did not inhibit AChE and was also a weak inhibitor of BChE. Paniculatin (**3**) closely resembles murranganone (**2**) with the only difference being the presence of a large butyric acid ester group in compound (**3**) at C-1′ instead of hydroxyl group in compound (**2**). The data suggested that the ketone functionality at C-2′ might be an important structural determinant of cholinesterase inhibition. Also, the isopropyl group in compounds (**2**) and (**3**) mediated the hydrophobic, alkyl-alkyl and π-alkyl interactions with aromatic amino acids such as tyrosine, tryptophan and phenylalanine showing importance of this

group in binding to the active sites of AChE and BChE proteins. The absence of isopropyl group in compound (**1**) decreased the interaction and thereby activity against AChE.

3.3 Molecular dynamics (MD) simulation

The MD simulation is a computational technique which provides the information about dynamic behavior of a molecular system (Hollingsworth and Dror, 2018). It is also useful in determining the time-dependent stability of protein-ligand complexes. To determine the most persistent interactions and to assess the stability of protein-ligand complexes over time, MD simulation studies were carried out for the coumarins. We employed 100 ns MD simulation of AChE in complex with the most potent compound paniculatin (**3**) and the standard drug Tacrine (Figure 5). During the course of simulation, the coumarin ring of paniculatin (**3**) helped in stacking between the Trp84 and Phe330 residues. The characteristic hydrophobic contacts of the compound with Leu127, Ile439, and Tyr442 residues were found to be persistent during simulation. Results of MD simulation testified the docking results since most of the docking interactions were shown to be consistent during the course of simulation. The root mean square deviation (RMSD) of the backbone atoms was plotted to determine the stability of the complex. As evident from Figure 5, the stability of AChE in complex with paniculatin (**3**) was very much comparable to Tacrine-AChE complex during the simulation. Both complexes showed RMSD of less than 0.32 nm with inconsiderable fluctuations. Similarly, both complexes showed similar patterns of root mean square fluctuation (RMSF) values and the average RMSF for both the complexes was found to be 0.14 nm. It was observed that the anionic site residues were more rigid and less flexible during the course of simulation. These observations indicated that paniculatin (**3**) significantly anchored in the active aromatic gorge of AChE and contributed to the stability of protein which pronounced the observed biological activity.

3.4 *In silico* absorption, distribution, metabolism and excretion (ADME) prediction

Most of the drug candidates fail during the clinical trials owing to their insufficient efficacy and/or toxicity. Apart from efficacy and toxicity, other important parameters leading to failure in drug development are pharmacokinetics and bioavailability (Thompson, 2000; Hassan et al., 2022). *In silico* ADME studies is an alternative to experimental techniques in the early process of drug discovery with an aim to increase the success rate in clinical trials. Herein, SwissADME was used to predict and evaluate the ADME profile of the tested coumarin derivatives and the results obtained are summarized in Table 2. To evaluate the oral bioavailability, a radar was plotted presenting six physiochemical properties including liphophilicity, size, polarity, solubility, saturation and flexibility. As evident from Figure 6, all six physiochemical chemical properties for the three tested compounds (**1–3**) fall into the acceptable range (pink region) and all the compounds were in significant agreement with the given criteria to be considered as drug-like.

The Brain or Intestinal Estimated Permeation predictive model (BOILED-Egg) was applied to estimate the gastrointestinal absorption and permeability to brain by computing the lipophilicity and polarity of the molecules. The WLOGP values (Log P calculated using Wildman and Crippen method) and topological polar surface area (TPSA) were estimated and placed in the BOILED-Egg predictive model. As evident in Figure 7, compound (**1**) was placed in the yolk (yellow) region of the egg suggesting high probability of this compound to cross blood-brain barrier (BBB) and permeate to the brain, while compound (**2**) and (**3**) fall into the white region of the egg indicating high probability of these compounds to be absorbed in the gastrointestinal region. The red dots in the figure suggest that these compounds are not predicted to be effluated from the central nervous system (CNS) by the P-glycoprotein (P-gp).

3 Conclusion

The 8-substituted-7-methoxy coumarins isolated from orange jasmine were identified as a new class of natural coumarins active against the cholinesterases enzymes; thereby can be regarded as potential candidates for AD. These coumarins showed non-selective moderate to good *in vitro* activity against both AChE and BChE by mixed-type inhibition mechanism. The structural features important for binding to the receptors were identified using molecular docking technique. It was observed that the hydrogen bonding by ketone and hydroxyl functionalities at different positions played an important part in binding to the receptor, whereas the isopropyl group at the C-3′ position was involved in the non-covalent hydrophobic interactions. The stability of drug-enzyme complex for the most active compound (**3**) as measured using the MD simulation studies was comparable to the standard drug Tacrine. These compounds showed optimal physicochemical properties to be regarded as drug-like molecules and can be explored further as lead molecules in order to improve the binding and efficacy.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effect of nutrition in Alzheimer's disease: A systematic review

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Background and objective: Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by declining cognitive ability. Currently, there are no effective treatments for this condition. However, certain measures, such as nutritional interventions, can slow disease progression. Therefore, the objective of this systematic review was to identify and map the updates of the last 5 years regarding the nutritional status and nutritional interventions associated with AD patients.

Study design: A systematic review.

Methods: A search was conducted for randomized clinical trials, systematic reviews, and meta-analyses investigating the association between nutritional interventions and AD published between 2018 and 2022 in the PubMed, Web of Science, Scopus, and Cochrane Library databases. A total of 38 studies were identified, of which 17 were randomized clinical trials, and 21 were systematic reviews and/or meta-analyses.

Results: The results show that the western diet pattern is a risk factor for developing AD. In contrast, the Mediterranean diet, ketogenic diet, and supplementation with omega-3 fatty acids and probiotics are protective factors. This effect is significant only in cases of mild-to-moderate AD.

Conclusion: Certain nutritional interventions may slow the progression of AD and improve cognitive function and quality of life. Further research is required to draw more definitive conclusions.

KEYWORDS

Alzheimer's disease, nutrition, microbiota, Western diet, Mediterranean diet

Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease characterized by decline in cognitive and functional abilities, episodic loss of memory and language skills, neuropsychiatric symptoms, and premature death. Currently, dementia affects approximately 25 million people worldwide, and it is estimated that due to the increase in life expectancy by the year 2050, there will be at least 115.4 million people with this disease. Within the group of all dementias, it is estimated that it occupies a frequency

between 60 and 80%, and is thus being the most common form of dementia. Although the cause of AD is not well-understood, is manifests at the biochemical level, it manifests as the accumulation of amyloid-beta peptide (Aβ) deposits and the formation of neurofibrillary tangles of tau protein in the brain. Currently, there is no effective treatment to reverse this situation. In addition, pharmacological treatments usually have adverse effects that can worsen patient. Research carried out in animal models shows that there is a relationship between nutrition and the biochemistry of Alzheimer's disease. However, the knowledge of the exact mechanisms is still scarce. It has been shown that adopting certain measures can slow down its progression, among which the nutritional approach has become increasingly important (Agahi et al., 2018; Samadi et al., 2019; Yilmaz and Arica Polat, 2019; Goncalves Tosatti et al., 2022; Shrestha et al., 2022; Simsek and Uçar, 2022; Miculas et al., 2023).

Recent findings suggest that AD onset and development are strongly correlated with lifestyle, including diet. Appropriate nutritional intervention may be a good approach for delaying neurocognitive decline and reducing the risk of AD onset and development. Following a healthy dietary pattern, a high intake of plant-based foods, probiotics, nuts, and omega-3 polyunsaturated fatty acids and a low intake of saturated fats, animal-based proteins, and refined sugars can decrease the risk of neurocognitive impairment (Pistollato et al., 2018; Abduljawad et al., 2022).

In addition, vitamins and minerals serve numerous vital functions, including modulation of brain health and cognitive function. Therefore, administering these micronutrient supplements can help maintain adequate cognitive activity or even prevent dementia (Karthika et al., 2022). However, the evidence in this field of research is still limited (Rutjes et al., 2018). Therefore, the objective of this review was to identify and map the updates of the last 5 years regarding the nutritional status and nutritional interventions associated with AD patients.

Materials and methods

Study design

Systematic review.

Search strategy and data sources

Between December 2022 and January 2023, a search was carried out for documents published in the last 5 years in the PubMed, Web of Science, Scopus, and Cochrane Library databases. The keywords used to search for articles were: Alzheimer Disease, Alzheimer Dementia, Alzheimer's Disease, Antioxidant, Caloric restriction, Carotenoids, Choline, DHA, Diet, Diet intervention, Dietary pattern, Docosahexaenoic, Eicosapentaenoic, Fatty acids, Fish oil, Green tea, Ketonic diet, Mediterranean diet, Microbiota, Micronutrient, Nutrient, Nutrition, Oil, Olive oil, Omega-3, Polyphenol, Prebiotic, Probiotic, PUFA and Resveratrol. The Boolean operator used were AND and OR. The exact search equation can be found in the **Supplementary material**.

Inclusion criteria

The selected documents were (1) randomized clinical trials, systematic reviews, and meta-analyses that explored the relationship between nutrition and Alzheimer's disease, (2) published between 2018 and 2022, (3) in English; (4) implemented in the over-18 years-old population, men, and/or women, and (5) full text available.

Exclusion criteria

The exclusion criteria were (1) studies that were not performed in humans and (2) studies that were not related to the topic of this review, such as other types of dementias or pharmacological interventions. Two researchers searched and screened the documents, and the consensus of the researchers resolved discrepancies regarding the selected documents.

Results

Study characteristics

A total of 955 documents were found, of which 38 articles were included in the study. **Figure 1** summarizes the selection process of the studies included in this review. Regarding the epidemiological design of the included studies, 17 studies were randomized clinical trials (RCT), and 21 were systematic reviews and/or meta-analyses. **Table 1** describes the main characteristics of the studies included in this review.

Role of diet in Alzheimer's disease

Protein-calorie malnutrition is strongly correlated with patients with cognitive decline and AD (Doorduijn et al., 2019). Wu et al. (2022) found that adequate nutritional support can significantly improve the quality of life, cognitive function, and psychological and nutritional status of elderly AD patients. Furthermore, nutritional support was associated with better sleep quality as measured by the Pittsburgh Sleep Quality Index (PSQI) (Wu et al., 2022).

An unhealthy diet pattern, such as a high-fat diet with a high glycemic load and high cholesterol or a Western diet, is an important risk factor for neurodegeneration because it increases Aβ peptide stores and other biomarkers of neurodegeneration in AD. Conversely, following a healthy dietary pattern, such as a DASH (Dietary Approaches to Stop Hypertension), Mediterranean, or low-fat diet, has neuroprotective effects in preventing AD. The main mechanisms are based on the reduction of oxidative stress and inflammation and a lower accumulation of Aβ peptides. Greater adherence to the Mediterranean diet decreased the levels of IL-6, TNF-α, CRP, and LDL. The DASH diet is characterized by low consumption of red and processed meat and high consumption of fruits, vegetables, and whole grains. The nutritional profile is high in potassium, calcium, magnesium, and fiber, whereas the sodium

