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Research Article

Identification of cholinesterases inhibitors from flavonoids derivatives for possible treatment of Alzheimer's disease: *In silico* and *in vitro* approaches

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ABSTRACT

Nowadays, one of the methods to prevent the progress of Alzheimer's disease (AD) is to prescribe compounds that inhibit the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. Researchers are actively pursuing compounds, particularly of natural origin, that exhibit enhanced efficacy and reduced side effects. The inhibition of AChE and BChE using natural flavonoids represents a promising avenue for regulating AD. This study aims to identify alternative flavonoids capable of modulating AD by down-regulating AChE and BChE activity through a molecular docking approach. Molecular docking analysis identified Ginkgetin and Kolaflavanone as potent inhibitors of AChE and BChE, respectively, among the selected flavonoids. Asn87 and Ala127 involved in the interactions of AChE-Ginkgetin complex through conventional hydrogen bonds. While in the BChE-Kolaflavanone complex, Asn83, Ser79, Gln 47, and Ser287 are involved. *In vitro* analysis further corroborated the inhibitory potential, with Ginkgetin exhibiting an IC_{50} of 3.2 mM against AChE, and Kolaflavanone displaying an IC_{50} of 3.6 mM against BChE. These findings underscore the potential of Ginkgetin and Kolaflavanone as candidate inhibitors for the treatment of AD through the inhibition of AChE and BChE enzymes. Nevertheless, additional *in vitro* and *in vivo* studies are imperative to validate the efficacy of these compounds.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a gradual and progressive decline in memory and cognitive functions (Breijyeh and Karaman, 2020). According to global Alzheimer's reports, the prevalence of AD was estimated at 36 million individuals in 2010, and projections suggest a substantial increase to 115 million by the year 2050 (Osman et al., 2014; Ozgun et al., 2016). This alarming rise underscores the urgent need for effective therapeutic strategies to address the escalating impact of AD on global public health.

Cholinesterases constitute an enzyme family responsible for catalyzing the hydrolysis of acetylcholine into acetic acid and choline, a crucial process essential for the restoration of cholinergic neurotransmission (Abbasi et al., 2018; Hassan et al., 2019; de Almeida et al., 2023). The two primary cholinesterase types are AChE (EC 3.1.1.7) and BChE (EC 3.1.1.8). AChE is predominantly distributed in the brain, muscle, and erythrocyte membrane, while BChE exhibits higher activity in the intestine, liver, kidney, lung, and heart (Mascarenhas et al., 2021; Türkan, 2021; Xu et al., 2023). Despite being products of distinct genes on human chromosomes, these enzymes share similar molecular forms and active sites. Inhibition of acetylcholine and butyrylcholine hydrolysis using cholinesterase inhibitors has been explored as a strategy to elevate the levels of these neurotransmitters in synapses (Liu et al., 2020; Wu et al., 2020). Although numerous ongoing research endeavors aim to develop treatments for AD, only a limited number of drugs, including Donepezil, Rivastigmine, and Tacrine have received approval from the FDA (Fig. 1) (Michels and Lehr, 2021; Malik et al., 2022). Consequently, the quest for novel cholinesterase inhibitors, particularly those derived from natural sources, remains of paramount significance in advancing therapeutic options for AD and related neurodegenerative disorders.

Over the preceding three decades, natural products derived from plant sources, particularly flavonoid derivatives, have garnered escalating attention from food experts, consumers, and nutritionists owing to

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their notable contributions to human health (Gil-Martín et al., 2022; Sulieman et al., 2023). Flavonoids, being biologically active secondary metabolites, are abundant in various plant-derived products, including seeds, fruits, and vegetables. Many flavonoids, characterized by the presence of more than three hydroxyl groups, exhibit potent antioxidant properties, imparting significant health benefits (Sadeghi et al., 2023; Li et al., 2023; Smyrska-Wieleba and Mroczek, 2023). Pharmacological investigations have revealed that flavonoids possess multifaceted activities, encompassing the regulation of inflammation, management of liver disorders, mitigation of oxidative stress, cardiovascular health, anti-tumor effects, and antimicrobial properties (Ferraz et al., 2020; Abou Baker, 2022; Taldaev et al., 2022). Noteworthy reports have also implicated flavonoids in exerting effects on AD. However, despite these findings, a comprehensive exploration of the inhibitory effects of flavonoids on AChE and BChE remains lacking. In recent studies, Durmaz et al. (2023) demonstrated that baicalin, a specific flavonoid, significantly inhibited the activity of both AChE and BChE in in vitro experiments. Molecular docking analyses further revealed that quercetin, another flavonoid, exhibited a stronger docking score with BChE compared to donepezil (Khan et al., 2009). Based on these observations, we propose the hypothesis that flavonoids may serve as potent inhibitors of AChE and BChE, suggesting their potential therapeutic relevance in the context of neurodegenerative disorders and cognitive decline.

