

Neuroprotective Effects of Certain Flavonoids as a Cholinesterase Inhibitor for the Management of Alzheimer's Disease – an *In silico* and *In Vitro* Study

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Abstract: This study used *in vitro* and *in silico* enzyme inhibition studies to examine the effects of certain commercially available flavonoids against the acetylcholinesterase enzyme. In order to compare the potential enzyme inhibitory effects of the chosen flavonoids, Donepezil, a known cholinesterase inhibitor, was employed as standard. Utilizing the AutoDock 4.2 program, the *in silico* docking evaluations were evaluated. Baicalein, Chrysin, Morin, Hesperitin, Rutin, and Theaflavin were studied computationally and were discovered to have superior docking scores and binding orientations with the acetylcholinesterase enzyme than the standard. AChE showed stronger inhibitory values (IC_{50}) to all the chosen flavonoids than the standard in *in vitro* enzyme inhibitory experiments. With a superior cholinesterase inhibition value of $10.18 \pm 0.68 \mu\text{g/ml}$ compared to the other compounds, Baicalein was identified as the most promising candidate among the chosen compounds. The *in silico* docking analyses concur with the *in vitro* AChE inhibitory findings and the potential use of Baicalein as a therapeutic drug to stop or delay the progression of AD. More research is required to fully understand the mechanism of action of Baicalein, which may offer a distinct therapeutic advantage in managing AD.

Keywords: acetylcholinesterase; Ellman's method; inhibition constant; molecular interactions; neurodegenerative diseases.

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1. Introduction

The progressive degeneration or death of nerve cells caused by neurodegenerative illnesses, which are incurable, is a serious social and medical issue. Despite the fact that versions of each of the disorders in childhood are recognized, they are the major problems of later life that occur in people with neurologically normal brains [1]. Alzheimer's disease (AD) is caused by the degeneration of the bulbar, spinal, and cortical motor neurons as well as inherited genetic changes and is characterized by the loss of cortical and hippocampal neurons, memory impairment, cognitive decline, and ALS [2, 3].

Alzheimer's disease is an organic brain ailment that affects memory, language, orientation, understanding, and learning ability. It is a chronic, debilitating condition [4]. Brain

atrophy, the production and deposition of hyperphosphorylated tau filaments known as neurofibrillary tangles, and the aggregation of β -amyloid ($A\beta$) molecules known as amyloid plaques are the three main morphological alterations seen in the brains of AD patients [5]. The cholinergic system, particularly in the cortex and hippocampus, is where these changes are most noticeable and are strongly associated with memory loss and cognitive dysfunction in AD [6].

According to neuronal and pharmacological findings, the severity of memory impairment is correlated with the cholinergic system's hypofunction. Patients with AD benefit greatly from cholinergic neurotransmission, which is mediated by the neurotransmitter acetylcholine. At the synapses of the cholinergic system, acetylcholine esterase (AChE), an enzyme, swiftly hydrolyzes the released acetylcholine into acetate and choline. Memory and learning deficits are caused by acetylcholine levels in the cortex and hippocampus being too low [7].

An important therapeutic strategy for AD has entailed initiatives to improve the brain's cholinergic system [8]. Using phosphatidylcholine and choline chloride as acetylcholine production precursors was one early strategy. A reversible AChE inhibitor called physostigmine enhances learning responses in animal models, and some studies have shown small, transient improvements in memory and learning in AD patients [9]. The selective AChE inhibitor donepezil has a limited impact on AChE in peripheral tissues. Patients with Alzheimer's disease who take Donepezil have had a modest improvement in their cognitive deficit, allowing for once-daily dosing [10].

Flavonoids are a class of pigment-containing, water-soluble plant compounds that are biologically active and found naturally in vegetables, fruits, and herbs. Although they are a natural component of both human and animal diets, animals and humans are usually unable to synthesize flavonoids [11]. Flavonoids are linked to various positive health outcomes [12-14]. They are crucial in numerous nutraceutical, medical, pharmacological, and cosmetic applications [15, 16]. Therefore, the goal of the current investigation is to determine whether commercially available flavonoids have the ability to inhibit AChE utilizing *in silico* and *in vitro* studies.

2. Materials and Methods

2.1. Software used.

The Molecular Graphics Laboratory (MGL) tools and AutoDock 4.2 were installed from www.scripps.edu, the Python 2.7 language was procured from www.python.com, Cygwin was obtained from www.cygwin.com, Discovery Studio Visualizer 2.5.5 was procured from www.accelerys.com, and ChemSketch was utilized from www.acdlabs.com. The translation of SMILES was done using the cactus.nci.nih.gov/translate/ website.