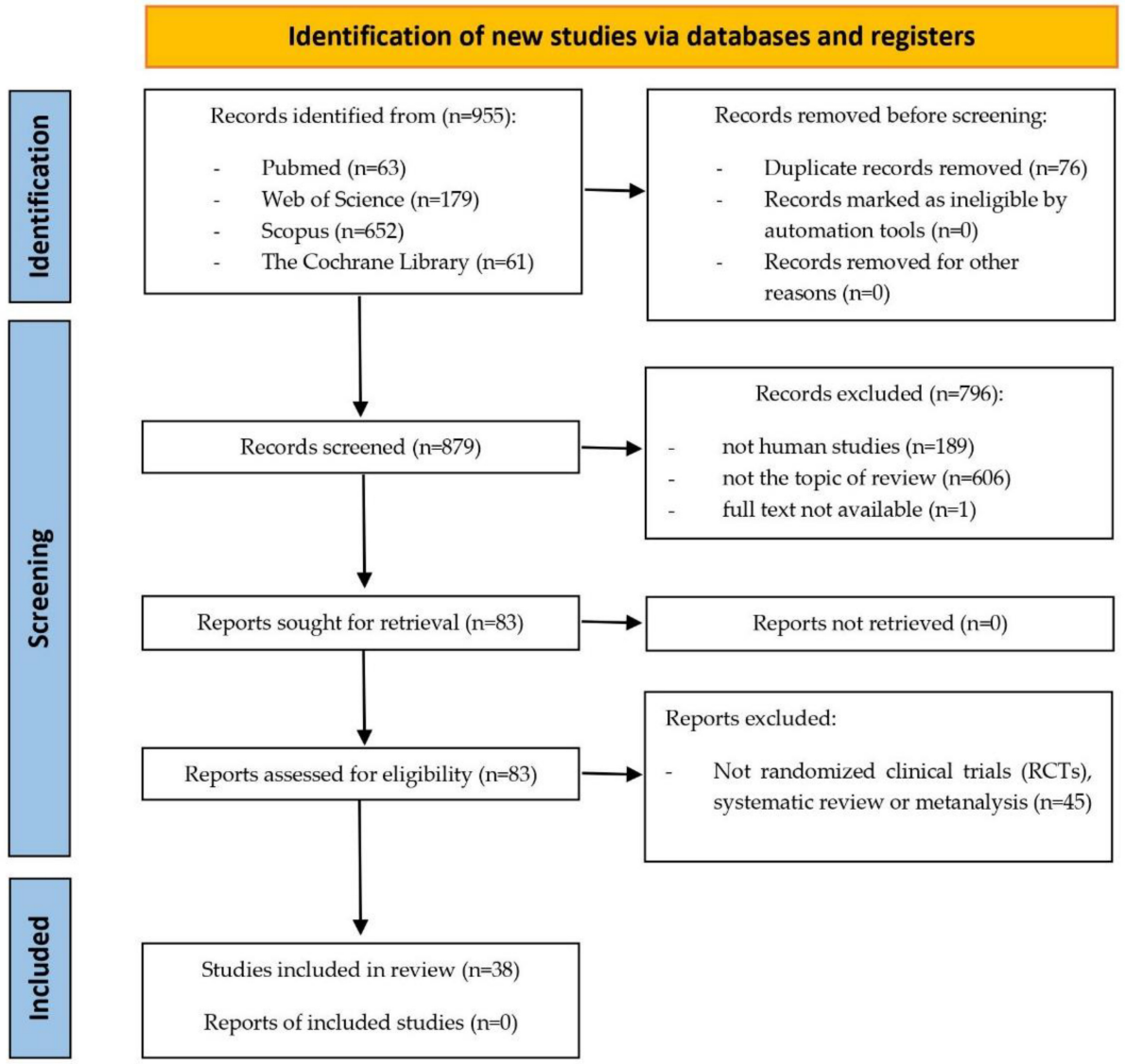

FIGURE 1
Flow chart.

and saturated fat contents are relatively low. For this reason, both the Mediterranean and DASH diets have anti-inflammatory effects and are capable of reducing oxidative stress, exerting a protective role against AD (Hill et al., 2019; Samadi et al., 2019; García-Casares et al., 2021). In addition, the Mediterranean diet provides numerous health benefits, including improved cerebral perfusion. However, this effect is more significant in patients with AD in the mild or early stages, whereas the effect in more advanced stages is not noticeable. In contrast, the Western dietary pattern increases the risk of AD by altering metabolic health and reducing cerebral perfusion and thus impairing cognition (Hoscheidt et al., 2022).

Patients with AD present with alterations in brain metabolism. Recent studies have argued that ketone bodies can help correct this situation. Ketone bodies are a direct source of cellular energy from fat metabolism and can be used as a source of energy for the brain when the glucose supply is limited. Recent research suggests that ketone bodies may improve episodic memory, temporary memory, semantic memory, and vitality in patients with early AD, and this effect is more evident in women. Ketone bodies have also been shown to improve functional capacity and quality of life, which are essential for AD patients. However, one of the most common problems with the ketogenic diet is that it is difficult to maintain over the long term because of its characteristics. Phillips et al. (2021) stated that high rates of retention, adherence, and safety can be achieved when it is well-established (de la Rubia Orti et al., 2018; Brandt et al., 2019).

Effect of fatty acids in Alzheimer's disease

The use of omega-3 fatty acid in AD has been widely studied. Nevertheless, omega-3 fatty acids did not improve cognitive and functional decline nor depressive symptoms in an RCT conducted

TABLE 1 Main characteristics of the studies included in this review.

References	Objectives	Method/Sample/ Duration	Measuring instruments	Intervention design	Results
Doorduijn et al. (2019)	To examine potential difference in energy and protein intake in patients with MCI and AD compared to controls as a possible mechanism for unintended weight loss.	Systematic review and meta-analysis *n* = 7 articles	Food diary, 24 h recall, survey of food intake	Case-control design (patients with AD and control)	No lower energy and protein intake of patients with AD compared to controls.
Wu et al. (2022)	To study the effect of nutritional support under the clinical nursing path on the nursing effect, quality of life, and nutritional status of elderly patients with AD.	RCT *n* = 110 patients	Nursing efficiency, QLI, PSQI, MSSNS, SDSS, MNA	Nutritional support (*n* = 55) control group (*n* = 55)	Nutritional support was associated with significantly QLI scores and significant lower PSQI, MSSNS, and SDSS scores, and fewer malnourished cases versus routine nursing ($p < 0.05$).
García-Casares et al. (2021)	To conduct a systematic review and meta-analysis to determine the effects of a higher adherence to MD on MCI and AD.	Systematic review and meta-analysis *n* = 11 articles	MDA, FFQ	OS (cross-sectional, case-control or longitudinal cohort studies) and RCT	Higher adherence to MD was associated with a significantly lower risk of MCI and lower risk of AD.
Hill et al. (2019)	To summarize the evidence relating diet and nutrition to the hallmark AD biomarkers (tau and β-amyloid).	Systematic review and meta-analysis *n* = 5 articles	Glycemic indices, MDA, PET imaging, CSF levels, plasma biomarkers of AD, αβ- amyloid and tau	RCT, cross sectional and longitudinal studies	Significant effect ($p = 0.002$) of diet on AD biomarkers. Adherence to a MeDi-styled dietary pattern was associated with a reduction in AD biomarkers and subsequent pathology. Adherence to a high-glycemic, high saturated fat diet was associated with an increase in AD biomarker burden.
Samadi et al. (2019)	To review the evidence on the relation between dietary pattern and AD.	Systematic review *n* = 26 articles	FFQ, food record	OS (prospective, retrospective, cross-sectional or case-control studies)	Adherence to healthy diet can decrease oxidative stress and inflammation and accumulation of amyloid-β and consequently can decrease the risk of AD.
Hoscheidt et al. (2022)	To compare the effects of the diet intervention on metabolic parameters, CSF biomarkers, cerebral perfusion assessed with MRI, and cognition.	RCT *n* = 87 participants 4 weeks	Lumbar puncture, blood biomarkers, cognitive tests, MRI	Med-diet (*n* = 44) West-diet (*n* = 43)	Dietary patterns are powerful modulators of metabolic function, cerebrovascular health, AD pathology, and cognitive function.
Brandt et al. (2019)	To establish the feasibility of implementing the MAD in older adults with early AD or MCI due to AD. To examine whether changes in participants′ cognition, behavioral, and emotional functioning are more favorable in those in the MAD than in those in a control diet.	RCT parallel, controlled trial *n* = 14 patients 12 weeks	MMSE-2-EV, POMS-Bi	MAD (*n* = 9) Control (*n* = 5)	The MAD may not be very easy to implement in the MCI/mild AD population. It may be useful for patients that agree to continue this diet.
de la Rubia Orti et al. (2018)	To detect changes in the main cognitive functions of patients with AD after following a coconut oil enriched MD, and to determine whether there are differences in function of stage or sex.	RCT *n* = 44 patients 21 days	7 min screen	Coconut oil enriched Mediterranean Diet (*n* = 22) Control (*n* = 22)	After intervention with coconut oil, improvement in episodic, temporal orientation, and semantic memory were observed, and it seems that the positive effect is more evident in women with mild-moderate state, although other improvements in males and severe state were also shown.

(Continued)

TABLE 1 (Continued)

References	Objectives	Method/Sample/ Duration	Measuring instruments	Intervention design	Results
Phillips et al. (2021)	To determine whether a 12 weeks modified ketogenic diet was well-tolerated and improved cognition, daily function, or quality of life in a hospital clinic of AD patients	RCT crossover n = 26 patients 12 weeks	ACE-III, ADCS-ADL, QLI	Modified ketogenic diet (n = 13) usual-ketogenic diet (n = 13)	Compared with a usual diet supplemented with low-fat healthy-eating guidelines, patients on the ketogenic diet improved in daily function and quality of life.
Lin et al. (2022)	To determine whether the n-3 PUFAs supplementation in different regimens could modulate the levels of pro-inflammatory cytokines and restore some cognitive and functional abilities, as well as mood status in patients with cognitive impairment.	RCT placebo-controlled 163 MCI or AD patients 24 months	Cognitive and functional abilities, biochemical, and inflammatory cytokines profiles	Placebo (n = 40) DHA (n = 41) EPA (n = 40) DHA + EPA (n = 42)	n-3 PUFAs supplements did not reduce cognitive, functional, and depressive symptom outcomes, but spoken language ability and constructional praxis subitems of ADAS-cog did.
Jernerén et al. (2019)	To investigate whether baseline levels of plasma tHcy, a marker of B vitamin status, modify the effects of n-3-PUFAs supplementation on cognitive performance in moderate AD.	RCT 171 patients 6 months	Plasma aminoacids, MMSE, ADAS-cog, Global CDR, CDRsob	n-3 PUFAs (n = 88) placebo (n = 83)	The effect of n-3 PUFAs supplementation on MMSE and CDR appears to be influenced by baseline tHcy, suggesting that adequate B vitamin status is required to obtain beneficial effects of n-3 PUFAs on cognition.
Nolan et al. (2022)	To build on the existing exploratory research and investigate the impact of the micronutrients on the natural progression of AD in a RCT.	RCT n = 77 patients 12 months	Blood analysis, MMSE, CDR	Fish oil, carotenoids, vitamin E (n = 50) placebo (n = 27)	The active group also performed better in objective measures of AD severity (i.e., memory and mood), with a statistically significant difference reported in the clinical collateral for memory ($p < 0.001$).
Albrahim (2020)	To find out the potential role of nutritional components in improving brain function among patients with AD.	Systematic review and meta-analysis n = 19 RCT	Omega-3, DHA, vitamin C, E, B12, folate, homocysteine	RCT	Chain-free SFA and TFA occur in greater amounts in the brains of individuals with AD than in those without AD.
Araya-Quintanilla et al. (2020)	To determine if there is scientific evidence of the effectiveness of omega-3 supplementation in improving cognitive function in patients with AD.	Systematic review and meta-analysis n = 6 articles	MMSE, ADAS-COG, CDR, NPI	RCT	No consistent evidence to support the effectiveness of omega-3 supplementation in improving cognitive function in AD patients in the short and medium term.
Zhu et al. (2021)	To provide new evidence on relationships between dietary fatty acid intake and cognition.	Prospective cohort meta-analysis n = 14 articles	Dietary fatty acids intake	Prospective cohort study	The intake of total fatty acids, SFAs, MUFAs, PUFAs, and omega-3 PUFAs was not significantly associated with dementia risk. However, omega-3 PUFA intake may be negatively associated with MCI risk.
Canhada et al. (2018)	To evaluate the effects of omega-3 fatty acids supplementation on cognitive function in AD patients.	Systematic review n = 7 articles	ADAS-cog, CIBIC-plus, MMSE, HDRS, MADRS, CDR, ADCS-ADL, ADCS-IADL, CGB, DAD, NPI, NTB, BADLS	RCT	Omega-3 fatty acids may be beneficial in disease onset, when there is slight impairment of brain function.
Moreira et al. (2020)	To evaluate the effect of dietary interventions on the cognitive performance of individuals with AD.	Systematic review n = 32 RCT	MMSE, ADAS-cog, CDR-SOB	RCT	Omega-3 fatty acid showed positive effects at different doses. Probiotic, ginseng, inositol and specialized nutritional formulas seemed to have a positive effect on cognition.

