



Integrated Experimental and Computational Investigation of Isoliquiritigenin as a Natural Acetylcholinesterase Inhibitor

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ABSTRACT

Natural products remain an important source of bioactive compounds for the discovery of novel acetylcholinesterase (AChE) inhibitors against Alzheimer's disease (AD). Previous structure-based virtual screening of natural products identified isoliquiritigenin, a naturally occurring chalcone, as a potential AChE inhibitor. However, the inhibitory activity and mechanism remain insufficiently evaluated. Therefore, this study aimed to experimentally assess the AChE inhibitory activity of isoliquiritigenin and elucidate its molecular mechanism using integrated computational analyses. Enzymatic assays confirmed the inhibitory activity of isoliquiritigenin with an IC₅₀ value of 1.078 μM, with donepezil as the positive control. A total of 100 independent molecular docking simulations predicted a favorable binding orientation of isoliquiritigenin and donepezil within the AChE active site. The 100-ns molecular dynamics (MD) simulations integrated with Molecular Mechanics/Poisson–Boltzmann Surface Area and per-residue energy decomposition analyses demonstrated that donepezil interacted stably in the AChE active site, while isoliquiritigenin maintained stability through hydrogen bonding with Tyr72 and Asp74, and aromatic interactions with Tyr341 in the peripheral anionic site (PAS) region. Density functional theory (DFT) further explained the electronic features underlying the observed hydrogen-bonding and aromatic interactions, highlighting the roles of the aromatic and hydroxyl moieties of isoliquiritigenin in stabilizing interactions within the PAS region of AChE. These results provide experimental and mechanistic evidence supporting isoliquiritigenin as a natural AChE inhibitor for further pharmacological investigation.

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Keywords: Isoliquiritigenin, Natural Products, Acetylcholinesterase, Enzymatic Assay, Molecular Modeling.

Introduction

Alzheimer's disease (AD) remains one of the major global health problems due to its increasing prevalence. The number of people living with AD is projected to increase substantially by 2060.¹ Several studies have demonstrated that natural products, including herbal extracts, phytochemicals, and other bioactive compounds, may offer alternative therapeutic options for AD because of their neuroprotective properties and ability to target multiple molecular pathways.^{2–4} Acetylcholinesterase (AChE) has been considered one of the potential therapeutic targets for AD, leading many researchers to investigate bioactive natural products and their extracts as potential AChE inhibitors.^{2–6} Additionally, AChE inhibitors compensate for the cholinergic deficit in AD patients by increasing acetylcholine levels in the brain.⁷ A previous study used a structure-based virtual screening (SBVS) campaign of 276,518 natural products available in the LOTUS database. Among the identified virtual hits, isoliquiritigenin emerged as a promising AChE inhibitor.⁶ Isoliquiritigenin is widely found in many foods and beverages,⁸ indicating that it is a readily available natural bioactive compound. Importantly, isoliquiritigenin crossed the blood-brain barrier (BBB),⁹ fulfilling a primary criterion for effective neurodegenerative treatments.¹⁰ Therefore, experimental validation of its inhibitory activity is essential. Molecular dynamics (MD) simulation

have been largely explored to evaluate the dynamic stability of ligand-protein complexes. In addition, Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA) analysis enables the quantitative estimation of binding free energies, while per-residue energy decomposition identifies key amino acid residues that contribute to ligand binding.^{11–13} Recently, the integration of MD simulations with quantum mechanical calculations, such as density functional theory (DFT), has emerged as a valuable strategy for gaining deeper insights into molecular interactions.^{14–16} Understanding the inhibitory potency of isoliquiritigenin requires an examination of its electronic properties and noncovalent interactions. Accordingly, DFT can provide complementary insights into the electronic features underlying ligand binding.¹⁴ This study aimed to assess the inhibitory activity of isoliquiritigenin against AChE using the modified Ellman enzymatic assay with donepezil as the positive control. The inhibitory mechanism was elucidated by integrating 100 independent molecular docking simulations, 100-ns MD simulations, MM/PBSA free energy calculations with per-residue energy decomposition, and DFT calculations. These findings provide experimental and theoretical insights supporting the development of isoliquiritigenin as a potential natural-product-derived therapeutic candidate.

Materials and Methods

Materials

The chemicals used in this study were Isoliquiritigenin (Sigma-Aldrich, St. Louis, MO, USA), Donepezil Hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), and QuantiChrom™ AChE Assay Kit (DACE-100) (BioAssay Systems, Hayward, CA, USA). All these chemicals were used to assess the AChE inhibitory activity of isoliquiritigenin and donepezil according to the manufacturer's procedure.

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Instrumentations

A computer with specifications of AMD Ryzen 7 5800X, GPU of NVIDIA GeForce GTX 1650, 32GB of RAM, and a 466 GB solid-state drive, embedded with YASARA Structure version 25.12.1 software (YASARA Biosciences, Austria), was employed to prepare the ligand-protein complex, perform molecular docking, and perform MD simulations. The 3D interactions were visualized using PyMOL 2.5.7 software (Schrödinger, USA). A computer server with a specification of Intel Xeon Gold 5218 CPU and 16 GB of RAM, installed with Uni-GBSA (DP Technology, China) and ORCA 6.1.0 (Frank Neese, Germany) software, was used to run MM/PBSA along with per-residue energy decomposition and quantum mechanical calculations, respectively.