Computational docking stands as an indispensable tool in the domain of structure-based strategies, offering a robust framework for the prediction and design of drugs or inhibitors with exceptional precision and reliability (Tousheh et al., 2013, 2015; Sadeghi and Miroliaei, 2022). By leveraging advanced algorithms and computational models, this methodology enables researchers to simulate the interactions between molecules, such as ligands and receptors, within a given biological context (Mohammadpour et al., 2024; Salehi et al., 2024). Through meticulous analysis of molecular structures and energetic properties, computational docking facilitates the identification of potential binding sites and the exploration of ligand-receptor interactions, elucidating crucial insights into the underlying mechanisms of drug action (Sadeghi et al., 2022a,b,c,d; Sadeghi et al., 2022a,b,c,d; Sadeghi et al., 2022a,b,c,d; Gayathiri et al., 2023). Despite its efficacy, the application of computer screening and molecular binding analyses focusing on flavonoids and their interactions with AChE and BChE remains relatively underreported. Consequently, the present study endeavors to fill this knowledge gap by conducting *in silico* and *in vitro* evaluations to assess the interactions between flavonoids and AChE as well as BChE. The outcomes of this investigation bear potential significance for drug design studies, particularly those targeting AD. The focus lies on identifying inhibitors that demonstrate substantial efficacy in inhibiting the activities of both AChE and BChE enzymes. The insights gained from these *in silico* and *in vitro* assessment may pave the way for the development of therapeutics aimed at addressing AD and related neurodegenerative conditions.

2. Materials and methods

2.1. Enzymes structure for docking

The three-dimensional structures of AChE (PDB ID: 4PQE) and BChE (PDB ID: 1P0I) were acquired from the Protein Data Bank (http://www.rcsb.org/pdb) (Fatullayeva et al., 2023). Subsequently, the selected structures (4PQE and 1P0I) were subjected to force field fixation using Chimera 1.7. This process involved the addition of partial charges, hydrogen atoms, and addressing any missing residues. These structural modifications were implemented to optimize the suitability of the proteins for subsequent molecular docking analyses.

2.2. Enzyme active site prediction

Amino acid residues within the active site of enzymes were identified utilizing CASTp (http://sts.bioe.uic.edu/castp/index.html?2pk9) (Dariya et al., 2021; Sadeghi et al., 2022a,b,c,d). The development of CASTp was informed by recent advancements in theoretical and algorithmic aspects of computational geometry. This method offers several advantages: (1) it accurately defines the boundary between the bulk solvent and the pocket, (2) employs analytical methods for pocket and cavity identification, and (3) calculates all rotationally invariant parameters. Importantly, CASTp does not necessitate discretization, dot surfaces, or the utilization of grid points.

2.3. Ligands

Initially, the three-dimensional structure of flavonoids was retrieved from the PubChem database (pubchem.ncbi.nlm.nih.gov) (Fig. 2 and Table 1). Subsequently, the obtained structure was converted to the PDB format using DS (Biovia Discovery Studio) (Alabbas, 2023). The resulting PDB format was then employed in the present study. Initially, nine established inhibitors underwent docking assessments using AutoDock. The obtained results, which included experimentally determined metrics such as inhibition percentage and docking score (-kcal/mol), exhibited a favorable positive correlation, as outlined in Table 1. Subsequently, a total of 62 flavonoids were subjected to docking simulations within the binding sites of 4PQE and 1P0I utilizing Autodock software embedded in DS 2.5. This software employs a shape-based methodology to precisely dock substrate into the active sites of enzymes. The resulting

