Identification of possible Anti-Alzheimer Mechanism of *ginkgo biloba* extract 'EGB761' using molecular docking technique

Sahu Pratap Kumar, Prusty Shakti Ketan, Acharjee Hyking and Bose Anindya*
School of Pharmaceutical Sciences, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar, Odisha-751029, INDIA
*anindyabose_in@yahoo.com

Abstract
Ginkgo biloba exerts (EGb761) is an effective and popular herbal medicine in patients with Alzheimer's disease; however, its exact mechanism of action remains uncertain. This study aims to investigate the role of phytoconstituents of G. biloba extract in treating Alzheimer's disease. The major Alzheimer's disease related target proteins are systematically investigated for their possible molecular interaction with major active constituents of G. biloba using molecular docking techniques in silico.

The results indicated that the protective effects of G. biloba extract on Alzheimer's disease may be a result of the synergistic effect of its various phytoconstituents on multiple Alzheimer's disease targets, with most of the active compounds most efficiently interacting with acetylcholinesterase and butyrylcholinesterase. The findings of this study enhance our understanding of the molecular mechanism of G. biloba extract in Alzheimer's disease and provide valuable insights for the future development of therapeutic strategies.

Keywords: Molecular docking, Flavonoids, Triterpene lactones, Acetylcholinesterase, Butyrylcholinesterase.

Introduction

Dementia is a collective term for several cognitive declines due to the dysfunction of the cerebral systems, resulting in impairment in memory, communication and thinking. Worldwide epidemiological studies have shown that about 15% of patients older than 65 years of age have dementia. Alzheimer's disease, a common neurodegenerative disorder, leads to dementia in around one in every four individuals over the age of 85[2].

The World Alzheimer report 2016 mentioned that currently, there are about 46.8 million persons around the world with dementia, with an estimated figure expected to double every 20 years nearly and will reach about 131.5 million by 2050[28]. Currently, available synthetic medicines, although they moderately diminish the symptoms, however, are unable to arrest the neurodegeneration process. In this context, several natural products have recently become popular as alternative remedies for preventing or controlling dementia. *Ginkgo biloba* is a popular herbal remedy for cognitive disorders and Alzheimer's disease[35]. *G. biloba*, also known as the maidenhair tree, has been a traditional Chinese medicine for about 5000 years. It possesses a range of health benefits including improvement of cognitive function. There are many bioactive memory enhancer phytoconstituents present in the leaves, including terpenoids (e.g. bilobalide and ginkgolides A, B, C, J and M) and flavonoids (e.g. kaempferol, quercetin, isorhamnetin)[29]. *G. biloba* leaf extract is one of the most popular herbal supplements that claim to prevent memory loss and dementia[4]. Standardized extract of *G. biloba* 'EGb761®' is marketed in more than 70 countries as medicine or healthy food[39]. Despite its beneficial effect in ameliorating dementia in Alzheimer's disease patients, its mechanism of action remains controversial.

The United States Food and Drug Administration (USFDA) has not approved this product due to inconsistent and insufficient scientific evidence of its neuroprotective activity. The presence of multiple bioactive phytoconstituent compounds with chances of synergism makes it challenging to confirm the exact mechanism of action. Hence, we attempted to investigate the pharmacological mechanism of *G. biloba* in treating Alzheimer's disease. In the present study, along with the evaluation of nootropic activity of the *G. biloba* extract, the reported significant Alzheimer's disease related target proteins were systematically investigated for their possible molecular interaction with major active constituents of *G. biloba* using molecular docking technique *in silico*.

Material and Methods

Ginkgo biloba extract: Standardized *Ginkgo biloba* leaf extract 'SEGb 761" manufactured by Dr. Willmar Schwabe Pharmaceuticals (Germany) was used for the pharmacological evaluation. The product was dissolved in distilled water containing 3% ethanol for oral administration in rats.

Animals: Wistar albino rats of either sex (150-200 g) were utilized in the study. All the experimental protocols were approved by the Institutional Animal Ethics Committee (Protocol No. IAEC/SPS/SOA/15/2019).

Spatial memory study: The rats were segregated into five groups, each of six animals. The control group (Group I) was treated with distilled water (10 ml/kg p.o.). Group II was administered with scopolamine (2 mg/kg i.p., Sigma, USA). Group III was treated with donepezil (5mg/kg p.o.). Group IV and V were treated with 5 mg/kg p.o. and 10 mg/kg p.o. of *Ginkgo biloba* extract. Groups III, IV and V were administered scopolamine (2 mg/kg i.p.) for 30 minutes

post-drug administration. Spatial working memory was studied by Y-Maze (INCO, India).