2.2. Chemicals used.

Sigma Aldrich, St. Louis, MO purchased Donepezil, acetylcholinesterase, Baicalein, chrysin, morin, hesperetin, rutin, and theaflavin. All other analytical-grade chemicals were commercially purchased and used for the present study.

2.3. Preparation of target enzyme.

Based on a literature search from the Brookhaven Protein Data Bank, the crystal structure of the mouse acetylcholinesterase (5HCU) protein was obtained (Figure 1). The 5HCU protein structure has structural sequence similarities to human acetylcholinesterase. The AutoDock Tools' optimization of the target enzyme included the inclusion of hydrogen atoms, an essential step for determining partial atomic charges. On the geometric center of the target molecule, a three-dimensional affinity grid with dimensions of 277 x 277 x 277 and 0.6 spacing was chosen [17].

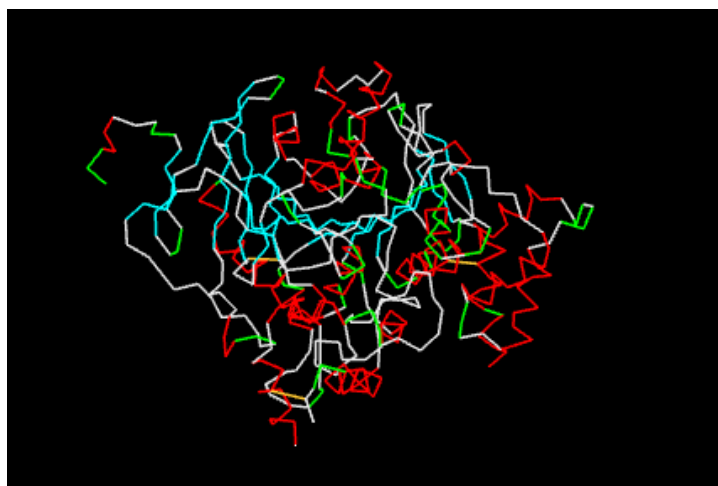


Figure 1. Refined crystal structure of mouse acetylcholinesterase (5HCU).

2.4. Drug likeness properties of the ligands.

The flavonoid ligands Baicalein, Chrysin, Morin, Hesperetin, Rutin, and Theaflavin were created in ChemSketch (Figure 2), and the flavonoids chosen were optimized in AutoDock 4.2. using the "Prepare Ligands" function [18].

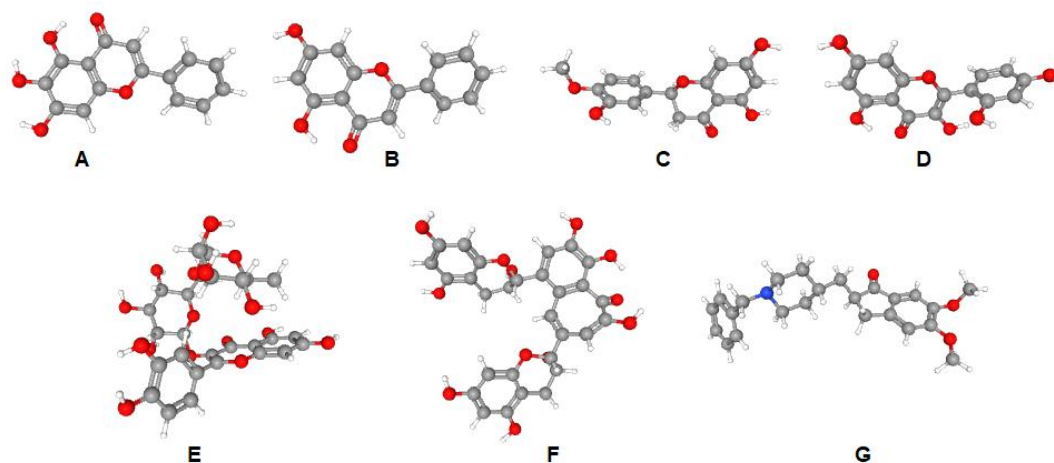


Figure 2. The optimized ligand molecules (A-Baicalein, B-Chrysin, C-Hesperetin, D-Morin, E-Rutin, and F-Theaflavin and G-Donepezil).

2.5. *In silico* acetylcholinesterase inhibition.

For the purpose of ligand conformational searching, the Lamarckian genetic algorithm combines genetic and local search algorithms. The method first creates a group of distinct fragments, each with a different docked molecular conformation. The local search algorithm

carried out energy minimizations when each ligand interacted with the target. Until the process is finished, the ligands with low binding energies to the target were obtained [19].