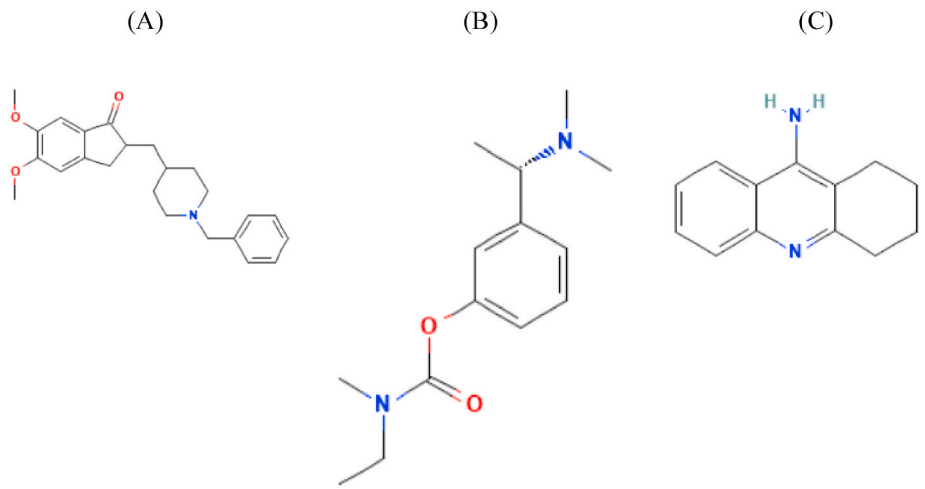

Fig. 1. Chemical structure of inhibitors for AChE and BChE: (A) Donepezil, (B) Rivastigmine, and (C) Tacrine.