Individual animals were placed at the center of the instrument and kept there for 5 minutes. The successive entries to separate arms were counted. Scopolamine was compared with the control and donepezil whereas *G. biloba* was compared with scopolamine[26]. Alternation was considered as the number of successive entries into these arms on overlapping triplet sets. The following formula determined the percentage of spontaneous alternation:

$$\% \text{ spontaneous alternation} = \frac{\text{Number of 3 out of 3 choices}}{\text{Total number of entries-degree of freedom}} \times 100$$

(Degree of freedom = 2)

Radial maze: Spatial working memory was studied by eight arm Radial maze (INCO, India). The rats were preselected by conducting a training trial. Rats were placed individually in the center of the hub and allowed to enter arms freely. The trial was considered complete when the rat visited all 8 arms. Then the rats were grouped into five groups of six each. Control (group I) animals were treated with distilled water (10 ml/kg p.o.). Group II was administered with scopolamine (2 mg/kg i.p., Sigma, USA). Group III was treated with donepezil (5mg/kg p.o.). Group IV and V were treated with *G. biloba* extract in doses 5 mg/kg p.o. and 10 mg/kg p.o. respectively. Groups III, IV and V were administered with scopolamine (2 mg/kg i.p.) 30 minutes' post-drug administration. Entry into an unvisited arm was considered a correct response, while re-entry was considered an error. The number of correct responses before the first mistake was measured[14].

Elevated plus maze: The effect on acquisition deficit induced by scopolamine was studied by elevated plus maze (INCO, India). The rats were divided into five groups of six animals and dosing was done similarly to the two methods described above. Thirty minutes post scopolamine administration, the rats were placed individually at the end of any one of the open arms and the time taken to move into the enclosed arm was measured as the transfer latency[26].

***In silico* docking studies**

Software: *In silico* studies were performed using software MGL Tools1.5.4, AutodockVina (version 1.1.2), Open Babel (Version 2.4.1) and Discovery Studio Visualizer (version 17.2.0.16349). The SwissADME Online server was utilized to estimate *in silico* pharmacokinetic parameters.

Selection of ligands and target proteins: Ten major bioactive phytoconstituents present in *G. biloba* extract (EGb 761) were selected for the study, which include four triterpene lactones (Bilobalide, Ginkgolide A, Ginkgolide B and Ginkgolide C) and six flavonoids (Isorhamnetin, Kaempferol, Myricetin, Quercetrin, Quercetin and Rutin)[5,8,9]. The chemical structures of these compounds were retrieved from the PubChem database for docking studies.

Target preparation: The seven major Alzheimer's diseases associated targets were downloaded from the research collaboratory for structural bioinformatics (RCSB) protein data bank (Table 1). The discovery studio was used to remove the native ligand (if present) and water molecules to avoid docking interference and saved in the PDB format.

Molecular docking studies: *In silico* docking studies were performed to predict the ligand-protein of *G. biloba* phytoconstituents at the atomic level to gain insight into their behavior in the target binding sites. All the selected compounds were docked with the seven Alzheimer's disease targets individually to determine the best binding affinity using AutodockVina; hence, a total of 70 docking studies were performed. As per the protocol, grid parameters for each protein were assigned using AutoDock Tools by creating a grid box large enough to cover the entire protein binding site with the grid points in *X*, *Y* and *Z* axes separated by 1000 A°.

The grid centers in X, Y and Z axes were specified independently for each receptor. For all the docking runs, the exhaustiveness was set at 8. The AutoDock Vina search algorithm predicted the binding energy of the ligand for the protein. The conformations of best binding modes of ligand-protein interactions were saved with their respective binding affinity.

Molecular interactions visualization: After docking was completed, Auto Dock preferences for the ligand were obtained in PDBQT format.

Open babel GUI was used to convert the preferred protein target (PDBQT) into PDB. AutoDockVina generated docking pairs of protein and ligands that were saved in PDB format and were visualized in the Discovery studio visualizer. The ligand-binding sites and surrounding amino acids were visualized and molecular interactions, including hydrogen bonding between proteins and ligands, were characterized.

Basic Pharmacokinetics Parameters: The online tool SwissADME (http://www.Swiss ADME.com/)[23] assessed basic pharmacokinetics parameters of the tested ligands. These predictions were made based on Lipinski's "Rule of Five" along with the topological polar surface area (TPSA) and the predicted percentage absorption (%ABS)[12,40].

Results and Discussion

The mechanisms of anti-Alzheimer's action of *G. biloba* extract are thought to be due to the effect of several of its phytoconstituents, which result in enhancement of brain blood supply by dilation of blood vessels, blood viscosity reduction, alteration in neurotransmitters and scavenging of free radicals[3]. However, its efficacy in the prevention and treatment of dementia remains controversial. In the present work, we have investigated the *in vivo* anti-Alzheimer's effect of *G. biloba* by rodent models to establish its

mechanism in declining Alzheimer's disease. The work further attempted to explore the interaction of major constituents of *G. biloba* with Alzheimer's disease receptors by docking studies to enlighten the contribution of these compounds in treating the disease.