Using AutoDock 4.2 and the optimized flavonoid PDB files, docking evaluation was done with the chosen target molecule. The affinity grid, positioned on the target molecule's geometric center, assisted in the AutoGrid calculation. The GLG file was generated following the successful conclusion of the AutoGrid evaluation. The AutoDock calculation in AutoDock 4.2 is carried out by the Lamarckian genetic method. Ten distinct docked simulations for a single flavonoid molecule against the target molecule were obtained at the conclusion of the AutoDock evaluation. For all of the chosen flavonoids, the docking parameters, including binding energy, inhibition constant, and intermolecular energy, were tabulated [20]. The flavonoids were selected for *in vitro* enzyme inhibition investigations based on the docking elements obtained from AutoDock 4.2.

2.6. *In vitro* acetylcholinesterase inhibition.

Based on Ellman's technique, the *in vitro* acetylcholinesterase inhibitory assay was carried out [21]. After adding 0.1 ml of the 0.28 U/ml acetylcholinesterase enzyme, 1.3 ml of the Tris-HCl buffer (pH 8.0; 50 mM) was combined with 0.4 ml of various doses of the flavonoid solution. After 15 minutes of incubation, 0.023 mg/ml of acetylthiocholine iodide and 3 mM of 1.9 ml DTNB solution were added to the solution. After 30 minutes of incubation at room temperature, this mixture's absorbance was finally measured at 405 nm. The experiment was carried out in triplicate, and mean \pm SEM findings were reported. For the flavonoids, the degree of acetylcholinesterase inhibition was determined [22].

2.7. Statistical analysis.

GraphPad was used for the statistical data analysis, consisting of a one-way variance analysis and a Dunnett's test. Results were expressed as Mean \pm Standard deviation (SD).

3. Results and Discussion

3.1. *In silico* acetylcholinesterase inhibition.

The docking characteristics for the chosen flavonoids were assessed using the acetylcholinesterase enzyme, including the binding energy, inhibition constant, and intermolecular energy. Compared to conventional Donepezil, all flavonoids showed favorable docking scores and orientations against the AChE enzyme.

According to the docking hypothesis, substances are found to have good ligand and enzyme binding capacity if the values obtained for binding energy are more negative. The binding energy of the chosen flavonoids ranged from -6.93 kcal/mol to -5.88 kcal/mol, as indicated in Table 1, while the standard has a binding value of -3.87 kcal/mol against AChE. The flavonoids' binding energy is decreasing, indicating a higher affinity for the AChE molecule. Baicalein was discovered to have a higher negative value for binding energy when compared to other flavonoids and the standard, which highlighted its strong affinity for the AChE molecule.

Table 1. Summary of the docking parameters of the flavonoids against the target molecule.

Name of the compound	Binding energy(kcal/mol)	Inhibition constant (μM)	Intermolecular energy (kcal/mol)
Baicalein	-6.93	8.32	-8.91
Chrysin	-6.30	45.39	-8.64
Hesperetin	-6.72	17.02	-8.81
Morin	-6.92	8.29	-8.90
Rutin	-6.51	23.27	-8.76
Theaflavin	-5.88	66.25	-8.52
Donepezil	-3.87	994.14	-5.46

The benchmark used to determine a compound's potency against the inhibition of a target enzyme is the inhibition constant. In our investigation, binding energy levels were directly correlated with the flavonoids' inhibitory constants. When the docked flavonoids' binding energy decreased, we noticed a considerable fall in the values of their inhibitory constants. When compared to the standard, which has an inhibition constant of 994.14 μM , all of the chosen flavonoids exhibited lower inhibition constants, ranging from 8.32 μM to 66.25 μM .

With a fall in binding energy, a significant reduction in intermolecular energy was found for the chosen flavonoids. In this study, the intermolecular energy of the flavonoids ranges from -8.91 to -8.52 kcal/mol, which is lower than the standard (-5.46 kcal/mol). Similarly, the results of the docking parameters, namely the inhibition constant and intermolecular energy, showed that the Baicalein was discovered to demonstrate a remarkable docking score towards the AChE molecule from the list of chosen flavonoids. The compounds' *in silico* AChE inhibitory actions were ranked by potency Baicalein > Morin > Hesperetin > Rutin > Chrysin > Theaflavin > Donepezil.

3.2. Amino acid interactions in the molecular docking studies.

When compared to the standard Donepezil, the chosen flavonoids showed improved binding orientations against the acetylcholinesterase enzyme. The interacting amino acid residues significantly influence the inhibitory affinity toward the target enzyme. According to the current study, flavonoids differ from the standard in that they may interact with amino acids to inhibit AChE.