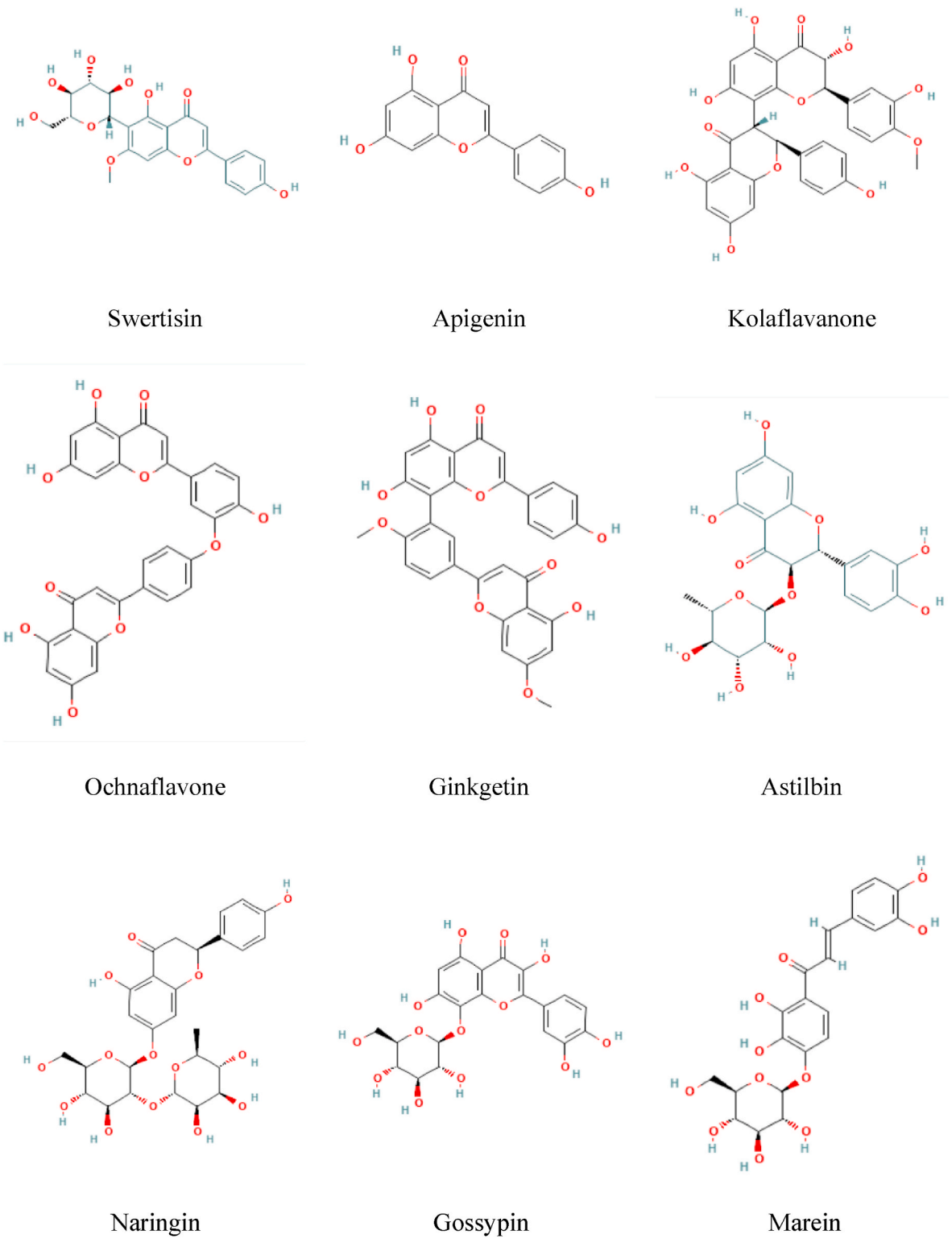

Fig. 2. The molecular structures of the nine docking energy of the flavonoids inhibitors against AChE and BChE.

docked poses were subjected to analysis, considering the efficiency of the potential docking energy.

2.4. Docking scoring

To elucidate the binding interactions between flavonoids and 4PQE and 1P0I, molecular docking analyses were conducted using AutoDock (version 1.5.6). The optimization of both the enzyme-substrate involved the removal of water, introduction of charges, and addition of polar hydrogens through methods such as Gasteiger and Kollman. A grid map of dimensions 110 × 125 × 110 with a spacing of 0.431 Å was generated to explore binding and active site residues. The Lamarckian Genetic Algorithm was employed for the docking simulations. The optimal conformation with the lowest docking score was selected, and a 2D diagram illustrating the ligand's interaction with binding site residues was generated using DS. For each ligand, a docking energy was calculated

Table 1
The chemical data of nine selected flavonoids (Molecular Weight: MW).

No.	Compound	Sources	MW (g/mol)	Molecular Formula	PubChem ID
1	Swertisin	*Gentiana orbicularis*	446.4	C22H22O10	124,034
2	Apigenin	*Verbascum lychnitis*	270.24	C15H10O5	5,280,443
3	Kolaflavanone	*Garcinia kola*	588.5	C31H24O12	155,169
4	Ochnaflavone	*Ochna integerrima*	538.5	C30H18O10	5,492,110
5	Ginkgetin	*Selaginella willdenowii*	566.5	C32H22O10	5271805
6	Astilbin	*Rhododendron simsii*	450.4	C21H22O11	119,258
7	Naringin	*Citrus latipes*	580.5	C27H32O14	442,428
8	Gossypin	*Rhodiola rosea*	480.4	C21H20O13	5281621
9	Marein	*Viguiera dentata*	450.4	C21H22O11	6441269

based on 15 conformations collected from each docking run.

2.5. AChE and BChE activity assay

The assessment of flavonoids' inhibitory effectiveness against BChE/AChE activities was conducted following the spectrophotometric protocol established by Taslimi et al. (2017). Butyrylcholine iodide and acetylthiocholine iodide compounds served as substrates for both reactions. In this procedure, 5,5′-dithio-bis(2-nitro-benzoic) acid (DTNB) was employed for the quantification of BChE/AChE activities. In a concise overview, 50 μL of buffer solution (pH 7.8, Tris/HCl, 0.5 M) and varying concentrations of sample solutions (25–100 μL) dissolved in deionized water were added to 25 μL of BChE/AChE solutions (2.5×10^{-2} U). The resulting mixture underwent a 15 min incubation period at 25 °C. Subsequently, 25 μL of DTNB (0.25 mM) and 20 mL of Butrylcholine iodide/Acetylthiocholine iodide were added to the incubated mixture. The enzymatic reaction was initiated with the addition of 25 μL of Butrylcholine iodide/Acetylthiocholine iodide. The activities of these enzymes were then assessed spectrophotometrically at a wavelength of 412 nm. The results for each concentration were done in three replicates.