Scopolamine, an anticholinergic drug that can abolish spontaneous alternation behavior (SAB), was used in our pharmacological studies to induce cognitive deficit[10]. The decline in cholinergic transmission in the hippocampal area of the brain (hippocampus) leads to a decline in cognitive function. Our results are in agreement with these studies[31]. Spontaneous alternation behavior (SAB) is a widely used test for evaluating spatial learning and working memory. Y-Maze is popularly used for the assessment of SAB[26]. Many parts of the brain, including the hippocampus, are involved in this task. The eight-arm radial maze is an excellent tool for assessing spatial memory[15]. Induction of hippocampal lesions in rodents can be used to evaluate impairment of spatial memory on the radial arm maze task[14].

Table 1
List of selected Alzheimer's disease-associated targets

S.N.	PDB ID	Protein Name (details)
1	4EY5	Acetylcholinesterase (AChE)[32]
2	4DJU	Beta Secretase-1 (BACE-1)[37]
3	4B0P	Butyrylcholinesterase (BChE)[24]
4	1EQG	Cyclooxygenase-1 (COX-1)[36]
5	1Q5K	Glycogen-synthase-kinase-3β (GSK-3β)[19]
6	1UDT	Phosphodiesterase-5 (PD-5)[17]
7	2FV5	TNF-α converting enzyme (TACE)[7]

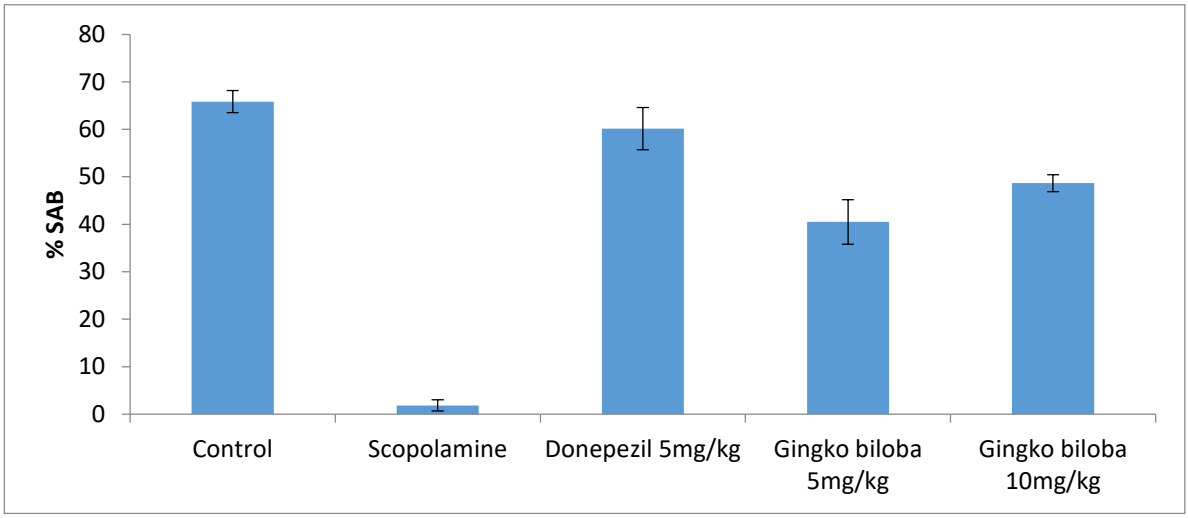


Figure 1: Effect of *Ginkgo biloba* extracts on the scopolamine-induced decrease in spontaneous alternation in Y-maze