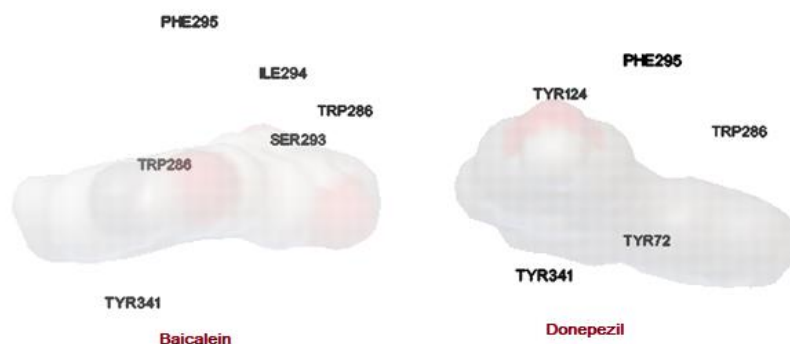


Figure 3. Binding orientations of acetylcholinesterase enzyme (5HCU) with Baicalein and Donepezil.

Trp 286, Ser 293, Ile 294, Phe 295, and Tyr 341 amino acid residues were found to be part of the binding site of the drug Baicalein (Figure 3), and Tyr 72, Tyr 124, Trp 286, Phe 295, and Tyr 341 amino acid residues were discovered to be part of the binding site of the drug Donepezil (Figure 3). Phenylalanine 295 and Tyrosine 341 were the two primary amino acid

residues in Baicalein and Donepezil that contributed to the target-ligand binding interactions. Compared to the Donepezil and other flavonoids, this study demonstrates that the Baicalein contained effective binding orientations. We conclude that the interactions seen using molecular docking calculations of the flavonoids will result in reversible AChE inhibitors based on the most stable conformations.

3.3. *In vitro* acetylcholinesterase inhibitory assay.

Based on the docking scores from the *in silico* computational investigations, the flavonoids were then added to *in vitro* acetylcholinesterase inhibitory experiments. The flavonoids' *in vitro* enzymatic assay highlights the *in silico* results and clarifies the function of flavonoids in treating AD.

We found that all of the chosen flavonoids have superior acetylcholinesterase enzyme inhibitory values than the standard. The concentration of the chosen flavonoids needed to inhibit the enzyme *in vitro* ranged from 10.92 to 21.48 $\mu\text{g/ml}$ (Figure 4). Comparing Baicalein's IC_{50} values to those of other chosen flavonoids and the standard, donepezil ($22 \pm 0.56 \mu\text{g/ml}$), Baicalein has an IC_{50} of $10.92 \pm 0.38 \mu\text{g/ml}$. The enzyme inhibitory potential is stronger when the IC_{50} values are lower. The *in vitro* AChE inhibitory activities of the compounds were in the order of Baicalein > Morin > Hesperetin > Rutin > Chrysin > Theaflavin > Donepezil.

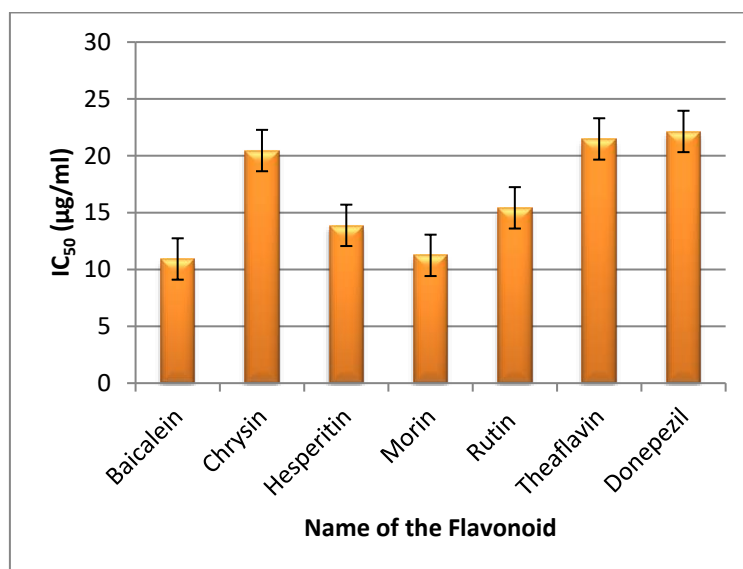


Figure 4. *In vitro* acetylcholinesterase inhibitory activity of the flavonoids. (Values are Mean \pm SEM of three parallel measurements).