Procedure

Acetylcholinesterase Activity Inhibition Assay

AChE inhibitory activity was assessed using a modified Ellman method according to the manufacturer's instructions. The assay kit contained enzymatic assay buffer, assay reagent, and calibrator.¹⁷ Before the assay, the working reagent was prepared by dissolving the assay reagent in the assay buffer at a concentration of 2 mg per 200 μ L. A total of 200 μ L of DMSO and 200 μ L of calibrator were added to separate wells of a clear 96-well plate. In addition, 10 μ L of isoliquiritigenin solution was mixed with 190 μ L of the working reagent in separate wells, designated as inhibitor wells. The assay was performed at a final isoliquiritigenin concentration of 0.5, 1.0, 2.5, 5.0, and 10 μ M in assay wells, obtained through a 20-fold dilution of stock solutions in DMSO. Absorbance at 412 nm was measured using a Synergy HTX-3 multimode reader was operated utilizing Gen5 3.08 software. The assay was performed at room temperature (25°C), while AChE activity was measured using Equation 1.

AChE Activity

$$= \frac{\text{Absorbance}_{10} - \text{Absorbance}_2}{\text{Absorbance}_{\text{calibrator}} - \text{Absorbance}_{\text{DMSO}}} \times 200 \text{ (U/L)} \quad (1)$$

Where Absorbance₁₀ and Absorbance₂ represent the absorbance values of the inhibitor wells at 10 and 2 minutes of incubation time, respectively. Absorbance_{calibrator} and Absorbance_{DMSO} represent the absorbance values of the calibrator and DMSO at 10 minutes of incubation time, respectively. All inhibitory assessments were conducted in triplicate, and DMSO served as the negative control for the calculation of inhibition percentages. Inhibition percentages were derived from the measured AChE activities to quantify the inhibitory effect of isoliquiritigenin on AChE across a range of concentrations. The IC₅₀ value was calculated using RStudio software 2026.05.1+225 version (Posit Software, USA), using nonlinear regression based on a four-parameter logistic model, with the lower and upper plateaus constrained to 0% and 100%, following previously reported methodology.¹⁸ To ensure the reliability of the enzymatic kit, donepezil was tested in triplicate at a concentration of 10 μ M in the assay wells. These concentrations served as positive assay controls because donepezil has a well-documented inhibitory profile against AChE.^{19,20}

Ligand-Protein Preparations, Redocking, and Molecular Docking Simulations

A three-dimensional crystal structure of human AChE complexed with native ligand donepezil (PDB ID: 7E3H) was retrieved from the Protein Data Bank using YASARA Structure. The crystal structure contains two identical AChE chains (chain A and B). Since both chains are structurally identical, only chain A was retained for subsequent simulations. Protein and ligand preparations were conducted according to a previously published protocol, including reconstruction of the missing amino acid residues, removal of crystallographic artifacts and water molecules, assignment of protonation states at physiological pH (7.4), and energy minimization.¹³ The simulation box used to perform both redocking and molecular docking simulations was defined as a cubic box with a distance of 5.0 Å from the outermost ligand atoms.¹³ Redocking and molecular docking simulations were subsequently performed according to a previously published protocol,¹³ consisting of 100 independent redocking simulations and 100 independent molecular

docking simulations to improve sampling of the ligand's best-redocked and best-docked pose. The best-redocked and best-docked poses were selected based on the lowest free energy of binding (FEB) value from each simulation. Furthermore, best-docked pose clustering was performed using previously published in-house developed YASARA plug-ins.¹³

Molecular Dynamics Simulations

MD simulations were configured to mimic physiological conditions, with a temperature of 310 K, a water density of 0.993 g/mL, system pH of 7.4, and NaCl concentration of 0.9%.¹³ In addition, the AMBER 14 force field and periodic boundary conditions were applied, while Particle-Mesh Ewald was utilized to calculate long-range Coulomb interactions. The total MD simulation time was set to 15 ns, comprising a 5-ns equilibration phase (NVT and NPT ensemble) followed by a 10-ns production phase. The initial 10-ns production phase was examined to evaluate the ligand-protein interactions, a simulation time previously reported as sufficient for preliminary ligand-protein stability evaluation.²¹ The simulations were performed in triplicate with different random seeds to account for stochastic variations, and the most stable replicate, which was characterized by the lowest RMSD backbone and ligand movement value, was extended to a 100-ns production simulation. The trajectory snapshots of the 100-ns production simulation were saved every 500 ps. The overall stability of ligand-protein interactions and AChE conformations were assessed by analyzing RMSD ligand movement, RMSD of backbone atoms, and root mean square fluctuation (RMSF) of AChE active site residues.

Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA) and Per-residue Energy Decomposition

Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA) calculations were performed using the recently published and open-source software Uni-GBSA.¹² Input file preparations were facilitated by our in-house-developed macro, which converts the trajectory snapshots of the production phase of MD simulations to ready-to-input files required by the Uni-GBSA pipeline.¹² MM/PBSA results were used to assess the stability of ligand-protein interactions, while the per-residue energy decomposition results provided insights into the key amino acid residues contributing significantly to the ligand binding.