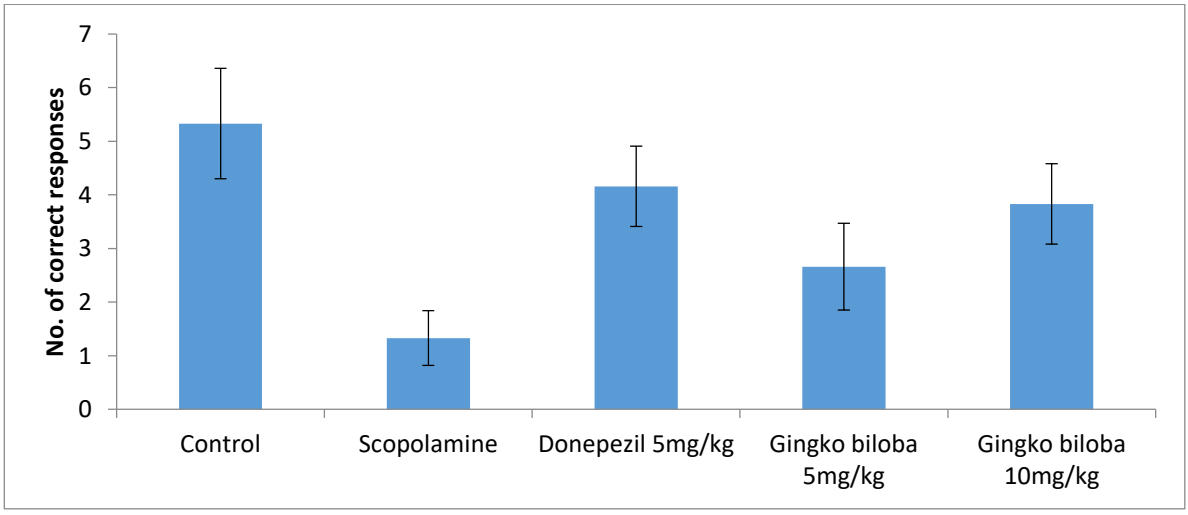


Figure 2: Effect of *Ginkgo biloba* extracts on the scopolamine-induced decrease in the number of correct responses in radial arm maze

In our study, scopolamine significantly reduced the spontaneous alternation in Y-maze and the number of correct responses in the radial maze. Both donepezil (5 mg/kg p.o.) and *Ginkgo biloba* extracts (5 and 10 mg/kg p.o.) significantly ($p<0.05$) prevented scopolamine-induced decrease in spontaneous alternation in Y-maze (Fig. 1) as well as improved the scopolamine-induced decline in number of correct responses in radial maze (Fig. 2). Our results with scopolamine are in agreement with previously reported studies[31] which signified the nootropic activity of *G. biloba* extract.

Elevated plus maze is a widely accepted model for the study of learning and memory in rodents where a decrease in transfer latency (the time elapsed between an animal's movements from the open area to a closed arm) is correlated with increased memory function[1]. Transfer latency is used as a parameter to assess the consolidation or retrieval mechanism of learning and memory. Scopolamine significantly increased the transfer latency in the elevated plus maze. Both donepezil and *G. biloba* extract significantly ($p<0.05$) prevented the scopolamine-induced increase in transfer latency (Fig. 3). It can, therefore, be concluded that similar to the claims of several preclinical and clinical studies, *G. biloba* exhibits profound anti-Alzheimer's effects[18,33,38].

Most chemicals fail in the drug discovery process due to poor pharmacokinetic parameters, mainly absorption, distribution, metabolism, excretion and toxicity (ADMET). These parameters are essential descriptors for the bioavailability of an orally administered compound. More preciously, they are the deterging factors for the ability of central nervous system (CNS) targeted drugs for crossing biological barrier and their brain access. The pharmacokinetics properties of natural compounds were predicted by the SwissADME web server using Lipinski's rule of five.

As per this rule, a molecule is likely to be a drug if it satisfies four conditions, namely, a molecular weight not more than 500, LogP (logarithm of partition coefficient) not more than five, hydrogen bond five or lesser and hydrogen bond acceptors ten or lesser[11]. SwissADME uses XLOGP3 to predict logP value (CLOGP), as XLOGP3 is known to provide similar predictions to CLOGP[21].

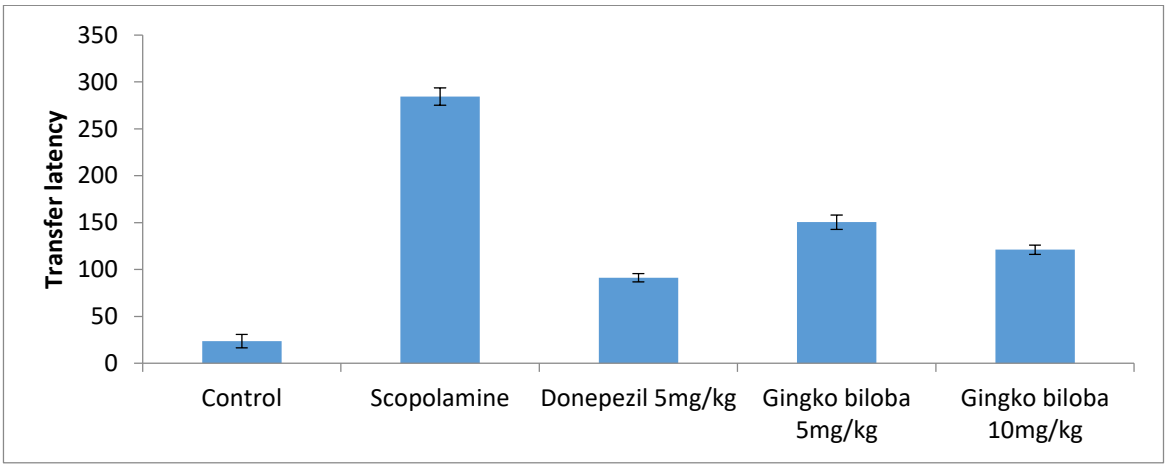


Figure 3: Effect of *Ginkgo biloba* extracts on the scopolamine-induced increase in transfer latency in the elevated plus maze