(Continued)

TABLE 1 (Continued)

References	Objectives	Method/Sample/ Duration	Measuring instruments	Intervention design	Results
Samieri et al. (2018)	To conduct a statistically powerful investigation of fish intake and decline in global cognition and episodic memory.	Systematic review $n = 5$ articles	FFQ, global cognition, episodic memory	Cohort studies	Higher fish intake was associated with slower decline in both global cognition and memory ($p < 0.031$).
Agahi et al. (2018)	To evaluate responsiveness of the inflammatory and oxidative biomarkers to the probiotic treatment.	RCT double blind 48 patients 12 weeks	Test Your Memory, serum concentrations	Probiotic ($n = 25$) control ($n = 23$)	Cognitive and biochemical indications in the patients with severe AD are insensitive to the probiotic supplementation.
Akbari et al. (2020)	To assess the effects of probiotic supplementation on cognitive function and metabolic status.	RCT double-blind controlled trial $n = 60$ patients	MMSE	Probiotics ($n = 30$) control ($n = 30$)	The probiotic treated patients showed a significant improvement in the MMSE score ($p < 0.001$).
Doulberis et al. (2021)	To investigate the accessible regarding possible association between AD and gastrointestinal microbiota.	Systematic review $n = 24$ articles	Gut microbiota analysis	OS	Positive association between gastrointestinal microbiota and deterioration of AD.
González Cordero et al. (2022)	To provide the best scientific evidence available on the relationship between the gut microbiota and AD.	Systematic review $n = 8$ articles	Gut microbiota analysis	OS	There is a high association between the decrease in the richness of the microbiota and the incidence of AD.
Liu et al. (2022)	To evaluate the clinical value of intestinal flora balance therapy supported by probiotics in improving cognitive function and symptoms in patients with AD.	Systematic review and meta-analysis $n = 5$ RCT	MMSE, instant memory score, ADAS-cog, ADL	RCT	Intestinal flora balance therapy based on probiotic support can effectively improve cognitive function, instantaneous memory, and ability of daily life in patients with AD.
Tamtaji et al. (2019)	To determine the effects of probiotic and selenium supplementation on cognitive function and metabolic status among patients with AD.	RCT double blind controlled trial 79 patients 12 weeks	MMSE, biochemical analysis, gene expression	Selenium + probiotics ($n = 27$) selenium ($n = 26$) placebo ($n = 26$)	Probiotic and selenium supplementation improved cognitive function and biochemical profiles.
Mullan et al. (2018)	To compare the plasma antioxidant status of individuals with AD and cognitively intact controls.	Meta-analysis of case-control studies $n = 52$ articles	α-carotene, β-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, vitamin A, C, E, uric acid	Case-control studies	Patients with AD had significantly lower plasma levels of α-carotene, β-carotene, lycopene, lutein, vitamin A, C, and E, and uric acid.
Qu et al. (2021)	To evaluate the associations of six major members of carotenoids with the occurrence of AD by conducting systematic review and meta-analysis.	Systematic review and meta-analysis $n = 16$ articles	Plasma and serum carotenoids analysis	Cross-sectional study, cohort study, case-control study	Lutein and zeaxanthin concentrations in plasma/serum were inversely related to the risk of AD.
Chen et al. (2021)	To evaluate the combined action of folic acid and vitamin B12 supplementation on cognitive performance and inflammation in patients with AD.	RCT single-blinded placebo-controlled trial 120 patients 6 months	Cognitive performance, blood folate, vitamin B12, inflammatory cytokine levels	Folic acid + vitamin B12 ($n = 60$) placebo ($n = 60$)	Folic acid and vitamin B12 supplementation showed a positive therapeutic effect in AD patients who were not on a folic acid-fortified diet.
Chai et al. (2019)	To comprehensively explore the associations between serum 25(OH)D deficiency and risk of dementia and AD.	Meta-analysis $n = 12$ articles	Vitamin D concentration	Prospective cohort, cross-sectional	There are significant associations between vitamin D deficiency and both dementia and AD. There are stronger associations between severe vitamin D deficiency (<10 ng/ml) and both dementia and AD compared to moderate vitamin D deficiency (10–20 ng/ml).

(Continued)

TABLE 1 (Continued)

References	Objectives	Method/Sample/ Duration	Measuring instruments	Intervention design	Results
Jayedi et al. (2019)	To test the dose-response association of serum 25(OH)D and risk of dementia and AD.	Meta-analysis $n = 8$ cohort studies	Vitamin D concentration	Prospective cohort, retrospective cohort	Higher levels of serum 25(OH)D were associated with a lower risk of dementia and AD.
Du et al. (2020)	To investigate if vitamin D supplementation can prevent AD.	Systematic review and meta-analysis of RCT $n = 9$ articles	MMSE	RCT	No significant difference in the MMSE, verbal fluency, verbal memory, visual ability, and attention scores between the vitamin D group and comparison group.
Yang et al. (2019)	To synthesize the association of serum vitamin D concentrations with AD in adults.	Meta-analysis of prospective cohort studies $n = 6$ articles	Vitamin D concentration	Prospective cohort	Serum vitamin D deficiency (<25 nmol/L) or insufficiency (25–50 nmol/L) was not statistically significant and associated with the risk of AD.
Jia et al. (2019)	To assess the effect of a 12 months vitamin D supplementation on cognitive function and amyloid β-related biomarkers in subjects with AD.	RCT double-blind placebo-controlled 210 patients 12 months	Test of cognitive performance and αβ-related biomarkers	Vitamin D ($n = 105$) placebo ($n = 105$)	Vitamin D group had significant increase in full scale IQ during follow-up period ($p < 0.001$).
Belitskaya-Lévy et al. (2018)	To examine the role of APOE genotypes on the effect of treatment in delaying the rate of functional decline of AD.	RCT $n = 415$ 6 months	ADCL-ADL	ε4 non-carriers ($n = 209$) ε4 carriers ($n = 206$)	Vitamin E group had slower functional decline than those receiving placebo.
Buglio et al. (2022)	To assess the effects of resveratrol on MCI and AD.	Systematic review $n = 5$ articles	Brain volume, MMSE	Interventional studies	In AD patients, the use of resveratrol improves brain volume, reduces the MMSE, and improves AD scores. In patients with MCI, this polyphenol prevents decline in Standard Volumes of Interest and increases the Resting-state Functional Connectivity score.
Fang et al. (2022)	To explore the effect of resveratrol combined with donepezil hydrochloride on inflammatory factor level and cognitive function level of patients with AD.	RCT $n = 90$ patients 12 months	MMSE, FIM, ADAS-cog	Resveratrol ($n = 45$) control ($n = 45$)	Resveratrol group obtained significantly higher good rate, MMSE score, and FIM score ($p < 0.05$) and lower clinical indicators and ADAS-cog score ($p < 0.001$).
Gu et al. (2021)	To investigate the antagonistic effect of trans-resveratrol on moderate to mild AD.	RCT $n = 30$ patients 52 weeks	MRI, CSF	Trans-resveratrol ($n = 15$) placebo ($n = 15$)	Neuroprotective role of trans-resveratrol in patients with mild to moderate AD.
Zhu et al. (2018)	To evaluate the safety, tolerability, and efficacy of an oral preparation of resveratrol, glucose, and malate (RGM) in slowing the progression of AD.	RCT double-blind placebo-controlled $n = 39$ patients 12 months	ADAS-cog, CIBIC-plus, MMSE, ADCS-ADL, NPI	Resveratrol ($n = 17$) placebo ($n = 15$)	Change scores on ADAS-cog, MMSE, ADCS-ADL, or NPI all showed less deterioration in the treatment than the control group; however, none of the change scores was significant.
Kakutani et al. (2019)	To examine the association between green tea intake and dementia, AD, MCI, or cognitive impairment.	Systematic review $n = 8$ articles	Food intake, FFQ	OS	Green tea intake might reduce the risk of dementia, AD, MCI, or cognitive impairment.

ACE-III, addenbrookes cognitive examination-III scale; AD, Alzheimer's disease; ADAS-cog, Alzheimer's disease assessment scale-cognitive section; ADCS-ADL, Alzheimer's disease cooperative study-activities of daily living; ADCS-IADL, Alzheimer's disease cooperative study-instrumental activities of daily living; BADLS, Bristol's activities of daily living scale; CDR, clinical dementia rating scale; CDR-SOB, clinical dementia rating scale-sum of boxes; CGB, caregivers burden scale; CIBIC-plus, clinicians global impression of change; CSF, cerebrospinal fluid; DAD, disability assessment for dementia scale; FFQ, food frequency questionnaire; HDRS, Hamilton depression scale; MAD, modified Atkins diet; MADRS, Montgomery Asberg depression rating scale; MCI, mild cognitive impairment; MD, Mediterranean diet; MDA, Mediterranean diet adherence; MMSE, mini-mental state examination; MNA, mini nutritional assessment; MRI, magnetic resonance imaging; MSSNS, mental status scale in non-psychiatric settings; NPI, neuropsychiatric inventory; NTB, neuropsychological test battery; tHcy, total homocysteine; OS, observational studies; PSQI, Pittsburgh Sleep Quality Index; QLI, quality of life index; RCT, randomized controlled trial; SDSS, social disability screening schedule.

by Lin et al. (2022) but spoken language ability did. It seems that this biomolecule must interact with other micronutrients to achieve this effect. Jernerén et al. (2019) suggested that adequate levels of B vitamins are needed for omega-3 fatty acids to effect cognition. On the other hand, other micronutrients, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), carotenoids, or vitamin E, can also help improve memory and mood. This effect is significant only in milder-to-moderate stages of the disease (Nolan et al., 2022).

Patients with AD tend to have higher serum saturated and trans-fatty acid concentrations than the general population. This is associated with alterations in the metabolomics of the tyrosine, tryptophan, purine, and tocopherol pathways. Albrahim's meta-analysis found that supplementation with these nutrients can improve cognitive decline, functional connectivity, and brain atrophy (Albrahim, 2020). We found more controversy regarding the effects of omega-3 fatty acids. Meta-analyses performed by Zhu et al. and Araya-Quintanilla et al. found no decreased risk of dementia or improved cognitive function with supplementation of these fatty acids (Araya-Quintanilla et al., 2020; Zhu et al., 2021). Other studies have indicated that omega-3 fatty acids can delay cognitive aging and memory decline; however, this effect is only demonstrable in cases of mild-to-moderate dementia or when brain function decline is still ongoing in the earliest stages of the disease (Canhada et al., 2018; Samieri et al., 2018; Moreira et al., 2020).

Microbiota-brain axis

The study of microbiota-health interactions has gained great importance in the last century. The composition of microbiota affects the development and evolution of numerous diseases, including AD. AD patients have been shown to have an altered microbiota, which promotes a pro-inflammatory state, affects cognitive function, and increases the risk of neurodegeneration through the gut-brain axis. Therefore, probiotic supplementation may improve metabolic abnormalities and attenuates inflammation and oxidative stress. Additionally, it can improve cognitive function. However, these effects depend on the formulation and dose of the probiotic bacterium, severity of the disease, and timing of probiotic administration. On the other hand, a deficit of certain nutrients, such as omega-3 PUFAs, decreases the resistance to neurotoxicity produced by this altered microbiota and affects the central nervous system. Therefore, in addition to correcting the altered microbiota with probiotics and prebiotics, it is necessary to correct the deficit of the nutrients that interact with the microbiota. Correction of altered gut microbiota using probiotics may improve cognitive function and instant memory in patients with AD (Agahi et al., 2018; Tamtaji et al., 2019; Akbari et al., 2020; Doulberis et al., 2021; González Cordero et al., 2022; Liu et al., 2022).

Vitamins and other antioxidants related to Alzheimer's disease

Vitamins play an important role in the pathogenesis of AD. Patients are observed to have lower plasma levels of α-carotene, β-carotene, lycopene, lutein, vitamins A, C, and E, and uric acid than the general population (Mullan et al., 2018; Qu et al., 2021). An RCT by Chen et al. (2021) found that combined folic acid and vitamin B_{12} supplementation significantly improved cognitive performance and inflammation in patients with AD. Vitamin D, it is observed that vitamin D deficiency is significantly associated with the development of dementia and AD. The stronger the association, the greater the vitamin D deficiency (<10 ng/ml). Conversely, when serum vitamin D concentrations increase, the risk of dementia decreases (Chai et al., 2019; Jayedi et al., 2019). However, other studies, such as those by Yang et al. (2019), Du et al. (2020) found no significant association between vitamin D deficiency and improvements in AD cognitive parameters. The RCT by Jia et al. (2019) found that vitamin D supplementation for at least 12 months significantly improved cognitive function and Aβ peptide-related biomarkers. The apolipoprotein E(APOE) genotype is a known risk factor for AD. Vitamin E administration may help delay cognitive decline by modulating the response to treatment in APOE genotypes (Belitskaya-Lévy et al., 2018).