Density Functional Theory Calculations

DFT calculations were performed using the Becke, 3-parameter, Lee-Yang-Parr (B3LYP) functional with the def2-TZVP basis set. These calculations were used to compute the electronic properties of the ligands, including molecular electrostatic potential (MEP), highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), energy gap, chemical softness, chemical hardness, electrophilicity, and electronegativity. In the ORCA input file, the spin multiplicity of each ligand was set to 1 (singlet), and the total charge was +1 for donepezil and 0 for isoliquiritigenin, representing the structure at the physiological pH state. In addition, DFT calculations were also performed on the most stable MD-derived complex selected from MM/PBSA results to evaluate the stability of ligand-protein interactions. The selected MD-derived complex was prepared by retaining only residues with high interaction occupancy and favorable binding free energy, as identified through MM/PBSA and per-residue energy decomposition analyses. The selected residues were subsequently capped at their termini with ACE and NME.^{22,23} Residue deletion was performed using the “DelRes” feature in YASARA Structure, while preserving the selected amino acid residues conformations and coordinates. Geometry optimization was performed using the B3LYP-D3BJ functional with the def2-TZVP basis set. RIJCOSX approximation and the conductor-like polarizable continuum model (CPCM) with water as the solvent were used to reduce computational cost.^{24–27} In the ORCA input file, heavy atoms of ACE and NME caps were constrained during the geometry optimization. The spin multiplicity was set to 1 (singlet), and the total charge was adjusted according to the net charge of the selected residues and the ligand.

Results and Discussion

Acetylcholinesterase Enzymatic Assay

AChE activity inhibition assay was performed to experimentally validate the previous SBVS study.⁶ This assay was conducted based on the modified Ellman method as mentioned in the manufacturer's instructions. The inhibitory activity is shown in Figure 1, showing a concentration-dependent relationship between isoliquiritigenin concentration and AChE inhibition. These findings were subsequently used to estimate the IC₅₀ value of isoliquiritigenin, resulting in a concentration of 1.078 μ M (95% CI: 0.590-1.566 μ M). Meanwhile, the positive control donepezil exhibited 88.2 % AChE inhibition at a concentration of 10 μ M in the assay wells, as this compound is a potent FDA-approved AChE inhibitor.²⁰ However, the IC₅₀ value obtained in the present study differed substantially from that reported in our previous study, in which isoliquiritigenin exhibited an IC₅₀ value of 126.22 μ M.²⁸ This discrepancy was primarily attributed to differences in the IC₅₀ calculation procedure. In the previous study, the IC₅₀ was estimated based on the isoliquiritigenin concentrations prepared from the stock solutions, whereas in the present study, the IC₅₀ was calculated

based on the final concentrations of isoliquiritigenin in the assay wells. In addition, slight variations in the working reagent concentration during assay preparation, despite following the intended preparation ratio, may have contributed to differences in the observed inhibitory activity. These methodological differences should therefore be considered when comparing the IC₅₀ values between the two studies. Although donepezil produced a higher percentage of AChE inhibition than isoliquiritigenin at the tested concentration, isoliquiritigenin exhibited a considerably lower IC₅₀ value compared to other bioactive compounds. Previous studies reported that naringenin exhibited an IC₅₀ value of 149.6 $\pm$ 4.6 μ M, while the most active flavonoid derivative exhibited an IC₅₀ value of 13.0 $\pm$ 1.9 μ M.²⁹ Therefore, these findings were consistent with previous reports, demonstrating that phenolic compounds inhibited AChE activity and may serve as natural AChE inhibitors. To further evaluate the inhibitory mechanism of isoliquiritigenin and donepezil, a total of 100 independent molecular docking simulations, 100-ns MD simulations, MM/PBSA with per-residue energy decomposition, and DFT calculations were subsequently performed to provide molecular-level insights into the inhibitory mechanism.

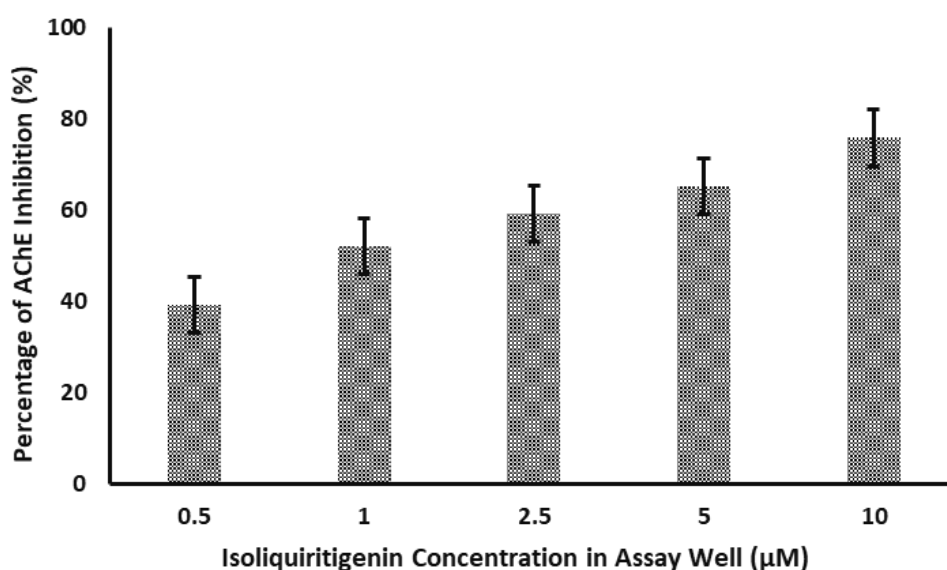


Figure 1: Concentration-dependent AChE inhibition (%) by isoliquiritigenin at various concentrations