Table 2
Pharmacokinetic properties of the selected *G. biloba* phytoconstituents

Ligand	Molecular Weight (g/mol)	H-Bond Acceptors	H-Bond Donors	XLOGP3	Molar Refractivity	Number of Violation (Lipinski)	TPSA total polar surface area	% ABS	nRB
Bilobalide	326.30	8	2	-0.27	71.20	0	119.36	69.074	1
GinkgolideA	408.40	9	2	0.59	92.13	0	128.59	65.986	1
GinkgolideB	424.40	10	3	-0.38	93.29	0	148.82	59.219	1
GinkgolideC	440.40	11	4	-1.36	94.45	1	169.05	52.452	1
Isorhamnetin	316.26	7	4	1.87	82.50	0	120.36	68.739	2
Kaempferol	286.24	6	4	1.90	76.01	0	111.13	71.827	1
Myricetin	318.24	8	6	1.18	80.06	1	151.59	58.293	1
Quercetrin	448.38	11	7	0.86	109.00	2	190.28	45.351	3
Quercetin	302.24	7	5	1.54	78.03	0	131.36	65.060	1
Rutin	610.52	16	10	-0.33	141.38	3	269.43	18.875	6

The total polar surface area (TPSA), the surface sum of all polar atoms in the molecule, was considered as another key property linked to bioactive molecule. The higher TPSA values are indicative of a decrease in the probability of permeability of the compound through biological membranes as most of the passively absorbed molecules with TPSA of more than 140 have low oral bioavailability[6,27].

As most of the compounds of *G.biloba* had a TSPA value less than 140, these compounds have a greater chance of crossing the BBB and having better oral bioavailability. The number of rotatable bonds (NRB) in a molecule expresses the flexibility of a molecule[34], which determines the ease with which the molecule transverses the membrane. The number of rotatable bonds of the majority of successful CNS drugs is less than 8[25]. There results of *in silico* prediction of bioavailability parameters using Bioinformatic stool SwissADME are given in table 2. These property predictions showed that the majority of the compounds qualify for drug-likeliness. Although both Ginkgolide C and myricetin showed one violation of Lipinski's rule, Quercetrin and rutin showed two and three violations.

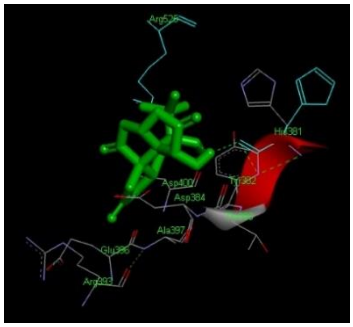
Molecular docking is a popular method in structure-based drug design that can predict the binding conformation of small molecules into the appropriate binding sites[22]. The molecules strongly binding to the receptor generally inhibit its function and thus can act as a drug[3,12]. Moreover, identifying the ligand's interacting atoms with specific amino acid residues of the therapeutic target has played an important role in drug discovery and protein function identification. The docking interactions' binding energies predict the drug's binding efficiency in the target protein. The greater is the energy released on binding i.e. the lesser is the binding energy, the better will be the affinity of the ligand for the target. In our docking studies, each compound was docked with seven Alzheimer's disease-associated targets individually. The binding affinity of the best binding configuration with the target protein was measured in Kcal/mol. The phytoconstituents of *G. biloba* extract showed good binding affinities with the selected Alzheimer targets. The binding affinities of the compounds ranged from between-6.3 Kcal/mol to -10.0 Kcal/mol (Table 3). It clearly showed the evidence of good interaction of these compounds in selected receptor sites. The variation in binding affinities could be attributed to the structures of the compounds and rotatable bonds present. The molecular visualization of best-docked poses of the compounds in active sites of protein receptors is represented in fig. 4 where green dash lines reflect hydrogen bonds with various amino acids involved in the interactions.