It is also suggested that resveratrol supplementation may improve cerebrovascular function and reduce the risk of developing dementia. The mechanism could be due to the fact that resveratrol is able to activate Sirt-1 and inhibit COX-2, 5-lipoxygenase and NFkB, resulting in less activation of proinflammatory pathways (Buglio et al., 2022). Other studies suggested that this is related to the Aβ peptide, which accumulates in the brains of people with AD. Resveratrol can act as an AD antagonist, fulfilling neuroprotective functions, improving inflammation levels, and promoting cognitive functions. The mechanism involves a reduction in Aβ accumulation and toxicity in the brain of these patients and a reduction in neuroinflammation. The neuroprotective effect has only been found to be significant in patients who are in the early stages of the disease (Zhu et al., 2018; Gu et al., 2021; Fang et al., 2022). In contrast, a systematic review by Kakutani et al. found that green tea intake can reduce the risk of dementia, AD, and general cognitive decline (Kakutani et al., 2019).

Discussion

Low dietary quality is a risk factor for the development AD, thus, worsening cognitive performance and verbal fluency (Hossain et al., 2019). Additionally, malnutrition and unintentional weight loss are associated with an increased risk of mortality in patients with AD (de Sousa et al., 2020). Consumption of refined carbohydrates or a diet with a high glycemic index is associated with increased accumulation of Aβ peptides in the brain. This effect is even worse in APOE-ε4 carriers, which is a genetic risk factor associated with AD and dementia, as well as insulin resistance. However, the exact mechanisms underlying this relationship remains unknown (Gentreau et al., 2020; Taylor et al., 2021). A Western diet pattern increases the risk of AD, as this diet increases inflammation levels (Wieckowska-Gacek et al., 2021). On the contrary, according to the Spain Dementia Cohort, adherence to the Mediterranean diet is associated with a 20% lower risk of dementia. The Mediterranean diet has been shown to improve cognitive outcomes, increase gray matter volume, improve memory, and decrease memory decline (Nutaitis et al., 2019; Ballarini et al., 2021; Encarnacion Andreu-Reinon et al., 2021). A ketogenic diet may also be useful in the treatment of AD, as it has

been shown to reduce oxidative stress and inflammation and reduce the negative effects of altered glucose metabolism in the brain. In addition, according to other clinical studies, this diet can improve verbal memory, attention, and overall cognitive function. However, long-term use of this diet may present risks; therefore, it should be monitored by an expert nutritionist (Simsek and Uçar, 2022).

Because AD presents with high levels of oxidative stress, adequate intake of antioxidants in the diet is a factor to be consider considered. Whether oxidative status is a cause or product of AD remains unknown (Socha et al., 2021); however, vitamin intake has been reported to help combat cognitive and memory decline (Alam, 2022). Lower levels of vitamin D are associated with worse cognitive performance scores in patients (Yilmaz and Arica Polat, 2019). However, according to the results of our review, the supplementation of vitamin D on improving AD state still lacks enough evidence (Chai et al., 2019; Jayedi et al., 2019; Jia et al., 2019; Yang et al., 2019; Du et al., 2020). Vitamin B_{12} deficiency is a fairly frequent condition in the elderly population and is a risk factor for AD (Shrestha et al., 2022). Vitamin E is a powerful antioxidant and an anti-inflammatory agent. Observational cohort studies have shown that compared to the general population, people with AD have significantly lower levels of tocopherols, tocotrienols, and total vitamin E (Casati et al., 2020). The same is true for choline (Yuan et al., 2022). On the contrary, we did not find any study that met the inclusion criteria for the nutritional intervention with vitamin C in patients with AD, which could be an interesting topic for future research. The studies included in our review indicated that the effects of nutritional interventions only work in patients with mild and moderate AD (Hoscheidt et al., 2022; Nolan et al., 2022). We did not find articles explaining why they are not useful in severe AD patients, although we think that a possible cause could be related to the high level of oxidative stress. Even though, studies with high quality done in humans about supplementation of vitamins on this topic are limited.

The study of fatty acids as nutritional factors for dementia is also a popular research topic. Adequate levels of omega-3 polyunsaturated fatty acids, especially EPA and DHA, are associated with slower rates of cognitive decline and reduced risk of AD. Therefore, it is important to advise AD patients to include fish, nuts, seeds, and vegetable oils in their diet (Gustafson et al., 2020; Chu et al., 2022; Li et al., 2022). However, the significant efficacy is still unknown, as demonstrated by our results (Lin et al., 2022). The microbiota, it is known to be a factor that must be considered in AD, as dysbiosis is a clear risk factor for the development of AD. This is because of the metabolites produced by the microbiota, which can modulate the biochemical state of the brain. This connection is called the "gut-brain axis." In addition, high-fat diets, the use of antibiotics, or the lack of probiotics and/or prebiotics can also change the composition of the microbiota and therefore be a risk factor for AD (Bello-Corral et al., 2021; Khedr et al., 2022; Szablewski, 2022). However, in this study, we found that AD was correlated with high levels of inflammation and oxidation (Goncalves Tosatti et al., 2022; Gu et al., 2022), which should be considered in future research and clinical treatments.

Most of the points included in our review are similar to those of other articles related to this field; thus, it seems that nutrition can protect and/or decrease the progression rate of AD. On the other hand, during the bibliographic search, a large number of articles investigated the relationship between microbiota and AD and malnutrition and AD, which suggests that these research topics have been topics of interest in recent years, since we filtered for the last 5 years. We also found a high percentage of studies carried out in postmortem humans, that evaluated the biochemical composition of the brain. This is one of the limitations of the study of AD, since the psychological and cognitive state is often not related to the physical and biochemical state of the brain. This is one of the reasons for the difficulty in conducting RCT with good methodological quality to study the relationship between nutrition and AD (Liu et al., 2022). Nutritional interventions are good non-pharmacological tools for the treatment of AD. However, more studies with effective methodological quality are needed to draw better conclusions.

Conclusion

The results showed that nutritional interventions are capable of slowing down the rate of progression of Alzheimer's disease, improving cognitive function, and improving the quality of life of these patients. However, many knowledge gaps remain to be investigated; therefore, a deeper study on the association between nutrition and AD is recommended.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1147177/full#supplementary-material

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Mapping new pharmacological interventions for cognitive function in Alzheimer's disease: a systematic review of randomized clinical trials

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Background and Objective: Alzheimer's disease (AD) is a progressive neurodegenerative disorder, that is, characterized by cognitive decline. To date, there are no effective treatments for AD. Therefore, the objective of this study was to map new perspectives on the effects of pharmacological treatment on cognitive function and the overall psychological state in patients with AD.

Methods: Two independent researchers searched for randomized clinical trials (RCTs) exploring new pharmacological approaches related to cognition in Alzheimer's disease in adults from 2018 to 2023 in PubMed, Web of Science, Scopus, and Cochrane Library databases. A total of 17 RCTs were included in this review.

Results: The results show that in recent years, new drugs have been tested in patients with Alzheimer's disease, including masitinib, methylphenidate, levetiracetam, Jiannao Yizhi, and Huannao Yicong formulas. Most studies have been conducted in populations with mild to moderate Alzheimer's disease.

Conclusion: Although some of the drugs found suggested improvement in cognitive function, the scarcity of available studies highlights the need for further research in this area.

Systematic review registration: [www.crd.york.ac.uk/prospero], identifier [CRD42023409986].

KEYWORDS

Alzheimer disease, cognition, pharmacology, treatment, latest research, beta-amyloid, immunotherapy

1 Introduction

Alzheimer's disease (AD) is a multifactorial progressive neurodegenerative disorder characterized by memory loss, disorientation, and gradual decline in intellectual ability (Korabecny et al., 2019). It affects approximately 46 million people worldwide and accounts for 60%–80% of all cases of dementia (Ghaffari et al., 2020). The etiology of the disease has

not yet been fully elucidated, and only one approved therapeutic approach currently exists for its treatment. The accumulation of beta-amyloid (Aβ) peptides is considered to be one of the fundamental neuropathological pillars of the disease (Dubois et al., 2023), and its dishomeostasis plays a crucial role in its onset (Jeremic et al., 2021; Torres-Mendoza et al., 2022; Tosatti et al., 2022). Researchers are investigating various therapies to combat this disease, including the modulation of targets such as Aβ aggregation, neuroinflammation, and oxidative stress, as well as the use of enhanced multiple biomarkers and risk prediction methods to detect the disease at early stages (Koh et al., 2021; Phadke et al., 2021; Levine et al., 2022). Additionally, research on miRNAs as a possible avenue for AD diagnosis, treatment, and prevention is being conducted (Ghaffari et al., 2020). The efficacy of pharmacological treatment may vary according to the characteristics of different population groups, such as age, disease severity, sex, and presence of other medical conditions (Cardinali et al., 2021).

AD is a progressive neurodegenerative pathology for which there is no definitive cure. However, there are different types of treatments that can help delay its progression and improve the quality of life of affected patients. Treatment options include medication, occupational therapy, cognitive stimulation therapy, physical exercise, massage therapy, music therapy, and nutritional supplements (Pisani et al., 2021). Pharmacological treatments available for AD include donepezil, rivastigmine, galantamine, and memantine (Miculas et al., 2023). These drugs help improve symptoms and delay the progression of the disease in some patients (Rong et al., 2020). Donepezil, rivastigmine, and galantamine are cholinesterase inhibitors used to treat mild to moderate symptoms, whereas memantine is an N-methyl-D-aspartate (NMDA) receptor antagonist used to treat moderate to severe AD symptoms (Birks and Harvey, 2018; Li et al., 2019; Thancharoen et al., 2019). The activity of cholinesterase inhibitors is characterized by the inhibition of the acetylcholinesterase enzyme, responsible mainly for the breakdown of acetylcholine in the nervous system. This allows for the prolonged action of the deficient neurotransmitter in the brain. Rivastigmine has a relatively low protein binding affinity and a more selective action with less possibility of interactions with other drugs. It is important to maintain the balance of different neurotransmitter systems, such as acetylcholine, norepinephrine, dopamine, serotonin, and glutamate, for proper brain function. Although AD is a chronic disease, most research has a limited duration of 6 months, which limits knowledge of the effectiveness of drugs in the long term (Marucci et al., 2021).

Despite advances in available treatments for AD, there is still no definitive cure. Therefore, new treatment approaches are constantly being investigated, such as immunotherapy, gene therapy, light therapy, diet, and physical exercise. Research on new approaches aims to find a more effective and specific therapy than the current treatment options, with the goal of finding a cure for this disease that affects millions of people worldwide (Liang et al., 2018a).

Several psychometric instruments have been used to assess the cognitive and functional performance of patients with AD and other related dementias. The Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-cog) evaluates memory, attention, reasoning, language, orientation, and praxis (Craft et al., 2020). The Clinical Dementia Rating (CDR) scale measures memory, orientation, judgment and problem-solving, community affairs, home and hobbies, and personal care (Gibson et al., 2020). The Neuropsychiatric Inventory (NPI) assesses a wide range of behaviors in patients with dementia (Gibson et al., 2020). The Alzheimer's Disease Cooperative Study-Activities of Daily Living (ADCS-ADL) scale is used to evaluate the patients' performance in basic and instrumental activities of daily living (Gibson et al., 2020). In addition, other global assessment scales, such as the Clinical Dementia Rating-Sum of Boxes (CDR-SB), Clinician's Interview-Based Impression Plus Caregiver Input (CIBI plus), and Clinical Global Impression (CGI), are used in patients with AD. The Mini-Mental State Examination (MMSE), ADAS-cog, and Severe Impairment Battery (SIB) scales are widely used to evaluate cognition in patients with this disease (Levine et al., 2021).

This study aimed to map out new perspectives on the effect of pharmacological treatment on cognition and overall psychological state in patients with AD.

2 Materials and method

2.1 Search strategy and data sources

From February 2023 to March 2023, a search was conducted across four databases, (PubMed, Web of Science, Scopus, and the Cochrane Library) to identify documents published within the past 5 years. To achieve the most comprehensive results possible, the search strategy employed was "Alzheimer Disease/drug therapy" [Mesh].

2.2 Inclusion criteria

The inclusion criteria for this search were as follows: 1) original articles of randomized clinical trials (RCT); 2) studies conducted in living humans with AD; 3) focusing on cognitive state or psychological aspects, such as memory, mood, etc.; 4) AD must be established at the start of the intervention; 5) adult population over 18 years, both men and women; 6) intervention must be a pharmacological treatment; 7) articles published in English; 8) published within the last 5 years, specifically from 2018 to 2023; 9) with full text available; and 10) methodological quality must score greater than three points on the JADAD scale (Jadad Bechara, 1996).