Molecular Docking Simulations

Initial protein preparation was performed to reconstruct a complete structure of AChE under physiological conditions before the redocking and molecular docking simulations.¹³ A total of 100 independent redocking and docking simulations were performed to improve conformational sampling and reduce stochastic bias during ligand pose prediction. The redocking simulations yielded an average free energy of binding (FEB) of -12.332 ± 0.043 kcal/mol for donepezil, indicating that donepezil interacted stably within the AChE active site. In addition, RMSD values obtained from the redocking simulations ranged from 0.370 to 0.478 Å, showing that the proposed molecular docking protocol used in the redocking simulations was reliable for determining the redocked conformation resembling the crystallographic conformation of donepezil.¹³ Although 100 independent molecular docking simulations of isoliquiritigenin produced a less favorable average FEB than that of donepezil, isoliquiritigenin still exhibited a favorable average FEB of -9.731 ± 0.012 kcal/mol. All ligands formed aromatic interactions with aromatic amino acid residues, including Trp286 in the peripheral anionic site (PAS) and Trp86 in the catalytic active site, as shown in Figure 2. The remaining interactions are summarized in Table 1. Best-pose clustering produced RMSD values ≤ 2.000 Å for both redocked and docked complexes, indicating that each ligand exhibited a single dominant binding cluster, as shown in Figure 2. Moreover, isoliquiritigenin was stabilized by Phe297 in the acyl-

binding pocket.^{19,30,31} To validate and further assess the interaction stability of the best-redocked and best-docked poses, MD simulations were subsequently performed to the lowest FEB value of the best-redocked and best-docked pose, as recommended in previous studies.^{13,32}

Evaluation of Conformational Stability and Reproducibility through Multi-Replica Molecular Dynamics Simulations

Preliminary stability evaluation of the ligand-protein complexes was performed using the best-redocked and best-docked poses, followed by triplicate 10-ns MD simulations with different random seeds. Previous studies revealed that a binding mode could be categorized as unstable if all five independent MD simulations could not retain the initial binding pose.²¹ Accordingly, the replicate that retained the initial binding pose while exhibiting the lowest RMSD ligand movement and the most favorable FEB was selected for further evaluation. Table 2 summarizes the average RMSD values of AChE backbone atoms, ligand movement, and FEB obtained from the 10-ns MD simulations. Although all replicates of AChE-donepezil possessed RMSD Backbone and ligand movement ≤ 2.000 Å, replicate 1 (Rep 1) showed the lowest values of average RMSD Backbone and RMSD ligand movement, indicating that AChE backbone atoms and donepezil conformations remained stable during the simulations. On the other hand, replicate 2 (Rep 2) of AChE-isoliquiritigenin exhibited the most stable AChE backbone atom and

isoliquritigenin conformations, with an average RMSD ligand movement of 1.662 ± 0.388 Å. Interestingly, replicate 1 of AChE-donepezil and replicate 2 of AChE-isoliquritigenin also exhibited the most favorable average FEB values among the three replicates, with values of -2.567 ± 0.102 and -1.945 ± 0.140 kcal/mol, respectively. Table 2 shows that the greater RMSD of ligand movement in replicate

3 of isoliquritigenin suggested partial displacement from its initial binding orientation, highlighting the importance of conducting multiple independent MD simulations.²¹ Therefore, these replicates were extended to 100-ns MD simulations to provide deeper insight into the ligand-protein interaction stability.

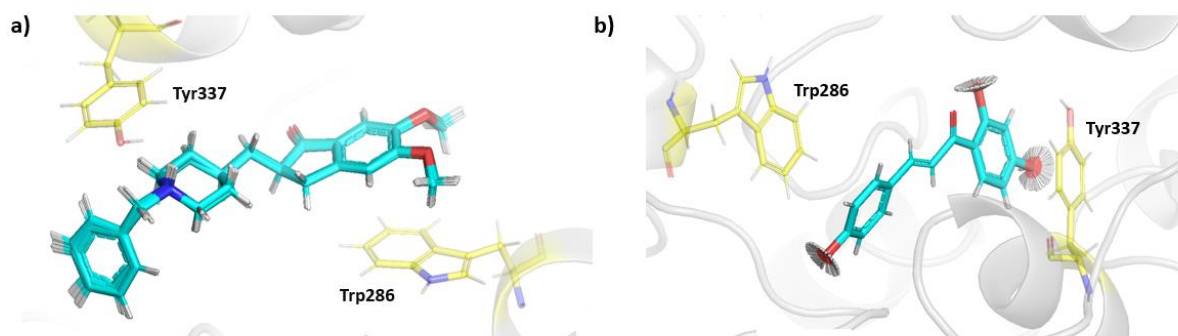


Figure 2: The superimposed single best-redocked poses of donepezil (a) and best-docked poses of isoliquritigenin (b) from the 100 independent redocking and molecular docking simulations. Carbon atoms of the ligands are shown in cyan color while carbon atoms of the visualized AChE residues are shown in yellow color

Table 1: Average free energy of binding and interactions formed by donepezil and isoliquritigenin in the AChE active site obtained from 100 independent redocking and 100 independent molecular docking simulations

Ligand	Free Energy of Binding (kcal/mol)	Electrostatic	H-bond	Aromatic face-to-face	Aromatic edge-to-face
Donepezil	-12.332 ± 0.043	-	-	Trp86, Trp286	Tyr341, His447
Isoliquritigenin	-9.731 ± 0.012	-	-	Trp286, Tyr337, Tyr341	Trp86, Phe297, His447