3. Results and discussion

3.1. Virtual screening of AChE inhibitors

The process of virtual screening involving small molecule libraries holds the potential to expedite the identification of novel lead compounds that are conducive to subsequent drug discovery studies. In contrast to alternative de novo design methods, virtual screening allows for the efficient identification of compounds with desired characteristics from commercially available sources for subsequent biological activity assays (Kumar and Ayyannan, 2023; Lyu et al., 2023; Singh et al., 2023). Hence, the application of virtual screening and molecular docking techniques to assess flavonoids from natural sources holds promise for rational design studies. In this context, we utilized molecular docking to discern and prioritize the most favorable flavonoid. The docking scores for the selected flavonoids interacting with AChE are presented in Table 2. These scores provide insights into the enzyme residues engaged with substrate atoms and contribute to the formation of the lowest energy conformation complex. The docking scores collectively indicate that all the chosen flavonoids exhibit favorable interactions with the AChE receptor. To elucidate further, the docking energy of Donepezil (utilized as a control inhibitor) with AChE was determined to be −7.92 kcal/mol 62 flavonoids were initially investigated and molecular docking was performed on all of them. Finally, nine flavonoids that had a better docking score than the control were selected. Our computational analysis, employing docking algorithms, underscored that among the docked flavonoids, Ginkgetin demonstrated superior docking energy compared to Donepezil. This observation suggests a heightened binding affinity, signifying robust interactions with AChE.

Table 2
Docking score and the total number of hydrogen bonds formed by the compounds during docking with AChE and BChE.

Compounds	AChE		BChE	
	Docking score (Kcal/mol)	Total no. of hydrogen bonds	Docking score (Kcal/mol)	Total no. of hydrogen bonds
Swertisin	−6.77	1	−6.13	1
Apigenin	−7.18	1	−5.99	2
Kolaflavanone	−6.94	1	**−7.48**	**4**
Ochnaflavone	−7.28	2	−6.59	1
Ginkgetin	**−8.72**	**3**	−6.77	2
Astilbin	−6.33	2	−6.14	1
Naringin	−7.63	1	−6.33	1
Gossypin	−6.85	2	−6.89	1
Marein	−7.34	1	−6.44	1
Donepezil	−7.92	2	−6.93	2

The Ginkgetin-AChE complex exhibited a minimum docking score of −8.72 kcal/mol. The interactions can be categorized into four classes: conventional hydrogen bonds, π-alkyl interactions, carbon-hydrogen bonds, and van der Waals forces. Specifically, three conventional hydrogen bonds were identified with Ala127 and Asn87 residues, while Asp 74, Leu 130, Pro88, Trp86, Tyr72, His447, and Trp439 formed carbon-hydrogen bonds. Additionally, π-alkyl interactions were observed with Gly120 and Tyr337 residues. Alongside these interactions, the bound Ginkgetin-AChE complex displayed 20 van der Waals interactions. Notably, Pro88, Trp86, Tyr72, His447, and Trp439 were located at the active site of AChE (Hassan et al., 2018). The binding of Ginkgetin to the active site of AChE corroborated with findings from previous studies (Fig. 3 A, C, E).

The Donepezil-AChE complex exhibited a docking score of −7.92 kcal/mol. Within the complex, several residues, including Gly120, Tyr133, Asn87, Thr83, and Gly 82, were observed forming carbon-hydrogen bonds. Furthermore, Trp86 and Tyr337 interacted with Donepezil through the formation of π-alkyl bonds. Complementing these interactions, the bound Donepezil-AChE complex displayed 20 van der Waals interactions (Fig. 3 B, D, and F).