2.3 Exclusion criteria

The exclusion criteria were as follows: 1) studies conducted in animal models, *in vitro*, *in vivo*, and/or post-mortem; 2) studies on biochemical composition or biomarkers; 3) studies on nutritional aspects, or other therapeutic alternatives; and 4) studies on the prevention of AD.

Two researchers conducted the search and screening of the documents, and any discrepancies in the selected documents were resolved through consensus between the researchers. This study was registered in the International Prospective Register of Systematic Reviews (PROSPERO) with the code [CRD42023409986].

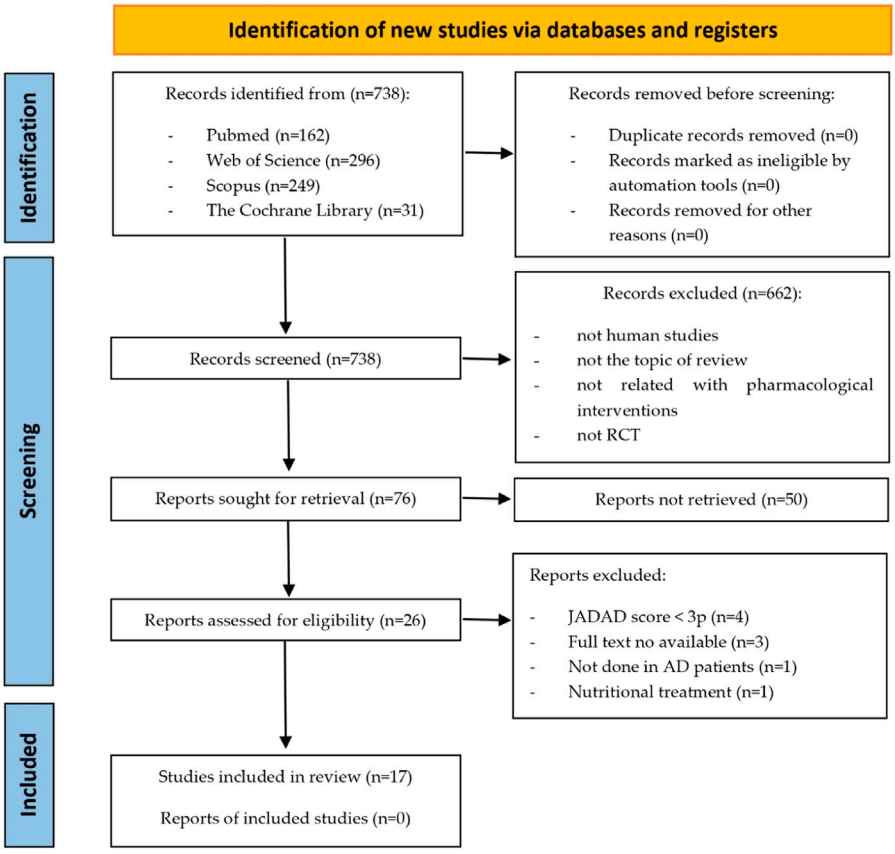

FIGURE 1
PRISMA flow chart.

3 Results

3.1 Study characteristics

A total of 738 documents were initially identified, 17 of which were included in the study. The selection process of the studies included in this review is summarized in Figure 1. The main characteristics of the studies included in this review are presented in Table 1. Additionally, Table 2 displays the JADAD scores of the included articles. Due to the high variability of interventions in the studies included in our systematic review, we have created Table 3 outlining the type of intervention, associated mechanism of action, and effect on cognition. Since the articles included in this systematic review investigate completely different drugs, they have been classified according to their mechanism of action and therapeutic use.

3.2 Synthesis of clinical trials investigating various treatments for Alzheimer's disease

3.2.1 Therapeutic potential of anticancer agents in Alzheimer's disease

Anticancer agents have garnered growing interest in the context of repurposed therapies for AD. To date, results have been controversial. For example, some anticancer drugs such as tyrosine kinase inhibitors and retinoid X receptor agonists can modulate cellular signaling pathways and reduce inflammation, which may be beneficial in the context of AD. Additionally, it has been shown that some anticancer drugs, such as histone deacetylase inhibitors, can improve cognitive function and synaptic plasticity in animal models of AD (Ancidoni et al., 2021). Masitinib is an anti-tumor drug that has been investigated for potential use in the treatment of AD. Its mechanism of action is believed to be related to its ability to inhibit certain enzymes that can contribute to neuronal damage and inflammation in the brain, which in turn may reduce AD symptoms. Masitinib is an inhibitor of tyrosine kinase, an enzyme that plays a role in the activation of inflammatory cells and some brain cells involved in AD progression. By inhibiting this enzyme, masitinib may reduce inflammation and activation of these cells, which could help protect brain cells and reduce AD symptoms (Piscopo et al., 2022; Villain, 2022). Masitinib has demonstrated neuroprotective effects in neurodegenerative diseases by inhibiting mast cell and microglia/macrophage activity and its ability to accumulate in the central nervous system at therapeutically relevant concentrations. Administration of Masitinib at doses of 4.5–6.0 mg/kg/day in patients over the age of 50 with mild to moderate AD-associated dementia has been shown to result in significant improvement in ADAS-cog scores ($p < 0.001$), indicating

TABLE 1 Main characteristics of the studies included in this review.

Reference	Objectives	Sample/ Duration	Measuring Instruments	Intervention Design	Results
Craft et al. (2020)	To examine the feasibility, safety, and efficacy of intranasal insulin for the treatment of persons with mild cognitive impairment and AD dementia in a phase 2/ 3 multisite clinical trial	n = 240 12 months	ADAS-cog-12, ADL-MCI, CDR-SB, blood collection, magnetic resonance imaging	Intranasal insulin 40 UI (n = 121) Placebo (n = 119)	No significant improvement in ADAS-cog-12 nor cerebrospinal fluid outcomes
Dubois et al. (2023)	To evaluate masitinib as an adjunct to cholinesterase inhibitor and/or memantine in patients with mild-to-moderate dementia due to probable AD.	n = 358 24 weeks	ADAS-cog, ADCS-ADL	Masitinib 4.5 mg/kg/day (n = 182) Placebo (n = 176)	$p < 0.001$ ADAS-cog $p = 0.038$ ADCS-ADL
Egan et al. (2018)	To evaluate verubecestat at doses of 12 mg and 40 mg per day, as compared with placebo, in patients who had memory impairment and elevated brain amyloid levels but whose condition did not meet the case definition of dementia	n = 1454 104 weeks	CDR-SB	Verubecestat 12 mg (n = 485) Verubecestat 40 mg (n = 484) Placebo (n = 485)	$p = 0.67$ CDR-SB (Verubecestat 12mg/placebo) $p = 0.01$ CDR-SB (Verubecestat 40mg/ placebo). Verubecestat did not improve clinical ratings of dementia. Cognition and daily function were worse in Verubecestat group than in placebo group
Frölich et al. (2019)	To assess the efficacy and safety of BI 409306 at doses of 10–50 mg daily over a 12-week treatment period in two phase II proof-of-concept studies in prodromal and mild AD.	n = 452 12 weeks	NTB, CDR-SB, ADAS-cog11, ADCS-ADL	BI 409306 10 mg QD (n = 77) BI 409306 25 mg QD (n = 74) BI 409306 50 mg QD (n = 76) BI 409306 25 mg BID (n = 76) Placebo (n = 149)	No changes in NTB, CDR-SB, ADAS-cog11, ADCS-ADL
Koch et al. (2020)	To investigate whether therapy with dopaminergic agonists may affect cognitive functions in patients with AD.	n = 94 24 weeks	ADAS-cog, ADCS-ADL, FAB, NPI	Rotigone (n = 47) Placebo (n = 47)	No effect on ADAS-cog. Significant changes in ADCS-ADL and FAB.
Koh et al. (2021)	To assess the effect of GV1001 on the cognition and activities of daily living in patients with moderate-to-severe AD.	n = 96 24 months	SIB, CDR-SB, ADCS-ADL, NPI, MMSE, GDS	GV1001 0.56 mg (n = 33) GV1001 1.12 mg (n = 32) Placebo (n = 31)	GV1001 1.12 mg effectively reduced the change in SIB compared with placebo ($p < 0.05$). Changes in ADCS-ADL and CDR-SB were not significant
Lang et al. (2021)	To determine the effects of 35 mg/day intepirdine *versus* placebo on cognition and activities of daily living in mild-to-moderate AD dementia patients on background donepezil	n = 1315 24 weeks	ADAS-cog, ADCS-ADL	Intepirdine 35 mg/day (n = 661) Placebo (n = 654)	No signficant changes in ADAS-cog ($p = 0.2249$) and ADCS-ADL ($p = 0.8260$)
Lawlor et al. (2018)	To determine if nilvadipine was effective in slowing cognitive decline in subjects with mild to moderate AD.	n = 511 18 months	ADAS-cog12, CDR-SB, DAD	Nilvadipine 8 mg (n = 253) Placebo (n = 258)	No significant changes
Mintzer et al. (2021)	To measure whether methylphenidate compared with placebo decreases the severity of apathy in individuals with AD.	n = 200 6 months	NPI apathy subscale, ADCS-CGIC	Methylphenidate 10 mg (n = 99) Placebo (n = 101)	Significant change in apathy
Padala et al. (2018)	To study the effects of methylphenidate on apathy in AD.	n = 60 12 weeks	Apathy Evaluation Scale-Clinician, MMSE	Methylphenidate (n = 30) Placebo (n = 30)	The methylphenidate group had significantly greater improvement in apathy than the placebo group. There was also greater improvement in cognition, functional status, caregiver burden, CGI scores, and depression in the methylphenidate group compared with the placebo group

(Continued on following page)

TABLE 1 (*Continued*) Main characteristics of the studies included in this review.

Reference	Objectives	Sample/ Duration	Measuring Instruments	Intervention Design	Results
Schneider et al. (2019a)	To assess the safety of ladostigil (10 mg/d) and to explore its effect on ameliorating progression from MCI to AD.	n = 210 36 months	NTB, DAD, GDS, CDR, MMSE	Ladostigil 10 mg (n = 103) Placebo (n = 107)	No significant effects on NTB, DAD, or GDS. Ladostigil did not delay progression to dementia
Schneider et al. (2019b)	To assess the efficacy, safety, and tolerability of edonerpic for patients with mild to moderate AD.	n = 484 52 weeks	ADAS-cog, ADCS-CGIC	Edonerpic maleate 224 mg (n = 166) Edonerpic maleate 448 mg (n = 158) Placebo (n = 158)	No clinical effect
Teng et al. (2022)	To evaluate the safety and efficacy of the monoclonal anti-tau antibody semorinemab in prodromal to mild AD.	n = 457 73 weeks	CDR-SB, ADAS-cog13, ADCS-ADL	Semorinemab 1500 mg (n = 94) Semorinemab 4500 mg (n = 136) Semorinemab 8100 mg (n = 92) Placebo (n = 135)	No clinical effect
Vossel et al. (2021)	To determine the ability of the antiseizure drug levetiracetam to improve cognition in people with AD.	n = 34 12 weeks	NIH-EXAMINER, Stroop, ADAS-cog, virtual route learning test, CDR-SB, ADCS-ADL, ADCS-CGIC, NPI	Levetiracetam (n = 17) Placebo (n = 17)	Levetiracetam did not improve cognition function, but improved performance on spatial memory and executive function tasks
Wangchan et al. (2020)	To investigate the long-term therapeutic effects of the Chinese medicine Jiannao Yizhi Formula (JYF) in the treatment of AD.	n = 51 6 months	ADAS-cog, CM-SS, MMSE, MoCA, ADL	JYF (n = 27) Donepezil Control (n = 24)	Compared with baseline, both JYF and donepezil increased the MoCA and MMSE scores and decreased the ADAS-cog and CM-SS scores ($p < 0.05$)
Yang et al. (2019)	To assess the effect and safety of Huannao Yicong Formula (HYF) in the treatment of patients with mild-to-moderate AD.	n = 52 6 months	ADAS-cog, CM-SS, MMSE, MoCA, ADL	HYF (n = 28) Donepezil Control (n = 24)	Compared with the baseline, HYF and donepezil significantly decreased the total scores of ADAS-cog and CM-SS, and significantly increased the scores of MoCA and MMSE ($p < 0.01$)
Yokoyama et al. (2019)	To investigate the therapeutic properties of teprenone in AD.	n = 96 12 months	ADAS-Jcog, MMSE	Donepezil + Teprenone (n = 48) Donepezil + placebo (n = 48)	ADAS-Jcog $p = 0.861$ MMSE $p = 0.044$