Table 2: Average RMSD of AChE backbone atoms, RMSD ligand movement, and free energy of binding of AChE-donepezil and AChE-isoliquritigenin complexes from 10-ns MD simulations

MD Parameters	Donepezil			Isoliquritigenin		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
RMSD Backbone (Å)	1.162 ± 0.137	1.192 ± 0.133	1.175 ± 0.112	0.981 ± 0.192	1.166 ± 0.226	1.118 ± 0.184
RMSD Ligand Movement (Å)	0.981 ± 0.192	1.166 ± 0.226	1.118 ± 0.184	2.031 ± 0.793	1.662 ± 0.388	4.244 ± 1.667
Free Energy of Binding (kcal/mol)	-2.567 ± 0.102	-2.558 ± 0.092	-2.535 ± 0.110	-1.922 ± 0.122	-1.945 ± 0.140	-1.695 ± 0.234

Molecular Dynamics Simulations, MM/PBSA, and Per-Residue Energy Decomposition Analysis

The interaction stability of donepezil and isoliquritigenin was further assessed through 100-ns MD simulations by analyzing RMSD Backbone, RMSD ligand movement, and RMSF of the active site amino acid residues (Figure 3). The average RMSD Backbone of the AChE-donepezil complex was 1.260 ± 0.118 Å, whereas the RMSD ligand movement was 1.908 ± 0.372 Å. Although one snapshot exhibited an RMSD ligand movement of 2.722 Å, donepezil remained in the active site based on trajectory inspection. In addition, isoliquritigenin also exhibited an average RMSD Backbone value of 1.316 ± 0.158 Å, retaining stable AChE conformations. In contrast, isoliquritigenin exhibited RMSD ligand movement of 7.364 ± 1.479 Å with a marked increase (7.912 Å) beginning within the first 2 ns of the simulations. Although isoliquritigenin adopted a different binding orientation after this transition, isoliquritigenin still formed aromatic interactions with Trp286 and Tyr341. Observations from trajectory inspection showed that isoliquritigenin still interacted at the PAS region, forming hydrogen bonds with Tyr72 and Asp74, along with other interacting residues including Leu76, His287, and Gly342, even at the snapshot

exhibiting the highest RMSD ligand movement (9.701 Å; 12 ns). Remarkably, all RMSF amino acid residues at the AChE active site remained stable with values of ≤ 2.000 Å in all complexes. These findings suggested that the higher RMSD ligand movement of isoliquritigenin reflected ligand orientation toward more stable hydrogen bonding interactions that were not captured by the initial best-docked pose. MM/PBSA calculations were also in good agreement with redocking and molecular docking results, indicating that donepezil possessed lower FEB than isoliquritigenin, with the average value of -24.902 ± 2.916 kcal/mol (Figure 3d). Although isoliquritigenin had a higher MM/PBSA free energy, with an average value of -7.408 ± 3.065 kcal/mol, the MM/PBSA profile became relatively stable after approximately 38 ns. Per-residue energy decomposition identified the amino acid residues contributing the most favorably to ligand binding. Table 3 shows that donepezil was stabilized by Asp74 (electrostatic) and Glu202 (anion- π) with 100% interaction occupancy. In addition, donepezil was also stabilized by aromatic (Trp86) and hydrophobic interactions (Ser293) throughout the simulations. These findings also correlated with previous studies, showing the importance of aromatic moieties of AChE inhibitors.⁶