The most common cause of Alzheimer's disease is the deterioration of acetylcholine (ACh) releasing nerve cells, which transmit messages between different areas of the central nervous system[13]. Acetylcholinesterase (AChE) enzyme aggravates the acetylcholine deficiency by hydrolyzing it. Inhibitors of AChE activity help to increase the concentration and duration of synaptic AChE action. AChE inhibition is thus considered an important strategy for treating Alzheimer's disease [30]. BChE, on the other hand, is associated with Aβ plaques and NFTs in the brain tissue of patients of Alzheimer's disease as well as in Aβ plaques in Alzheimer's disease mouse models. The role of BChE in Alzheimer's disease pathology is not fully established; however, it may be responsible for the maturation of these structures into some neurotoxic species, which can lead to neurodegeneration and subsequent clinical manifestations of Alzheimer's disease[20].

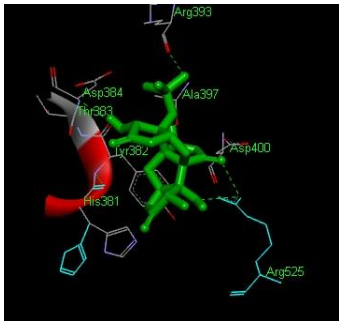
As indicated by the binding affinity, two of the selected Alzheimer's disease receptor targets, namely AChE and BChE, most efficiently interacted with selected phytoconstituents. Flavonoids such as Kaempferol, myricetin, ishormnetin and quercetin, showed similar interactions with the AChE receptor (Fig. 4). The common interactions occur at LEU 289, VAL 294, TRP 286, TYR 124, TYR 337, TYR 341, GLU 291, GLU 292, ARG 296, SER 293, PHE 295, PHE 338 and ASP 74. On the other hand, terpenic lactones bilobalide, ginkgolide A, ginkgolide B and ginkgolide C showed similar interactions (Fig. 4) with common interactions at GLU 396, HIS 381, THR 383, ARG 393, ARG 525, ASP 384, ASP 400, ALA 397, TYR 382.

Table 3
Binding energies (kcal/mol) of all the *G. biloba* phytoconstituents docked with Alzheimer's disease-associated targets

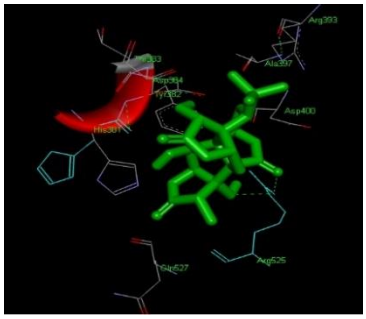
Compounds	Receptors						
	4EY5 (AChE)	4DJU (BACE-1)	4B0P (BChE)	1EQG (COX-1)	1Q5K (GSK-3β)	1UDT (PD-5)	2FV5 (TACE)
Bilobalide	-7.0	-7.2	-8.8	-7.0	-7.5	-6.3	-7.3
Ginkgolide A	-8.4	8.7	-9.4	-8.0	-8.1	-6.7	-7.6
Ginkgolide B	-8.8	-8.9	-8.9	-8.0	-7.9	-7.1	-7.8
Ginkgolide C	-8.9	-8.7	-8.6	-8.2	-7.7	-6.5	-7.8
Isorhamnetin	-8.1	-8.3	-9.2	-7.6	-8.6	-6.4	-9.0
Kaempferol	-9.1	-8.3	-9.3	-8.0	-8.5	-7.5	-7.1
Myricetin	-8.8	-7.9	-9.6	-7.6	-8.6	-9.5	-8.3
Quercetrin	-8.7	-7.5	-8.6	-9.0	-8.8	-7.8	-7.9
Quercetin	-9.1	-8.2	-9.6	-7.1	-8.6	-6.4	-9.1
Rutin	-9.6	-8.9	-10.0	-9.1	-8.8	-7.2	-7.9