AD, Alzheimer's disease; ADAS-cog, Alzheimer's disease assessment scale—Cognitive subscale; ADCS-ADL, Alzheimer's disease cooperative study—Activities of daily living; ADCS-CGIC, Alzheimer's disease cooperative study—Clinical global impression of change; ADL, Activities of daily living; ADL-MCI, Activities of daily living—Mild cognitive impairment; CDR, Clinical dementia rating; CDR-SB, Clinical dementia rating—Sum of boxes; CM-SS, Chinese medicine symptom scale; DAD, Disability assessment for dementia; FAB, Frontal assessment battery; GDS, Geriatric depression scale; MMSE, Mini-mental state examination; MoCA, Montreal cognitive assessment; NIH-EXAMINER, National institutes of health—Toolbox cognitive battery—Executive function; NPI, Neuropsychiatric inventory; NTB, Neurobehavioral symptom inventory; SIB, Severe impairment battery.

improved cognition. Masitinib also improved overall function, as assessed by ADCS-ADL ($p = 0.038$). However, this drug has potential safety concerns, including maculopapular rash, neutropenia, and hypoalbuminemia (Dubois et al., 2023). GV1001 is a peptide composed of 16 amino acids corresponding to a fragment of the catalytic site of human telomerase reverse transcriptase. This peptide possesses neuroprotective properties, as it protects neural cells against neurotoxicity, apoptosis, and reactive oxygen species (ROS) induced by Aβ and oxidative stress. These neuroprotective effects are mediated through a variety of mechanisms, such as anti-apoptotic effects, mitochondrial stabilizers, anti-inflammatory, anti-aging, and antioxidant properties. In a clinical study conducted on patients with moderate to severe AD, a dose of 1.12 mg of GV1001 was administered for 24 weeks, resulting in a significant reduction in changes in SIB scores compared to placebo treatment ($p < 0.05$). However, changes in the ADCS-ADL and CDR-SB scores were not significant. Furthermore, GV1001 was well-tolerated without safety issues (Koh et al., 2021).

3.2.2 A novel therapeutic agent for Alzheimer's disease

The Aβ protein is produced through the sequential action of the β-site amyloid precursor protein (APP) cleaving enzyme (BACE-1) and γ-secretase on the amyloid precursor protein (APP). Inhibition of BACE-1 in preclinical models has been shown to reduce Aβ production and amyloid plaque deposition, which may potentially delay the progression of AD. Verubecestat is a selective inhibitor of BACE-1 that reduces Aβ levels in the cerebrospinal fluid of healthy individuals and AD patients by more than 60% (Egan et al., 2018). However, clinical studies have shown that this inhibitor is ineffective and produces numerous negative side effects, such as rash, dermatitis, sleep disorders, weight loss, and coughing (Miranda et al., 2021). In Egan et al. (2018) RCT, it was found that verubecestat administration for 104 months did not improve clinical dementia symptoms in patients between 50 and 85 years of age. In addition, cognitive and daily function scores were worse in the intervention group than in the placebo group, as measured by the CDR-SB. Adverse effects were also more frequent in the

TABLE 2 JADAD score of included articles.

	Randomized? yes 1p, not 0p	Method of randomization is adequate? yes 1p, not 0p, not suitable -1p	Double-blind? yes 1p, not 0p	Method of blinding is adequate? yes 1p, not 0p, not suitable -1p	Description of loss of follow-up and abandonments? yes 1p, not 0p	Total score
Craft et al. (2020)	1	1[d]	1	0[b]	1	4
Dubois et al. (2023)	1	1[c]	1	0[b]	1	4
Egan et al. (2018)	1	1[c]	1	1[a]	1	5
Frölich et al. (2019)	1	0[b]	1	1[a]	1	4
Koch et al. (2020)	1	0[b]	1	1[a]	1	4
Koh et al. (2021)	1	1[c]	1	1[a]	1	5
Lang et al. (2021)	1	1[c]	1	1[a]	1	5
Lawlor et al. (2018)	1	1[e]	1	0[b]	1	4
Mintzer et al. (2021)	1	1[c]	0	1[a]	1	4
Padala et al. (2018)	1	1[e]	1	0[b]	1	4
Schneider et al. (2019a)	1	1[e]	1	1[a]	1	5
Schneider et al. (2019b)	1	1[e]	1	1[a]	1	5
Teng et al. (2022)	1	1[e]	1	0[b]	1	4
Vossel et al. (2021)	1	0[b]	1	1[a]	1	4
Wangchan et al. (2020)	1	1[d]	1	1[a]	1	5
Yang et al. (2019)	1	1[d]	1	1[a]	1	5
Yokoyama et al. (2019)	1	1[c]	1	0[b]	1	4

[a]Identical-appearing tablets
[b]Not specified
[c]Computer generated randomization
[d]Stratified random method
[e]Block randomization.

intervention group compared to the placebo group (Egan et al., 2018).

Ladostigil is a novel therapeutic agent that acts as an inhibitor of both monoamine oxidase (MAO) and acetylcholinesterase (AChE) in the brain. It has also been found to possess neuroprotective and antiapoptotic properties by preventing oxidative-nitrate stress and gliosis (Uddin et al., 2020). MAO is an enzyme that degrades important neurotransmitters such as dopamine, noradrenaline, and serotonin. Inhibition of MAO by ladostigil increases the amount of these neurotransmitters in the brain, which could improve cognitive function. Ladostigil also prevents the decline of mitochondrial potential caused by oxidative stress and the release of proinflammatory cytokines from activated microglia. In patients with mild cognitive impairment (MCI), treatment with ladostigil may reduce ROS and proinflammatory changes, suggesting a potential slow action in disease progression. However, in the clinical trial by Schneider et al. (2019b), a dose of 10 mg of ladostigil was found to have no significant effects on cognitive function, daily activity, or depressive symptoms in patients with dementia, as evaluated by NTB, DAD, and GDS, respectively.

TABLE 3 Summary of the results obtained in the systematic review.

Type of intervention	Associated mechanism of action	Effect on cognition
Insulin Craft et al. (2020)	Insulin resistance	No effects
Masitinib Dubois et al. (2023)	Inhibitor of tyrosine kinase	Positive effects
GV1001 Koh et al. (2021)	Telomerase	Low effect
Verubecestat Egan et al. (2018)	Inhibitor of BACE-1	No effects
Ladostigil Schneider et al. (2019a)	Inhibitor of MAO	No effects
Semorinemab Teng et al. (2022)	IgG4 antibody	No effects
Rotigotine Koch et al. (2020)	Dopaminergic agonist	Low effect
BI 409306 Frölich et al. (2019)	Inhibitor of PDE9	No effects
Intepirdine Lang et al. (2021)	5-HT6 receptor antagonist	No effects
Nilvadipine Lawlor et al. (2018)	Calcium channel blocker	No effects
Methylphenidate Mintzer et al. (2021)	Increase availability of neurotransmitters	Positive effects
Methylphenidate Padala et al. (2018)	Increase availability of neurotransmitters	Positive effects
Edonerpic maleate Schneider et al. (2019b)	Activation of sigma-1 receptor	No effects
Levetiracetam Vossel et al. (2021)	Positive modulator of SV2A protein	Low effect
Teprenone Yokoyama et al. (2019)	Upregulation of HSP70	Low effect
Jiannao Yizhi Formula Wangchan et al. (2020)	Unknown	Positive effects
Huannao Yicong Formula Yang et al. (2019)	Unknown	Positive effects

However, total brain volume and hippocampal volume decreased significantly less in the ladostigil-treated group than in the placebo group, suggesting a potential effect on cerebral atrophy (Schneider et al., 2019a).

Semorinemab is a humanized monoclonal IgG4 antibody that targets the N-terminal domain of tau protein. Its mechanism of action is based on its ability to bind and eliminate Aβ protein fragments, which accumulate as plaques in the brains of patients with AD. Semorinemab was selected for development because of its high affinity and specificity for all known isoforms of full-length tau. Neurofibrillary tangles composed of aggregated tau protein are a hallmark neuropathological feature of AD and are correlated with the clinical severity of the disease. Monoclonal antibodies targeting tau protein may have the potential to slow or stop the spread and accumulation of pathological tau, thereby improving the progression of AD. However, a 73-week treatment with semorinemab in patients with prodromal to mild AD did not result in significant improvements in CDR-SB, ADAS-cog13, and ADCS-ADL. The treatment also did not reduce the rate of cerebral tau accumulation or clinical decline in patients with prodromal-to-mild AD. The safety profile of semorinemab was found to be acceptable and well-tolerated (Teng et al., 2022).

3.2.3 Mitochondrial electron transport inhibitors for Alzheimer's disease

Dopamine is a crucial neuromodulator that influences several distinct synaptic processes and plays an important role in controlling higher cognitive functions such as memory, learning, and decision-making. Dopaminergic dysfunction may contribute to cognitive impairment in patients with AD. Intervention with a transdermal rotigotine patch, a dopaminergic agonist, did not have an effect on global cognitive dysfunction in patients with mild to moderate AD, as evaluated by the ADAS-cog. However, a significant improvement was observed in the deterioration of activities of daily living, as measured by the ADCS-ADL. This effect appears to be related to minor cognitive dysfunction in the frontal lobe (Koch et al., 2020).

3.2.4 The serotonin 5-HT6 receptor as a therapeutic target for Alzheimer's disease

One of the key features of AD is an abnormality in glutamatergic neurotransmission related to the function of the N-methyl-D-aspartate (NMDA) receptor in the cortex and hippocampus. Activation of the NMDA receptor signaling pathway produces postsynaptic signaling events through the elevation of second messengers such as cyclic guanosine monophosphate (cGMP). In conditions of NMDA receptor hypofunction, such as in AD, it is hypothesized that inhibition of phosphodiesterase type 9 (PDE9), which hydrolyzes cGMP, may increase cGMP levels and improve NMDA receptor signaling. This could lead to increased plasticity and synaptic stabilization through enhanced long-term potentiation (LTP), thus potentially improving cognitive functions. BI 409306 is a selective and potent inhibitor of PDE9 that has been investigated for the symptomatic treatment of AD. However, administration of this drug at various doses for 12 weeks did not improve NTB, CDR-SB, ADAS-cog11, or ADCS-ADL scores in patients with

mild AD. It has not been demonstrated to be effective in improving cognition in patients with prodromal or mild AD (Frölich et al., 2019).

The serotonin 5-HT6 receptor, which is found in critical areas of the brain involved in memory, learning, mood, and behavior, has been studied as a potential therapeutic target for AD. Inhibition of this receptor has been shown to improve the release of important neurotransmitters in AD, which could improve cognition in preclinical models. Specifically, the 5-HT6 receptor antagonist, Intepirdine, has been evaluated in phase 2 clinical trials in AD patients and has been suggested as a possible oral treatment to improve cognition. Since the 5-HT6 receptor is primarily located in the central nervous system, antagonism of this receptor may increase the release of important neurotransmitters and minimize peripheral side effects (Lang et al., 2021; Nirogi et al., 2023). However, Lang et al. (2021) RCT, which evaluated intervention with 35 mg/day of Intepirdine for 24 weeks in patients with mild to moderate AD receiving donepezil as the baseline treatment, did not show significant improvements in ADAS-cog scores (p = 0.2249) or ADCS-ADL scores (p = 0.8260). Nevertheless, it was observed that Intepirdine demonstrated a favorable safety profile, similar to placebo (Lang et al., 2021).

3.2.5 The neuroprotective mechanisms of calcium channel blockers in Alzheimer's disease

Nilvadipine is a dihydropyridine calcium channel blocker drug used to treat hypertension (Lawlor et al., 2018; Ling et al., 2021; Dhapola et al., 2022). In addition to its direct blocking action on calcium channels and maintenance of intracellular calcium homeostasis, nilvadipine has been shown to have a number of neuroprotective mechanisms of action. These include reducing the production of amyloid beta 40 and 42 amino acid peptides (Aβ40 and Aβ42) *in vitro* and *in vivo* in transgenic mouse models of AD, and improving Aβ clearance across the blood-brain barrier *in vivo* mouse models (Lawlor et al., 2018). It is believed that these protective effects could have a dual effect on AD pathogenesis, reducing both mitochondrial dysfunction and beta-amyloid accumulation (Ling et al., 2021; Dhapola et al., 2022). However, the results of a RCT conducted by Lawlor et al. (2018) indicate that nilvadipine did not produce significant changes in cognitive decline in patients with mild to moderate AD after receiving 8 mg of nilvadipine for 18 months (Lawlor et al., 2018).