Cognitive function is gradually impaired but relentlessly deteriorates as a result of Alzheimer's disease. The initial clinical sign of the disease is typically a decline in short-term memory, but retrieval of distant memories is generally maintained. Additional cognitive functions like the ability to calculate, visuospatial skills, and use everyday objects and equipment are hampered as the illness worsens [23]. Nicotine and other ACh receptor agonists, such as AChE inhibitors, have been used to treat AD. The efficacy of this strategy indicates that additional significant abnormalities contribute to cognitive impairment and ACh shortage [24].

Senile plaques, NFT, oxidative stress, inflammatory cascade, glutamatergic, and cholinergic insufficiency are a few of the pathogenic pathways that contribute to AD development. The U.S. Food and Drug Administration-approved cholinesterase inhibitors and <https://nanobioletters.com/>

NMDA receptor antagonists that are now used to treat AD symptoms have also been shown to ameliorate behavioral and psychological signs of the disease [25].

Molecular docking is a method used in the rational drug design process to investigate the interactions between the target and the ligand. It calculates the characteristics of the target and ligand's interactions as well as their binding orientations [26]. Many software programs are available for molecular docking evaluations, including AutoDock 4.2, GLIDE, GOLD, SLIDE, and FlexX [27]. Due to its improved docking speed and accessibility, AutoDock 4.2 is the docking program that receives the most mentions and is the most frequently used for virtual screening [28].

Saeedi *et al.*, 2017 evaluated the *in vitro* ChEI activity of a number of plants, including betel nuts (*Areca catechu* L.), clove buds (*Syzygium aromaticum* L.), dodder aerial parts (*Cuscuta chinensis* Lam.), common polypody rhizomes (*Polypodium vulgare* L.), and turpeth roots (*Ipomoea turpethum* R. Br.). *A. catechu* L. aqueous extract was discovered to be a powerful anti-AChE ($IC_{50} = 32.00 \mu\text{g/mL}$) and anti-BuChE ($IC_{50} = 48.81 \mu\text{g/mL}$) agent among them. The flavonoids in *A. catechu* L., including catechin, quercetin, and epicatechin, may be the cause of its cholinesterase inhibitory activities [29].

Hajlaoui *et al.* (2016) examined the chemical makeup of Tunisian *Origanum majorana* essential oil and assessed its antioxidant, antibacterial, cytotoxic, and anti-acetylcholinesterase capabilities. The results showed that the oil had higher IC_{50} values than butylated hydroxyl toluene and displayed high activity, notably in reducing power and β -carotene bleaching. When the oil's anti-AChE properties were assessed, it was discovered to have substantial activity, with IC_{50} values as high as $150.33 \pm 2.02 \mu\text{g/mL}$. The neuropharmacological effects of this plant may be due to terpenoids like Terpinen-4-ol and terpinenes [30].

Asokkumar *et al.* (2017) used *in vitro* and *in silico* research to investigate the inhibitory binding potential for certain flavonoids against the crystal structure of the acetylcholinesterase enzyme. Scopoletin was discovered to be a powerful and precise target inhibitor, with an IC_{50} value of $10.18 \pm 0.68 \mu\text{M}$, according to the study. To inhibit the acetylcholinesterase enzyme, scopoletin demonstrated a variety of hydrogen bonds to numerous important amino acid residues [31].

In the current study, we discovered that the minimal binding energy of the flavonoids against AChE showed that the compounds have better binding interactions than the typical Donepezil. According to the findings, Baicalein has potential AChE inhibitory binding sites compared to other flavonoids and the standard, and its structural characteristics are responsible for its AChE inhibitory ability.

The absorbance of the chosen flavonoids was reduced with increasing concentrations in a dose-dependent manner, according to an *in vitro* AChE inhibition assay. According to the findings, flavonoids might be a source of cholinergic inhibition. The future discovery of effective AChE inhibitors for the treatment of AD may result from this *in vitro* enzyme inhibitory and *in silico* docking research. A strong correlation was found between *in vitro* enzyme inhibitory studies and *in silico* investigations. Baicalein was identified as a possible natural chemical for treating AD based on the current study's findings.

4. Conclusions

In silico studies, Baicalein had better binding interactions and docking scores with the AChE enzyme than the standard. Baicalein was shown to be effective in inhibiting the ChE enzyme in an *in vitro* enzyme inhibition assay, which is thought to be connected to memory

impairment. Baicalein demonstrated lower IC₅₀ values against AChE in light of these results, and it may be beneficial to investigate whether it has *in vivo* memory-improving potential for use in treating AD.

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Conflicts of Interest

The authors declare that there are no conflicts of interest for this work.

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