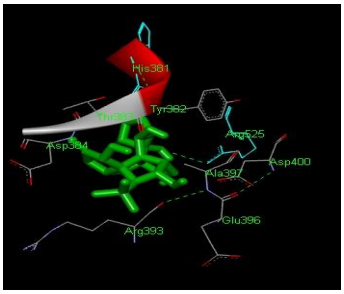
Bilobalide



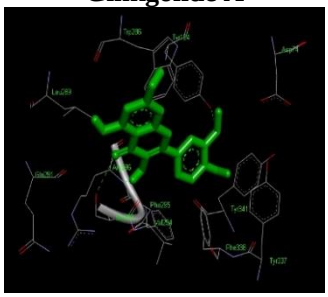
Ginkgolide A



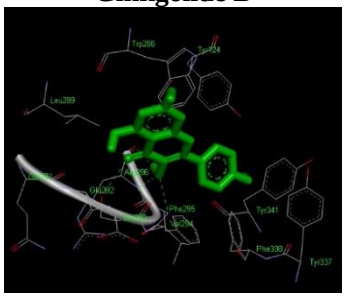
Ginkgolide B



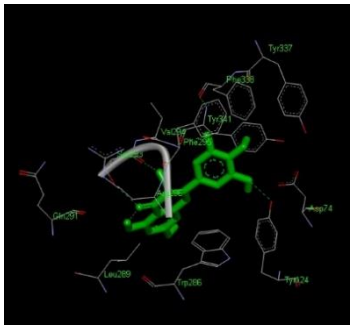
Ginkgolide C



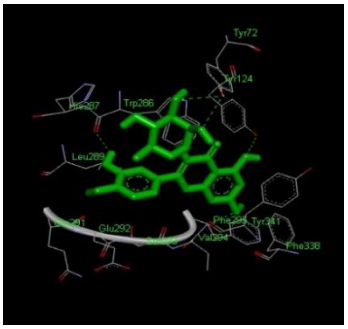
Isorhamnetin



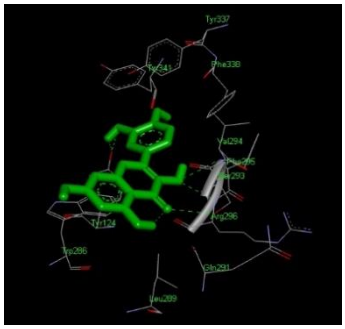
Kaempferol



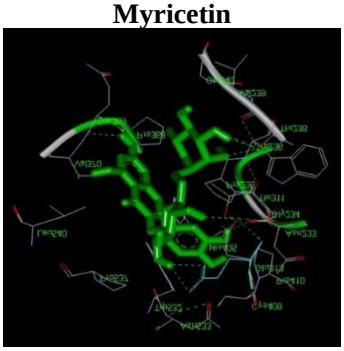
Myricetin



Quercetrin



Quercetin



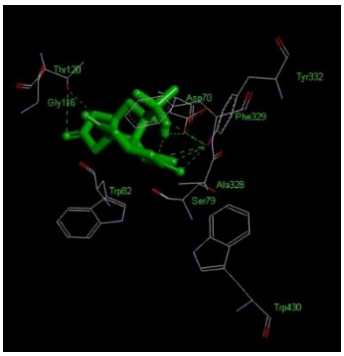
Rutin

Figure 4: Predicted docking interactions between phytoconstituents of *Ginkgo* biloba and Acetylcholinesterase (PDB ID: 4EY5)

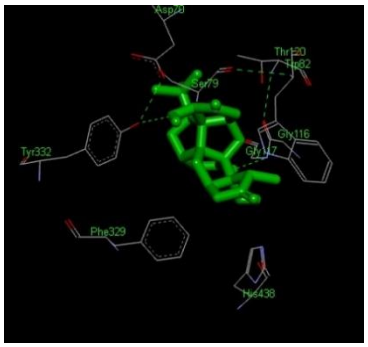
In case of BChE receptor, flavonoids kaemferol, myricetin, isohamnetin and quercetin showed similar interactions with common interactions (Fig. 5) from ASP 70, ASN 68, ASN 83, GLU 197, GLY 439, HIS 438, ILE 69, ILE 442, THR 120, TRP 82, TYR 332, TYR 440 and PRO 84. However, quercetrin and rutin interacted with a different set of amino acids. The terpenes understudy did not show similarities in binding among them (Fig. 5).

The docking results further indicated that the different phytoconstituents of *G. biloba* interacted with multiple Alzheimer's disease receptors with strong binding affinities. These results suggest that the compounds of *G. biloba* may be considered to possess a kind of multi target directed ligands for treating Alzheimer's disease. Among terpenes, Ginkgolide B showed effective interactions with all seven receptors while the other three of the lot i.e. Bilobalide, Ginkgolide A and Ginkgolide C interacted each with six receptors. The best docking among terpenes was observed for Ginkgolide A with BChE, with a score of -9.4 Kcal/mol. Although Ginkgolide C showed only one violation of Lipinski's rule and a TPSA value of more than 140, the other three terpenes passed through Lipinski's filter.

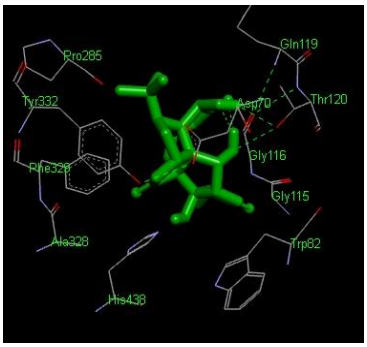
Hence, these terpenes may be the main contributors to the effectiveness of *G. biloba* in treating Alzheimer's disease. Among flavonoids understudy, kaempferol, myricetin, quercetrin and rutin showed effective interactions with all seven receptors. In comparison, quercetin and isorhamnetin showed effective docking of six receptors. Comparing these flavonoids, rutin showed the highest binding affinity score of -10.0 Kcal/mol with BChE. However, considering three violations of Lipinski's rule and high TPSA value, it may not be effective in Alzheimer's disease. Isorhamnetin, kaempferol and quercetin did not show violations of Lipinski's rule. These flavonoids showed a good binding affinity with AChE and BChE receptors and may be effective contributors in the Alzheimer's disease of *G. biloba* by inhibiting these two enzymes.