3.2.6 The role of central nervous system stimulants (methylphenidate) treating apathy in AD patients

There are studies suggesting that methylphenidate, a medication used to treat ADHD, may have beneficial effects on patients with AD, such as improving memory and cognition. However, further research is needed to determine its efficacy and safety in individuals with AD. Additionally, it is important to note that methylphenidate is not suitable for all patients and may have side effects. The exact mechanism of action of methylphenidate is not fully known, but it is believed to act by increasing the availability of neurotransmitters such as dopamine and norepinephrine in the brain. These neurotransmitters are involved in the regulation of attention, mood, and cognition. By increasing their availability, methylphenidate may improve attention and memory in patients with ADHD and possibly in those with AD. Furthermore, it is believed that methylphenidate may have neuroprotective effects, protecting nerve cells from oxidative damage and inflammation (Sassi et al., 2020; van Dyck et al., 2021; Andrade, 2022). Regarding apathy in AD, although no treatment has been shown to be effective, catecholaminergic agents such as methylphenidate are promising. It has been proposed that methylphenidate could act as a cognitive enhancer by increasing dopaminergic and noradrenergic neurotransmission, which are diminished in AD (Padala et al., 2018; Mintzer et al., 2021). Although there is still not enough evidence to confirm its efficacy in this regard, studies have shown that intervention with 10 mg methylphenidate for 6 months significantly improved apathy in AD patients, with no significant differences in safety profiles between treatment groups (Mintzer et al., 2021). Additionally, after 12 weeks of treatment with methylphenidate, apathy also significantly improved in patients with mild AD compared to the placebo group, with improvements in cognition, functional status, caregiver burden, CGI scores, and depression (Padala et al., 2018).

3.2.7 The therapeutic effect of neurotransmitter modulators in AD patients

The edonerpic maleate can exert its effects through different mechanisms, including activation of the sigma-1 receptor, modulation of microglial function, and interaction with the collapsin response mediator protein 2 (CRMP2), which facilitates the administration of the AMPA synaptic receptor. According to Schneider et al. (2019a), edonerpic maleate can protect against Aβ-induced neurotoxicity and memory deficits, promote neurite growth, and preserve hippocampal synapses and spatial memory. However, intervention with edonerpic maleate for 52 weeks in patients with mild to moderate AD had no significant effects on the ADAS-cog or ADCS-CGIC scales (Schneider et al., 2019b).

Levetiracetam is an antiepileptic drug that acts as a positive modulator of the SV2A protein, which is a member of the SV2 protein family involved in neurotransmission and is found in most nerve terminals. Levetiracetam binds to the SV2A protein and modulates its function to inhibit neurotransmitter release and reduce neuronal activity (Stout et al., 2019). Decreased levels of SV2A in the brains of patients with AD have been demonstrated, which may contribute to synaptic dysfunction and neuronal loss in AD. SV2 is an important target for new PET tracers that have been developed to visualize synaptic density in the brain (Stout et al., 2019; Carson et al., 2022; Samudra et al., 2023). Although the precise function of SV2 is not fully understood, some possible functions include vesicular transport, stabilization of vesicular neurotransmitter load, anchoring of vesicular proteins, regulation of calcium sensitivity, and interaction with the extracellular matrix. The hypothesis is discussed that SV2 does not directly transport calcium but makes prepared vesicles more sensitive to calcium (Stout et al., 2019). The mechanism of action of Levetiracetam is not fully understood, but it is believed to act by inhibiting the release of glutamate, a neurotransmitter that has been linked to neuronal death in AD. In addition, Levetiracetam has been shown to have neuroprotective effects, helping to prevent brain cell death and neuroinflammation (Musaeus et al., 2021; Vossel et al., 2021). Although Levetiracetam did not improve cognitive function in AD patients in a 12-week intervention study, it improved performance in spatial memory tasks and executive function. It

has been studied as a possible treatment to improve cognition in AD patients, but more studies are needed to confirm its effectiveness in this field. Levetiracetam is considered safe and well-tolerated at low doses (Vossel et al., 2021).

3.2.8 Antidiabetics in AD

Currently, research is being conducted to determine the effectiveness of antidiabetic treatments in improving AD due to the possible relationship between type 2 diabetes and AD. Type 2 diabetes is a metabolic disease that affects insulin activity in regulating blood glucose levels. Insulin resistance in the brain, which is an important factor in the development of type 2 diabetes, has also been shown to be related to a higher risk of developing AD (Chen et al., 2022; Malin et al., 2022). Several epidemiological and clinical studies have suggested a possible shared pathophysiology between diabetes and AD, and the administration of certain antidiabetic medications, such as intranasal insulin, metformin, incretins, and thiazolidinediones, has been shown to improve cognition and memory in patients with mild cognitive impairment and AD. As a result, the term "type 3 diabetes" has been proposed for AD, considering it a metabolic disease caused by insulin resistance and insulin-like growth factor in the brain (Čater and Hölter, 2022). Additionally, antidiabetic drugs such as metformin have been observed to improve insulin sensitivity and reduce inflammation in the brain, suggesting that they could slow or reverse the process of cognitive decline in patients with AD. It has been shown that insulin signaling is reduced in the brains of patients with AD, known as cerebral insulin resistance, and is related to the accumulation of beta-amyloid plaques and the formation of neurofibrillary tangles (Michailidis et al., 2022). Although brain glucose metabolism does not depend on insulin, it can alter its use through interactions with the neuronal glucose transporter type 4 (GLUT4) in key cognitive circuits and by promoting glycogen uptake in astrocytes, processes that are considered important during times of high energy demand. Additionally, insulin improves synaptic viability and dendritic spine formation, and modulates levels of key neurotransmitters such as dopamine. Although previous studies have demonstrated that intranasal insulin administration improves performance in the ADAS-cog test and brain glucose metabolism in patients with AD, a randomized clinical trial by Craft et al. (2020) did not find significant effects of intranasal insulin administration on ADAS-cog-12 scores in AD patients, possibly due to the inadequate use of some devices in the study (Craft et al., 2020).

3.2.9 Gastric protectors HSP70 overexpression and teprenone administration as a neuroprotective strategies for AD

It has been reported that elevating HSP70 levels in the brain via genetic modification or teprenone administration in mouse models of AD inhibits the accumulation of Aβ, senile plaque formation, neuronal death, and neurodegeneration, while significantly enhancing memory capacity. Its mechanism of action is believed to involve multiple protective effects, such as reducing Aβ protein production and decreasing cerebral inflammation. HSP70 overexpression leads to positive regulation of Aβ degrading enzyme and TGF-β1 expression, both *in vitro* and *in vivo*. Additionally, teprenone is an antiulcer agent that can inhibit Aβ increase, senile plaque formation, neuronal degeneration, and improve memory. However, a 12-month intervention study in patients with mild to moderate AD who received a combination of donepezil and teprenone did not significantly affect the ADAS-Jcog score ($p = 0.861$), but did impact the MMSE score ($p = 0.044$) (Yokoyama et al., 2019).

3.2.10 Traditional Chinese medicine: a natural alternative for treating Alzheimer's disease

Traditional Chinese Medicine (TCM) employs a variety of herbal medicines to treat various diseases, including AD, and is considered a natural alternative to synthetic drugs. The mechanisms of action of these herbal medicines have been investigated, and it is believed that they may have beneficial effects in the prevention and treatment of AD. Some herbal medicines may reduce inflammation in the brain, protect nerve cells from damage, and improve cognitive function by modulating different signaling pathways such as NF-κB, Nrf2, JAK/STAT, ubiquitin-proteasome pathway, AMPK/mTOR related to the autophagy-lysosome pathway, GSK-3/mTOR, and PI3K/Akt/mTOR, as well as the SIRT1 and PPARα pathways (Soheili et al., 2021; Ding et al., 2022; Tan et al., 2022). Herbal medicines can also modulate multiple signaling pathways associated with Aβ deposition, protein tau phosphorylation, and chronic inflammation. Some herbal medicines may prevent excessive apoptosis and reduce AChE activity (Fang et al., 2020; Pei et al., 2020; Li et al., 2021). A 6-month study with the Jiannao Yizhi formula increased MoCA and MMSE scores and decreased ADAS-cog and CM-SS scores ($p < 0.05$) in patients with AD. There were no significant differences in the group receiving donepezil, suggesting that the effect of the Jiannao Yizhi formula is not inferior to that of donepezil. The Jiannao Yizhi formula had a favorable safety profile, and no serious adverse effects were found (Wangchan et al., 2020). Similarly, a 6-month study with the Huannao Yicong formula increased MoCA and MMSE scores and decreased ADAS-cog and CM-SS scores ($p < 0.01$) in patients with mild to moderate AD. No serious adverse effects were observed during the study. The Huannao Yicong formula may prevent neuronal apoptosis in the CA1 area of the hippocampus, inhibit secretase activity, and reduce neurotoxicity caused by Aβ peptide in rat models of AD (Yang et al., 2019).

4 Discussion

Our systematic review was limited by the scarcity of studies available for discussion of the findings. However, we presented some of the discoveries obtained during the literature search, which could not be included in the systematic review because their failure to meet the established inclusion criteria.

Lithium is considered to be a potential treatment for improving neurotrophic responses and protecting the brain. In patients with amnestic cognitive impairment, treatment with lithium carbonate showed cognitive and functional stability for 2 years, with better performance in memory and attention tests compared to the placebo group (Forlenza et al., 2019). Unlike the expensive aducanumab approved by the FDA in 2021 for patients with mild dementia caused by AD, lithium is more cost-effective and has been shown to be effective for both mild cognitive impairment and AD. In addition,

a recent meta-analysis found that lithium is more effective than aducanumab in reducing cognitive decline, as measured by MMSE (Terao et al., 2022). However, some studies indicate that lithium has no significant effect on cognitive performance, and it is important to carefully monitor its administration and follow-up due to its toxicity (Restrepo-Martínez et al., 2022).

Insulin not only regulates glucose homeostasis, but it also has functions in the brain. It has been shown to improve synaptic viability, modulate neurotransmitter levels, such as dopamine, and protect against the toxic effects of Aβ peptide (Craft et al., 2020; Kellar et al., 2022). Unlike the results reported by Craft et al. (2020) (Craft et al., 2020), low insulin levels could be related to AD, and intranasal insulin administration has been shown to improve in cognition in patients with this disease. Intranasal insulin can also reduce the progression of white matter hyperintensity and improve verbal memory (Avgerinos et al., 2018; Zhang et al., 2018; Kellar et al., 2021). Antidiabetic drugs such as intranasal insulin, pioglitazone, rosiglitazone, metformin, sitagliptin, and liraglutide can significantly improve the cognition of patients with AD and mild cognitive impairment; However, metformin does not seem to reduce the risk of AD, and its consumption in the Asian population is associated with a higher risk of this disease, although causality is unknown (Cao et al., 2018; Munõz-Jiménez et al., 2021; Luo et al., 2022).

In the last decade, research has being conducted to evaluate the effect of vaccination and immunotherapy in the treatment of AD (Foroutan et al., 2019; Vaz and Silvestre, 2020). Most of these investigations are in the safety and tolerability phase or in phase I. However, the efficacy of this type of treatment for managing neurodegenerative disease is still unknown (Lacosta et al., 2018). On the other hand, intravenous administration of immunoglobulins has been shown to be ineffective in the treatment of AD, according to reports from previous studies (Okuya et al., 2018; Manolopoulos et al., 2019). Currently, one of the most investigated goals is the treatment of AD with monoclonal antibodies. Despite promising results from clinical trials, the risk-benefit profile of these drugs remains uncertain (Lacorte et al., 2022). Monoclonal antibodies aducanumab and solanezumab have been investigated for their effect on Aβ accumulation and cognitive function. It has been shown that these antibodies can improve cognitive outcomes, evaluated by the ADAS-cog scale (Avgerinos et al., 2021; Budd Haeberlein et al., 2022). Lecanemab (BAN2401) is a humanized monoclonal IgG1 antibody that selectively targets soluble aggregated Aβ species, including oligomers, protofibrils, and insoluble fibrils. Phase II clinical trials have demonstrated that this antibody can reduce brain amyloid burden and clinical decline. However, given that the available evidence is still limited, further studies are needed to fully evaluate its efficacy and safety (Swanson et al., 2021; Dhadda et al., 2022).