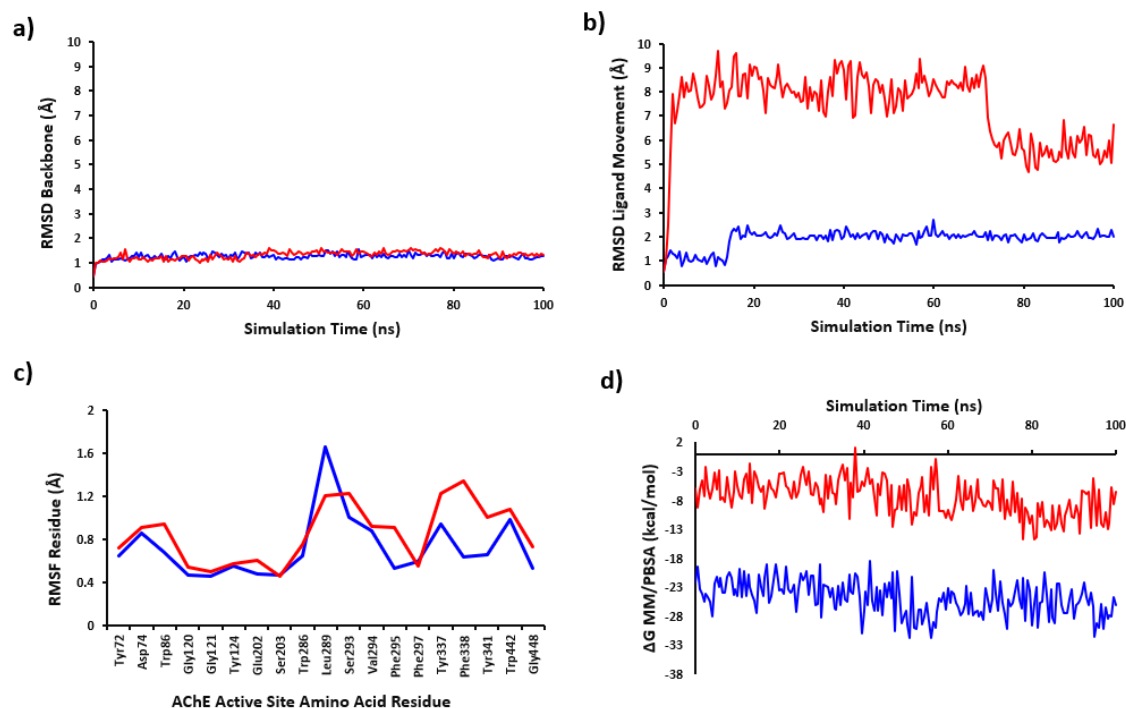


Figure 3: Results of the 100-ns MD simulations showing RMSD Backbone profiles (a), RMSD ligand movement trajectories (b), RMSF profiles of AChE active site residues (c), and MM/PBSA free energy of binding of AChE-isoliquritigenin and AChE-donepezil complexes (d). AChE-isoliquritigenin and AChE-donepezil complexes are presented in red and blue color, respectively

Table 3: Individual energy decomposition and occupancy of key binding site residues of AChE for donepezil and isoliquritigenin calculated from 100-ns MD snapshots

Ligand	Amino Acid Residue	Average Per-residue Energy Decomposition (kcal/mol)	Occupancy (%)	Observed Interaction
Donepezil	Asp74	-17.614 ± 1.166	100.00	Electrostatic
	Trp86	-2.668 ± 0.434	100.00	Aromatic face-to-face
	Glu202	-17.923 ± 0.885	100.00	Anion-π
	Trp286	-9.249 ± 0.461	4.98	Aromatic face-to-face
	His287	-2.179 ± 0.421	100.00	Hydrophobic
	Ser293	-8.054 ± 0.629	99.00	Hydrophobic
	Phe295	-1.540 ± 0.307	98.51	Hydrophobic
	Arg296	-1.403 ± 0.257	99.00	Hydrophobic
	Phe338	-3.325 ± 0.727	99.50	Hydrophobic
	Leu339	-2.827 ± 0.513	100.00	Hydrophobic
Isoliquritigenin	Gly342	-4.352 ± 0.422	100.00	Hydrophobic
	Tyr72	-1.722 ± 0.627	96.52	Hydrogen bond
	Asp74	-8.887 ± 2.591	100.00	Hydrogen bond
	Leu76	-1.133 ± 0.341	80.60	Hydrophobic
	His287	-2.149 ± 0.985	98.01	Hydrophobic
	Tyr341	-0.747 ± 0.142	34.83	Aromatic edge-to-face and aromatic face-to-face
	Gly342	-1.711 ± 0.632	96.52	Hydrophobic

Trajectory inspection further showed that the hydroxyl group of isoliquiritigenin was predominantly stabilized in the PAS region by hydrogen bonding with Tyr72 and Asp74 with 96.52% and 100% occupancy, respectively. Interestingly, Tyr341 underwent reversible face-to-face and edge-to-face aromatic stacking interactions at several MD snapshots due to its flexibility. These findings are supported by previous studies, showing that AChE inhibitor hits formed hydrogen bonds with Tyr72 and Asp74, along with aromatic interactions with Tyr341.³³ Previous studies also reported that hydrogen bond and aromatic interactions with those amino acids were crucial in AChE activity inhibition.³⁴ These findings suggest that the robust interactions with PAS region residues, particularly the high-occupancy hydrogen bonds and dynamic aromatic stacking, likely contribute to the inhibitory mechanism of isoliquiritigenin against AChE.

Quantum Mechanical DFT Calculations

Frontier molecular orbital (FMO) analysis, particularly E_{HOMO} and E_{LUMO} , provides important descriptors of molecular electronic properties and chemical reactivity,³⁵ showing that isoliquiritigenin exhibited higher E_{HOMO} and E_{LUMO} levels than donepezil. E_{HOMO} is associated with the molecule's tendency to attack electrophiles, while E_{LUMO} shows the molecule's tendency to be attacked by nucleophiles. The spatial distributions of E_{HOMO} and E_{LUMO} orbitals are shown in

Figure 4. E_{HOMO} distributions of both compounds were primarily localized over the aromatic rings, although in donepezil the orbital was mainly concentrated on the substituted aromatic ring. Table 4 shows that the HOMO-LUMO energy gap of donepezil (3.467 eV) was lower than that of isoliquiritigenin (3.802 eV), indicating that donepezil was electronically more reactive. These observations are consistent with MM/PBSA results, which showed that donepezil exhibited a more favorable FEB than isoliquiritigenin, consistent with findings from previous studies.³⁶ Chemical hardness indicates a molecule's capability to withstand changes in its electron configuration. Meanwhile, chemical softness indicates a greater tendency toward electronic polarization. The higher chemical hardness (1.901 eV) and lower chemical softness (0.526 eV) of isoliquiritigenin indicated lower reactivity than donepezil. The electrophilicity index indicated that donepezil was more electrophilic than isoliquiritigenin, likely reflecting its protonation state at physiological pH. Both compounds exhibited high electrophilicity indices (>1.5 eV), indicating pronounced electrophilic character.³⁷ Donepezil exhibited a higher electronegativity value (5.967 eV) than isoliquiritigenin (4.182 eV), confirming their electron-attracting capabilities, which may be attributed to the protonated amine. Molecular electrostatic potential (MEP) plots further illustrated the electron-rich and electron-deficient regions of donepezil and isoliquiritigenin, as shown in Figure 5.