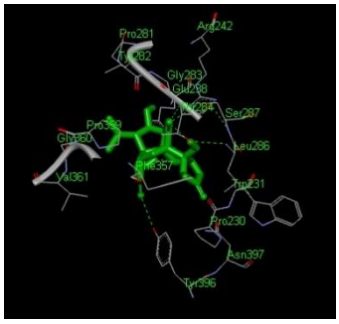
Bilobalide



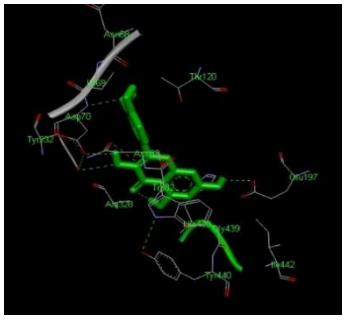
Ginkgolide A



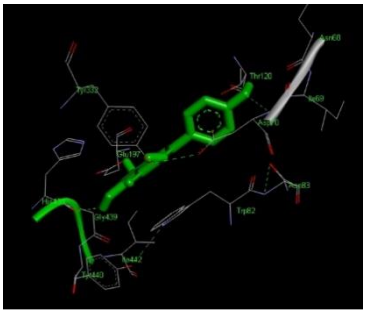
Ginkgolide B



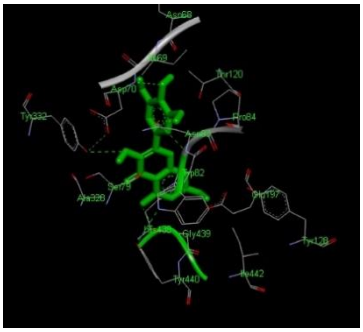
Ginkgolide C



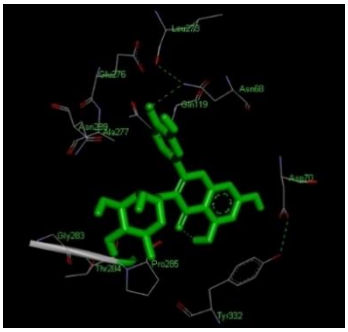
Isorhamnetin



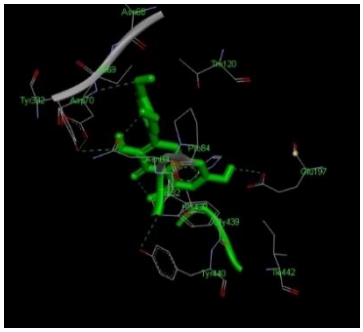
Kaempferol



Myricetin



Quercetrin



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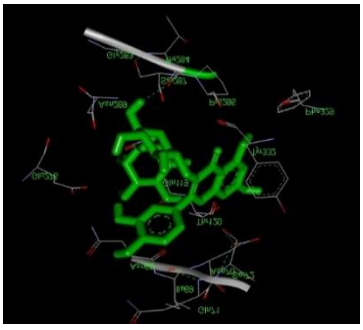

Rutin

Figure 5: Predicted docking interactions between phytoconstituents of *Ginkgo biloba* and Butyrylcholinesterase (PDB ID: 4B0P)

Conclusion

The present study investigated the mechanism of action of *G. biloba* by examining the binding mode of its best-docked phytoconstituents in Alzheimer's disease targets. The results suggest that the tested phytoconstituents of *G. biloba* potentially interacted with the Alzheimer's targets. Both molecular docking and *in silico* pharmacokinetic studies showed that most of the compounds could serve as an ideal anti-Alzheimer drug.

The correlation between Alzheimer's disease protein targets and phytoconstituents of the *G. biloba* extract may help to explain the mechanisms of *G. biloba* extract in treating Alzheimer's disease. Further studies are needed to establish the target specific signaling pathways of these natural compounds, their mode of action in various brain regions and the mechanism behind their synergistic action on the Alzheimer's disease targets.

Acknowledgement

Authors are thankful to Siksha 'O' Anusandhan (Deemed to be University), India for providing all necessary facilities for carrying out this research work.

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(Received 04th December 2024, accepted 08th February 2025)