Given that pharmacology and nutrition have some points in common, we will also discuss some data we have found regarding the relationship between nutrition and AD. Various nutrients and nutraceuticals have been linked to improvements in cognition and other psychological aspects related to AD (Guzman-Martinez et al., 2021; Abduljawad et al., 2022; Mahnashi et al., 2022; Xu Lou et al., 2023) Some examples include Gingko Biloba (Liao et al., 2020), Melissa Officinalis (Noguchi-Shinohara et al., 2020; Noguchi-Shinohara et al., 2022), Ginseng (Ahmad et al., 2023), anti-inflammatory fatty acids (Albrahim, 2020), medium-chain fatty acids (Juby et al., 2022), ketone bodies (Avgerinos et al., 2020a), saffron (Avgerinos et al., 2020b; Talebi et al., 2021), fenugreek seed (Foroumandi et al., 2023), genistein (Viña et al., 2022), sodium oligomannate (Xiao et al., 2021), anthocyanin (Suresh et al., 2022), microbiota and probiotics (Den et al., 2020; Maitre et al., 2021; Liu et al., 2022; Naomi et al., 2022), benfotiamine (Gibson et al., 2020), omega-3 fatty acids (Canhada et al., 2018; Jernerén et al., 2019), resveratrol (Gu et al., 2021; Buglio et al., 2022; Fang et al., 2022; Tosatti et al., 2022), melatonin (Tseng et al., 2022), citicoline (Bonvicini et al., 2023), folic acid, vitamin B12 (Chen et al., 2021), vitamins and minerals (Mccleery et al., 2018; Karthika et al., 2022), selenium (Pereira et al., 2022), vitamin D (Jia et al., 2019), and mangosteen (Muangpaisan et al., 2022). However, some studies do not support the efficacy of certain nutrients (Zhu et al., 2018; Thancharoen et al., 2019; Araya-Quintanilla et al., 2020; Burckhardt et al., 2020; Du et al., 2020; Prabhakar et al., 2020; Shim et al., 2021; Tofiq et al., 2021; Takada et al., 2022). The effects of these nutrients and micronutrients appear to be related to their anti-apoptotic, antioxidant, and anti-inflammatory properties (de Andrade Teles et al., 2018; Summers et al., 2018; Tamtaji et al., 2019; Zamanian et al., 2021; Rasi Marzabadi et al., 2022). The response to nutritional interventions is greater in the early stages of AD (Moreira et al., 2020), and this response is linked to the APOE genotype (Xu et al., 2020). The maximum benefit of probiotics has been observed in individuals with early cognitive dysfunction and no effect has been found in those with advanced disease or no apparent disease (Akhgarjand et al., 2022; Sánchez-De-Lara-Sánchez and Sánchez-Pérez, 2022). Caprylic acid is a ketone that, when metabolized, produces beta-hydroxybutyrate and acetoacetate ketones, that can cross the blood-brain barrier. Caprylic acid improves cognition in patients with mild-to-moderate AD. This is associated with an increased blood flow in specific brain regions. However, only patients who lack the APOE ε4 allele benefit from this effect (Torosyan et al., 2018).

AD is commonly treated with drugs such as donepezil, rivastigmine, galantamine, and memantine (Li et al., 2019). Although these drugs do not generally cause serious adverse events (Hong et al., 2019), common side effects include headache, diarrhea, nausea, and vomiting (Tricco et al., 2018). Although donepezil is the first-line drug for AD treatment (Birks and Harvey, 2018; Thancharoen et al., 2019), high doses should be administered with caution due to an increased risk of gastrointestinal and cardiac problems (Espiritu and Cenina, 2020; Wang et al., 2022). Both donepezil and memantine are widely used for the treatment of moderate AD (Matsunaga et al., 2018; Rozankovic et al., 2021; Youn et al., 2021). It has been observed that memantine may have a more significant effects on cognition than other commonly used AD medications (Liang et al., 2018b). However, a meta-analysis suggested that the efficacy of memantine is limited in some cases and does not differ significantly compared to placebo (Blanco-Silvente et al., 2018). Although memantine is associated with a lower incidence of AD progression, it also increases the incidence of somnolence (Kishi et al., 2018). Additionally, it is important to note that most available studies have a duration of less than 6 months, and participants usually have mild AD. Optimal pharmacological treatmenst often includes multiple drugs (McShane et al., 2019).

Zolpidem and Zopiclone have been studied for their use in AD patients with insomnia, as insomnia is a frequent problem in these patients (Louzada et al., 2022). Depression is a condition associated with dementia and AD, and sertraline and mirtazapine are antidepressant drugs that have been evaluated in RCTs in patients with AD who also suffer from depression. However, they have not been shown to be effective in these cases (Zuidersma et al., 2019). In contrast, vortioxetine could have beneficial effects on the cognition and mood in elderly patients with AD (Cumbo et al., 2019). While in animal models antidepressants have been shown to delay cognitive decline in animal models, there is still insufficient evidence to support these results in humans (Qin et al., 2022). Agitation and aggression are common symptoms in patients with dementia (Ruthirakuhan et al., 2019; Supasitthumrong et al., 2019), but there are no effective drugs for their treatment. Typical and atypical antipsychotics are commonly used to treat agitation and psychosis in dementia, although their effect on psychosis is insignificant (Mühlbauer et al., 2021). Nabilone may improve agitation; however, more studies are needed to confirm these results (Herrmann et al., 2019). On the other hand, pimavanserin may improve both agitation and aggression in patients with AD (Ballard et al., 2020).

Compared to donepezil, TCM shows no significant difference in effectiveness for treating AD (Hang-kun et al., 2018), although some of its formulas may help improve disease progression (May et al., 2018). For example, Danggui-Shaoyao-San has been found to significantly reduce symptoms in patients with vascular dementia (Kim and Cho, 2020). Furthermore, some studies suggest that a combination of TCM and Western medicine may offer greater benefits than using only one of them (Huang et al., 2021). Traditional Chinese and Japanese medicines have become important sources for drug discovery, and their efficacy in modern drug discovery needs to be investigated (Paudel et al., 2020).

AD is a common type of dementia that has caused a significant global economic and health burden, and there has been a wide debate on the use of statins as a treatment for this disease. Although a systematic review by Mejias-Trueba et al. (2018) did not find statins to improve cognition in AD patients (Mejias-Trueba et al., 2018), a more recent meta-analysis by Xuan et al. (2020) found that statins used in AD patients had beneficial short-term effects on MMSE scores, delaying the deterioration of neuropsychiatric status and significantly improving activities of daily living. However, no benefits were found in the ADAS-cog scores (Xuan et al., 2020).

Animal studies have suggested that TNF-α inhibitors may improve cognition and behavior. However, human studies have been limited (Ekert et al., 2018). Sodium benzoate has been shown to be a cognitive enhancer in patients with AD, schizophrenia, or late-life depression (Lane et al., 2022). On the other hand, estrogen has been shown to delay disease progression and minimize cognitive decline in AD patients, especially in women. However, hormone replacement therapy should be carefully considered due to its potential side effects (Zhou et al., 2020). The new selective glycine transporter-1 inhibitor, BI 425809, has not shown significant clinical improvement in patients with probable AD dementia (Wunderlich et al., 2023). Anti-inflammatory drugs may be beneficial in preventing dementia, although there is no evidence to support the use of aspirin or other NSAIDs (Jordan et al., 2020; Davis et al., 2021). According to epidemiological and laboratory studies, anti-inflammatory drugs may delay or prevent the onset of AD. In observational studies, the use of NSAIDs is significantly associated with a lower risk of AD, especially in long-term users. However, there is no support from RCTs. Neuroinflammation participates in the pathogenic cascades of AD. One possible mode of action for the effectiveness of NSAIDs is through the blocking of COX-2 in the brain. In addition, NSAIDs can also function by activating peroxisome proliferator-activated nuclear receptors, a group of nuclear hormone receptors that act to negatively inhibit the transcription of proinflammatory genes such as IL-6, TNF-α, COX-2, NOS, and cytokines (Wang et al., 2015).

Benzodiazepines and related drugs have been associated with an increased risk of AD in old age and adverse events in patients with mild to moderate AD (Dyer et al., 2020). Eszopiclone may improve sleep quality and cognitive function in elderly patients with AD and sleep disorders (Huo et al., 2022). The combination of various treatments has a better effect than the use of a single treatment or monotherapy in patients with AD, both in moderate and severe stages (Glinz et al., 2019; Guo et al., 2020; Knorz and Quante, 2022). Although our results suggest that methylphenidate has positive effects on apathy associated with AD (Padala et al., 2018; Mintzer et al., 2021), longer follow-up studies are needed to evaluate its efficacy (Lee et al., 2022). Idalopirdine, a selective 5-hydroxytryptamine6 receptor antagonist, has not demonstrated significant efficacy in patients with AD and has been associated with a higher incidence of adverse events (Matsunaga et al., 2019). Current evidence suggests that anti-tau drugs have little impact on slowing cognitive decline (Zheng et al., 2022). The presence of the APOE ε4 genotype, the main genetic risk factor for AD, does not influence the therapeutic effect of acetylcholinesterase inhibitors, but its relationship with other types of drugs is unknown (Cheng et al., 2018). Preclinical studies in transgenic models have suggested that DHP1401 has neuroprotective effects and improves memory. Although studies in humans with this drug are limited (Shim et al., 2022). According to a systematic review by Fink et al. (2018), the use of pharmacological treatments for cognitive protection in individuals with normal cognition or mild cognitive impairment has not been supported. Instead, most studies have been conducted in mild to moderate AD populations, and very few studies have been conducted in more advanced stages (Fink et al., 2018). Verubecestat and lanabecestat have been shown to worsen the cognitive status of patients with AD, although they may improve verbal fluency tasks (Wessels et al., 2020). A relationship has been found between hypertension and an increased risk of AD. Antihypertensive drugs can improve cognition and behavioral symptoms in patients with AD and reduce the incidence of cognitive disorders. Angiotensin receptor blockers are associated with a lower risk of AD, although their potential mechanisms remains unknown (Oscanoa et al., 2021; Rahimi et al., 2021). Epichaperomes play an important role in neuronal pathology, and their inhibition is a promising therapeutic approach for treating neurodegenerative diseases, including AD. However, drugs of this type are still in phase I, and their efficacy is unknown (Silverman et al., 2022). The effectiveness of thiazolidinediones in treating AD is influenced by APOE gene polymorphisms (Iketani et al., 2018). Finally, cannabis-based

drugs may inhibit the progression of AD by modulating Aβ modifications. However, more research is needed to determine their efficacy in treating psychiatric manifestations of AD (Farkhondeh et al., 2020; Paunescu et al., 2020).

5 Limitations of the systematic review

Although RCTs are methodologically sound according to the JADAD scale, there is still significant variability in the types of interventions used. Only two studies (Padala et al., 2018; Mintzer et al., 2021) have investigated the same active ingredient, highlighting the need for further exploration in this area of research. Additionally, the majority of studies on drugs for AD are conducted in Caucasian populations, despite ethnicity being a factor that can affect treatment efficacy. Ethnic diversity in AD clinical trials remains inadequate (Franzen et al., 2022). Presently, there are no pharmacological therapies that modify the natural progression of AD. Clinical trials in this field typically involve only patients in the early stages of the disease, with those in the advanced stages underrepresented (Ruiz et al., 2022). Symptomatic anti-Alzheimer's drugs are commonly employed in the treatment of the disease (Watanabe et al., 2019). Overall, our findings suggest that Alzheimer's pharmacology is a constantly evolving field with significant implications for the development of new pharmacological therapies. We emphasize the critical need for further investigation in this area to advance our understanding of this disease. The findings of our systematic review on the latest advances in Alzheimer's research indicate that there is still no definitive cure for this disease, at best, its progression can be slowed down. We believe that research into biogenetics and bioengineering could possibly pave the way for new lines of research for the treatment and cure of currently incurable diseases. Many of the drugs included in this systematic review are still in phase III, and therefore, little is currently known about them. Our review opens up new avenues for research into the treatment of AD. While we acknowledge that some of the studies included in our analysis have shown no significant difference between pharmacological intervention and placebo and, in some cases, even worsened the situation, we believe that reporting both positive and negative outcomes is crucial in scientific research to provide a reliable representation of reality.

6 Conclusion

In conclusion, although a potential improvement in cognitive function has been observed with some of the evaluated drugs, the limited number of available studies necessitates further research to determine their effectiveness and safety in treating cognitive impairments in Alzheimer's disease.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

All the authors listed have made substantial, direct, and intellectual contributions to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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