The Kolaflavanone-BChE complex demonstrated a docking score of −7.48 kcal/mol. Conventional hydrogen bonds were established between Kolaflavanone and Asn83, Ser79, Gln67, and Ser287 residues. Additionally, Ala 287 formed a π-kation bond, and the π-alkyl bond was observed with Phe 329. Notably, Asn83, Ser79, Gln67, and Ser287 were situated at the active site of BChE. The binding of Kolaflavanone to the active site of BChE corroborated with findings from a prior study (Silva et al., 2020). Comparative analysis suggests that the number of van der Waals and conventional hydrogen bonds in the Kolaflavanone-BChE complex may surpass that in the Donepezil-BChE complex (Fig. 4A–F). Donepezil-BChE complex demonstrated the docking energy of −6.93 kcal/mol (Table 2). In the 2D interaction method, Donepezil-BChE complex via one conventional hydrogen bonds (Thr120) as well as two carbon-hydrogen bonds.

Consequently, considering the significance of conventional hydrogen bonds and van der Waals interactions compared to other bonds, it is anticipated that the docking score of the Kolaflavanone-BChE complex will be higher than that of the Donepezil-BChE complex.

3.2. Inhibitory activity (%) of the selected flavonoids on AChE and BChE

The inhibitory efficacy of flavonoids on AChE and BChE is presented in Table 3. Enzyme inhibition results indicate that Ginkgetin and Kolaflavanone exhibited notable inhibitory effects on AChE and BChE, respectively, which was consistent with molecular docking results. At a