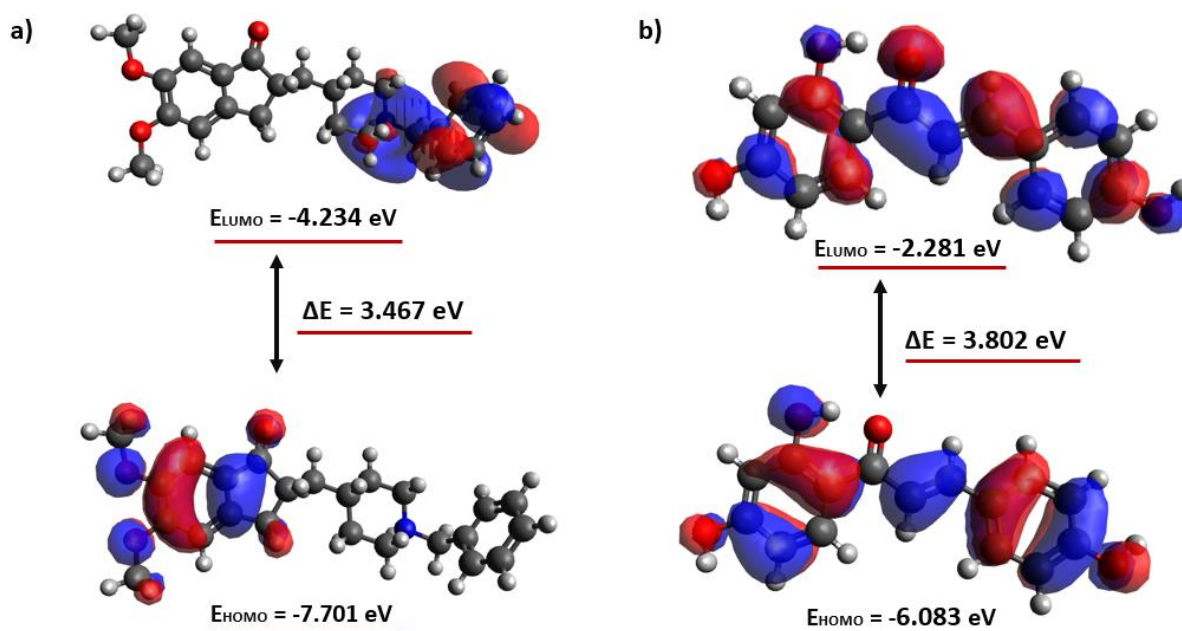


Figure 4: HOMO and LUMO orbital distributions of donepezil (a) and isoliquiritigenin (b) calculated using DFT/B3LYP/def2-TZVP level of theory. The corresponding HOMO-LUMO energies and $E_{\text{HOMO-LUMO}}$ gap values are indicated

Table 4: Frontier molecular orbital parameters of donepezil and isoliquiritigenin calculated using DFT/B3LYP with def2-TZVP level of theory

Electronics parameters	Ligand	
	Donepezil (eV)	Isoliquiritigenin (eV)
E_{HOMO}	-7.701	-6.083
E_{LUMO}	-4.234	-2.281
$E_{\text{HOMO-LUMO}}$ gap	3.467	3.802
Chemical softness	0.576	0.526
Chemical hardness	1.733	1.901
Electrophilicity	30.865	16.623
Electronegativity	5.967	4.182

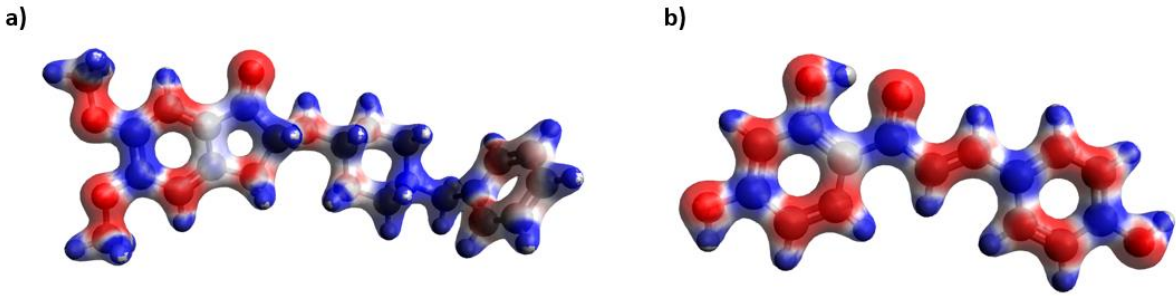


Figure 5: Molecular electrostatic potential of donepezil (a) and isoliquiritigenin (b) calculated using DFT/B3LYP/def2-TZVP level of theory. Regions of high and low electron density are shown in red and blue, respectively

Consistent with their molecular structures, the oxygen atoms in the hydroxyl and carbonyl groups exhibited regions of high electron density, suggesting that these functional groups may participate in hydrogen-bond interactions with nearby amino acid residues. FMO and MEP analyses were in good agreement with 100-ns MD simulation results, showing that the electron-deficient protonated nitrogen atom and the higher electrophilicity of donepezil formed electrostatic interactions with the deprotonated carboxyl group of Asp74. Meanwhile, the phenolic hydroxyl group of isoliquiritigenin formed persistent hydrogen bonds with Tyr72 and Asp74, while its aromatic

ring established aromatic face-to-face interactions with Tyr341. The interaction stability of the most stable MD-derived complex was further assessed using DFT/B3LYP-D3BJ with the def2-SVP basis set. The modeled interaction distance comparison, derived from the high interaction occupancy and favorable FEB from per-residue energy decomposition, is presented in Table 5. As shown in Figure 6, the optimized-geometry interactions preserved the interactions of donepezil with the deprotonated carboxyl group of Asp74 (electrostatic interaction), Trp86 and Trp286 (aromatic face-to-face), and Glu202 (anion- π).