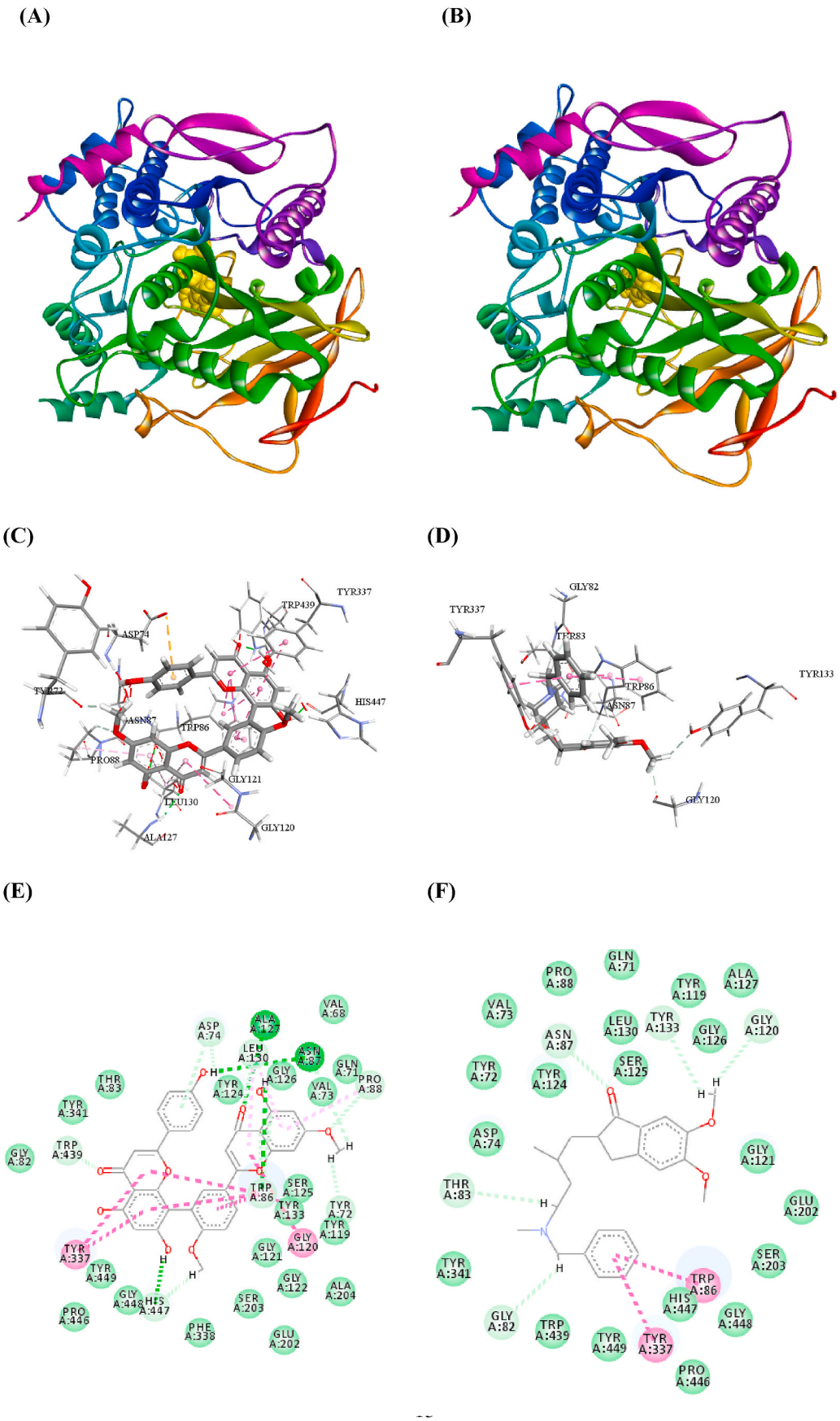

Fig. 3. (A) Whole structure of the Ginkgetin-AChE, (B) Whole structure of the Donepezil-AChE, (C) 3D interaction of Ginkgetin-AChE, (D) 3D interaction of Donepezil-AChE, (E) 2D interaction of Ginkgetin-AChE, (F) 2D interaction of Donepezil-AChE.

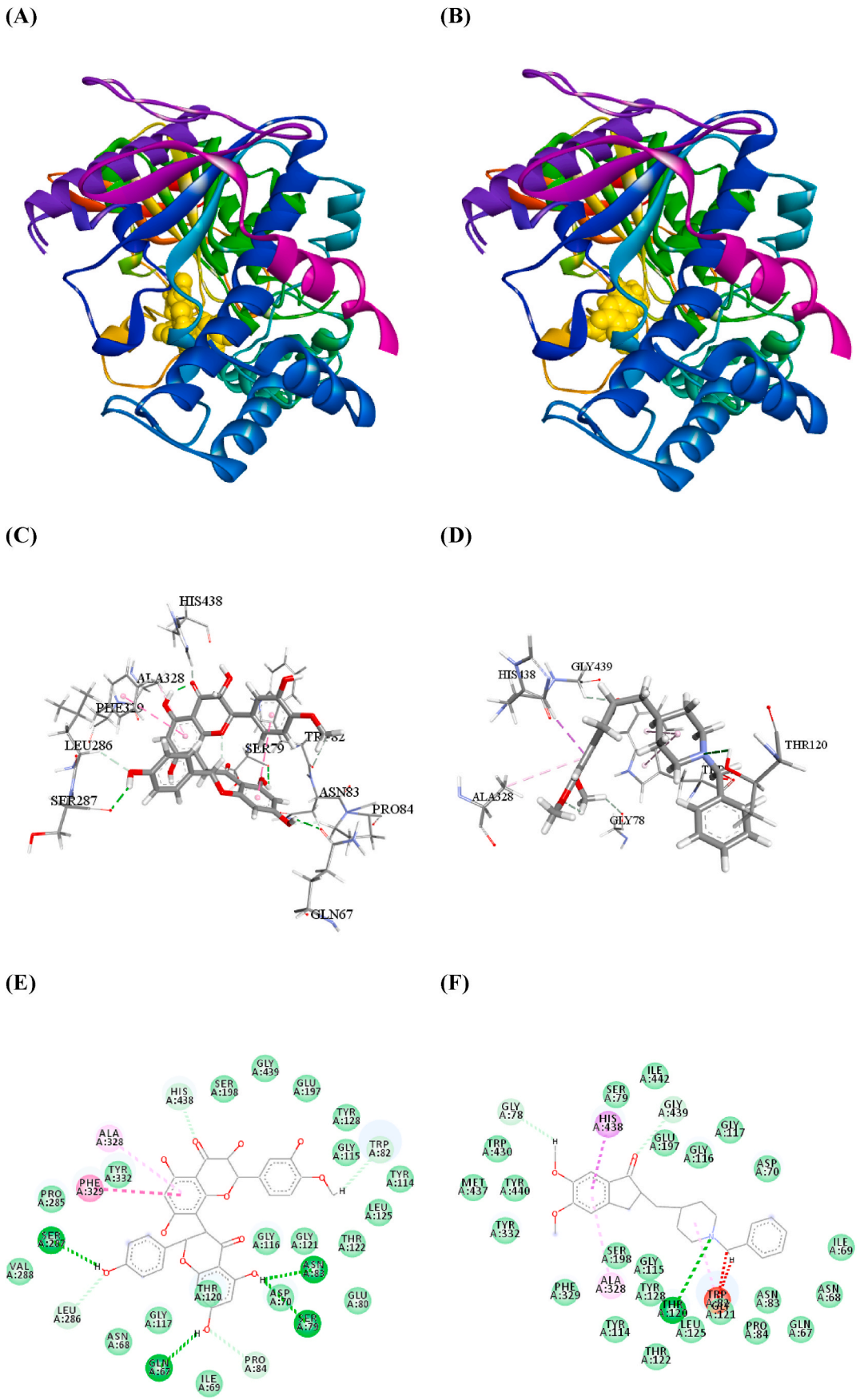

Fig. 4. (A) Whole structure of the Kolaflavanone-BChE, (B) Whole structure of the Donepezil-BChE, (C) 3D interaction of Kolaflavanone-BChE, (D) 3D interaction of Donepezil-BChE, (E) 2D interaction of Kolaflavanone-BChE, (F) 2D interaction of Donepezil-BChE.

Table 3
The Inhibition and IC_{50} value of nine flavonoids on AChE and BChE.

Compounds	AChE		BChE	
	Inhibition (%)	IC_{50} (mM)	Inhibition (%)	IC_{50} (mM)
Swertisin	53 ± 1.3	5.3	65 ± 1.2	6.2
Apigenin	48 ± 1.2	–	63 ± 1.8	4.2
Kolaflavanone	64 ± 1.7	4.5	**85 ± 1.7**	3.6
Ochnaflavone	NI	–	52 ± 1.2	4.8
Ginkgetin	**78 ± 1.6**	3.2	58 ± 1.3	4.3
Astilbin	55 ± 1.2	4.3	NI	–
Naringin	61 ± 1.7	4.1	64 ± 1.5	5.9
Gossypin	51 ± 1.6	5.8	NI	–
Marein	NI	–	71 ± 1.6	5.2
Donepezil	93 ± 1.9	2.7	88 ± 1.2	2.4

Inhibition at 80 mM.
The value is the mean ± SD (n = 3).
No inhibition; NI.

concentration of 80 mM, Ginkgetin and Kolaflavanone demonstrated inhibition rates of 78% ± 1.6% and 85% ± 1.7%, respectively. Additionally, the calculated IC_{50} values for Ginkgetin and Kolaflavanone were 3.2 and 3.6 mM, respectively, indicating their inhibitory potential against the enzymes. Despite the lower inhibitory effects compared to Donepezil, a common flavonoid, Ginkgetin, and Kolaflavanone may still hold promise for AD control due to their inhibitory activity against AChE and BChE.

4. Conclusion

The present study showed the potential of flavonoids in inhibiting the activity of AChE and BChE enzymes. We have tried to develop a set of 62 selected flavonoids containing natural antioxidant properties in the design of enzyme inhibitors. With the help of detailed computational analysis, it was determined that Swertisin, Apigenin, Kolaflavanone, Ochnaflavone Ginkgetin, Astilbin, Naringin, Gossypin and Marein bind more favorably to the active sites of AChE and BChE (based on docking energies). Ginkgetin showed significant inhibitory activity against AChE with IC_{50} of 3.2 mM, while compared to other flavonoids, Kolaflavanone exerted strong inhibitory effect on BChE (IC_{50} = 3.6 mM). Remaining challenges include the need to develop assays that specifically determine the association of flavonoids and/or their analogues with AChE and BChE, as well as their binding mechanisms. Though, bioinformatics approaches are presently facing some hurdles, evaluating the potential of flavonoids for the treatment of AD using "*in silico*" methods could pave the way for the identification of valuable "lead molecules".

CRediT authorship contribution statement

Morteza Sadeghi: Data curation & analysis, Writing original draft, Methodology. **Seyedehmasoumeh Seyedebrahimi:** Manuscript preparation. **Mustafa Ghanadian:** Enzymes & Flavonoid supplying. **Mehran Miroliaei:** Project Supervisor, Revision and editing the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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