Table 5: Quantum mechanical interaction profiles of the most stable MD-derived complex for donepezil and isoliquiritigenin complex calculated using DFT/B3LYP-D3BJ with def2-TZVP level of theory

Ligand	Interactions	Intermolecular distance (Å)	
		MD-derived	B3LYP-D3BJ/ def2-TZVP
Donepezil	Asp74 (electrostatic)	7.023	4.544
	Trp86	3.806 and 3.406	4.040 and 3.999
	(aromatic face-to-face)		
	Trp286	5.830 and 4.449	3.563 and 3.808
	(aromatic face-to-face)		
	Glu202 (anion- π)	3.516	6.665
Isoliquiritigenin	Tyr72 (hydrogen bond)	1.890	1.989
	Asp74	1.899	1.636 (side-chain) and
	(hydrogen bond)	(side-chain)	1.780 (backbone)
	Tyr341	4.879	3.831
	(aromatic face-to-face)		

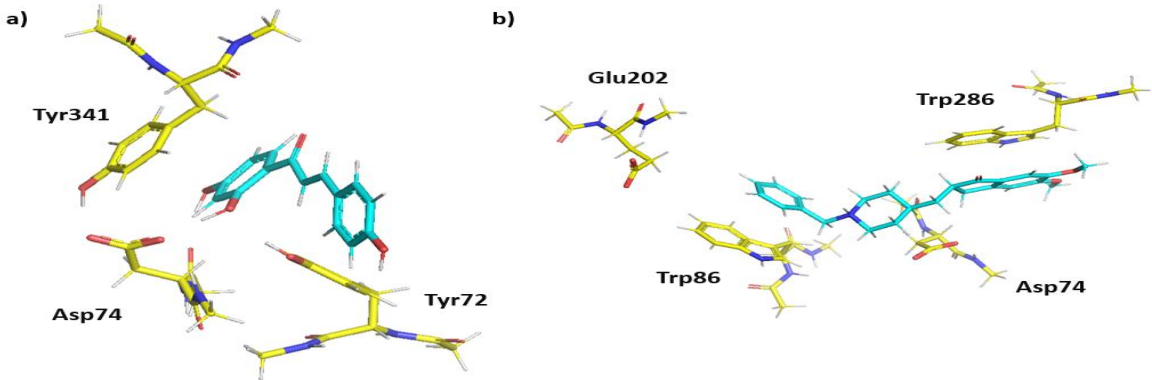


Figure 6: Interaction stability evaluations calculated using DFT/B3LYP-D3BJ with def2-TZVP level of theory depicting stable interactions derived from per-residue energy decomposition for isoliquiritigenin (a) and donepezil (b), respectively. The carbon atoms of the ligands are visualized in cyan color while the carbon atoms of AChE residues are visualized in yellow color

These findings indicated that aromatic interactions were formed with both the pyrrole and benzene rings of the Trp residues, including the pyrrole ring (3.563 Å and 4.040 Å in Trp286 and Trp86, respectively) and the benzene ring (3.808 Å and 3.999 Å in Trp286 and Trp86, respectively). Meanwhile, isoliquiritigenin retained stable interactions with Tyr72 and Asp74 (hydrogen bond), together with Tyr341 (aromatic face-to-face). Although there was only one hydrogen bond observed in the MD-derived complex, a new hydrogen bond was formed with the backbone carbonyl group of Asp74, likely due to local conformational flexibility, which tended to reinforce the overall interaction stability. These quantum mechanical calculations therefore verified the stability of several pivotal interactions of the most stable MD-derived complex and provided robust computational insights into ligand binding during AChE inhibition.

Conclusion

In conclusion, this study demonstrated the inhibitory potential of isoliquiritigenin against AChE through the integration of an experimental enzymatic assay and complementary computational analyses. The enzymatic assay confirmed that isoliquiritigenin exhibited an IC_{50} value of 1.078 μ M. Furthermore, 100-ns MD simulations, MM/PBSA, per-residue energy decomposition, and DFT calculations consistently showed that isoliquiritigenin preferentially interacted with the PAS of AChE, forming hydrogen bonds with Tyr72 and Asp74, and aromatic interactions with Tyr341 across multiple MD simulation snapshots. DFT analyses further highlighted the contribution of the phenolic moiety of isoliquiritigenin to ligand binding. Overall, the findings provide experimental and mechanistic evidence supporting isoliquiritigenin as a promising phenolic natural product for further investigation as a lead compound for AD treatment. Further studies are needed to explore the potency of isoliquiritigenin through in vivo evaluations and to assess its pharmacokinetic properties. In addition, structural modifications of isoliquiritigenin could be explored to design new derivatives with more optimal AChE inhibitory activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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