RESEARCH ARTICLE

Neuroprotective Activity of Flavonoids from *Peperomia pellucida* by *Invitro* and *Insilico* Studies

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ABSTRACT

Introduction: One of the neurodegenerative disorders that causes irreversible mild cognitive impairment is Alzheimer's disease(AD). As AD becomes more prevalent, the need for the development of innovative technologies and medications to prevent and treat neurodegeneration becomes increasingly critical. The plant *Peperomia pellucida* possesses neuroprotective properties.

Material & Methods: *Invitro* Acetylcholinesterase Inhibitory Activity performed in *Peperomia pellucida* extract and *insilico* evaluations conducted using online tools such as Autodock, PASS, Swiss ADME, and ProTox-II.

Result: *Peperomia pellucida* ethanolic extract inhibits cholinesterase more effectively than other extracts, according to the present *invitro* investigation. Acacetin, Isovitexin, and Apigenin are the significant flavonoids found in *Peperomia pellucida.* The effectiveness of these flavonoids against Alzheimer's disease was proven by *insilico* and toxicity profile studies.

Conclusion: Acacetin and Apigenin exhibit comparable docking scores to Isovitexin, whereas the former demonstrates superior results in ADME parameters and safety. The potential therapeutic applications of flavonoid constituents derived from *Peperomia pellucida* for Alzheimer's disease are underscored in this work.

Keywords: *Peperomia pellucida*, Alzheimer's disease, *insilico* study Acacetin, Apigeninin

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INTRODUCTION

Alzheimer's disease is classified as a neurodegenerative disorder[1], marked by the accumulation of amyloid plaque and neurofibrillary tangle[2,3]. Prompt identification and full recovery constitute the main challenges associated with AD. It was frequently misdiagnosed as age-related dementia. The confirmation of Alzheimer's disease is exclusively possible through clinical diagnosis and neuroimaging techniques, including Magnetic Resonance Tomography (MRI)[4], Computed Tomography (CT) and Positron Emission Tomography (PET)[5]. Older patients who had been exposed to COVID had a 50–80 percent greater probability of getting Alzheimer's disease than healthy individuals who had not been impacted by COVID-19. Women who are 85 years of age or older are particularly susceptible to this particular hazard. Recent research has established a correlation between COVID-19 and neurological problems as well[6].

The drug development process can benefit from natural plant sources. Active phytoconstituents found in plants are the area of focus in present drug research. *Peperomia pellucida* is a fleshy herb, which belongs to the Piperacea family, with significant ethnopharmacological value. It possesses various therapeutic properties, including analgesic, anti-inflammatory[7], anticancer, antioxidant[8], hypotensive, immunostimulatory[9], anti-ulcer, antipyretic, and neuropharmacological effects[10,11]. Phenolic chemicals, flavonoids, glycosides, alkaloids, sugars, and tannins are all present in *Peperomia pellucida*[12]. Some of the prominent flavonoid constituents present in *Peperomia pellucida* are Apigenin, Acacetin, Isovitexin and pellucidatin[13]. Hence, the current investigation centers on the *invitro* and *insilico* utilisation of flavonoids found in *Peperomia pellucida* to treat Alzheimer's disease and offer a novel therapeutic alternative for Alzheimer's disease clinical practice.

MATERIALS AND METHODS

Plant Materials Collection

The whole parts of *Peperomia pellucida* plant was acquired

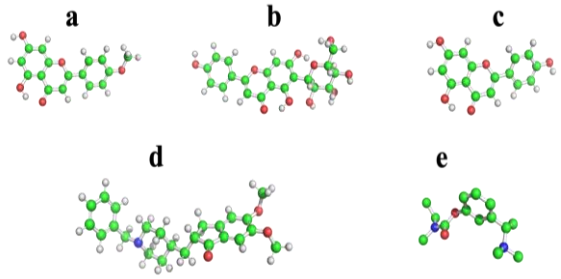

Figure 1: Structure of ligands used in the current study (a) Acacetin, (b) Isovitexin, (c) Apigenin, (d) Donepezil, (e) Rivastigmine

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from the southwestern portion of Thiruvananthapuram district in Kerala, India, in April 2022. To eliminate sticky contaminants and grit, the gathered plants were washed under running tap water. Subsequently, the shade-dried substance was pulverised.

Extraction of *Peperomia pellucida*

Extraction was performed on the powdered material (100 g) using ethanol (500 ml) in a Soxhlet device at 65 degrees Celsius for 72 hours. The acquired extract was concentrated and filtered via a rotary vacuum evaporator set at a temperature of 50^0C. The resultant mass was further extracted using ethanol (3x11 ml), petroleum ether (3x11 ml), and ethyl acetate (3x11 ml). Subsequently, the solvents were evaporated at reduced pressure to obtain residues of the respective extracts[10].

Preliminary phytochemical screening for flavonoids

Shinoda test

Plant extracts (1 ml) were combined with fragments of magnesium ribbon and diluted HCl. A few minutes later, the pink tint showed the presence of flavonoids.

Alkaline Reagent Test

The extract of the plant, when combined with 2 ml(2.0 %) NaOH mixture resulted in an intense yellow tint which was neutralised by addition of two drops diluted acid to the mixture.

***Invitro* anti-cholinesterase activity and Determination of IC50**

In-vitro Anti-cholinesterase activity of *Peperomia pellucida* extract was estimated using Ellman's method. Thiocholine is produced when acetylthiocholine is hydrolysed by the enzyme; this thiocholine then interacts with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate. The optical absorbance was detected at 412 nm. In addition to 100µL of 3mM DNTB, 20µL of 0.26 U/mL AChE, and 40µL buffer (50 mM Tris pH 8.0), a 96-well plate was filled with 20µL of various plant extracts dissolved in buffer at concentrations of 25, 50, 100, 200, and 400 µg/mL. After a 15-minute incubation at 25 °C and thorough mixing of the components, the absorbance is measured at 412 nm. Following this, 20µL of acetylthiocholine iodide (ATCI) was added to initiate the reaction. The absorbance was measured at two time intervals following mixing (5 minutes, and 20 minutes). After measuring concentration

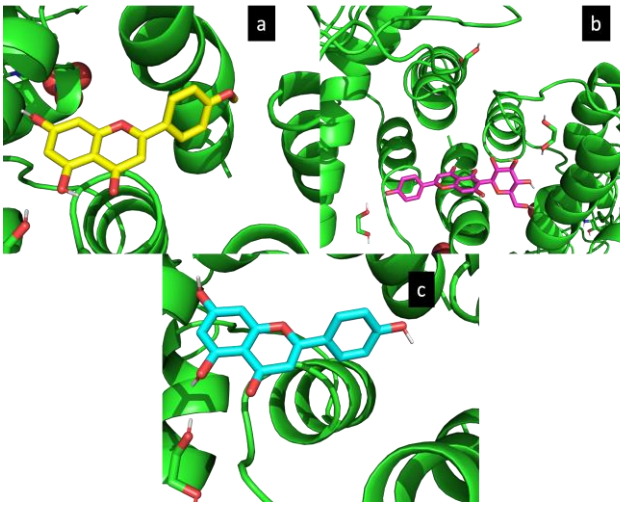

Figure 3: Molecular Docking view of selected flavonoids with Acetylcholinesterase: A) Acacetin B) Isovitexin, and C) Apigenin

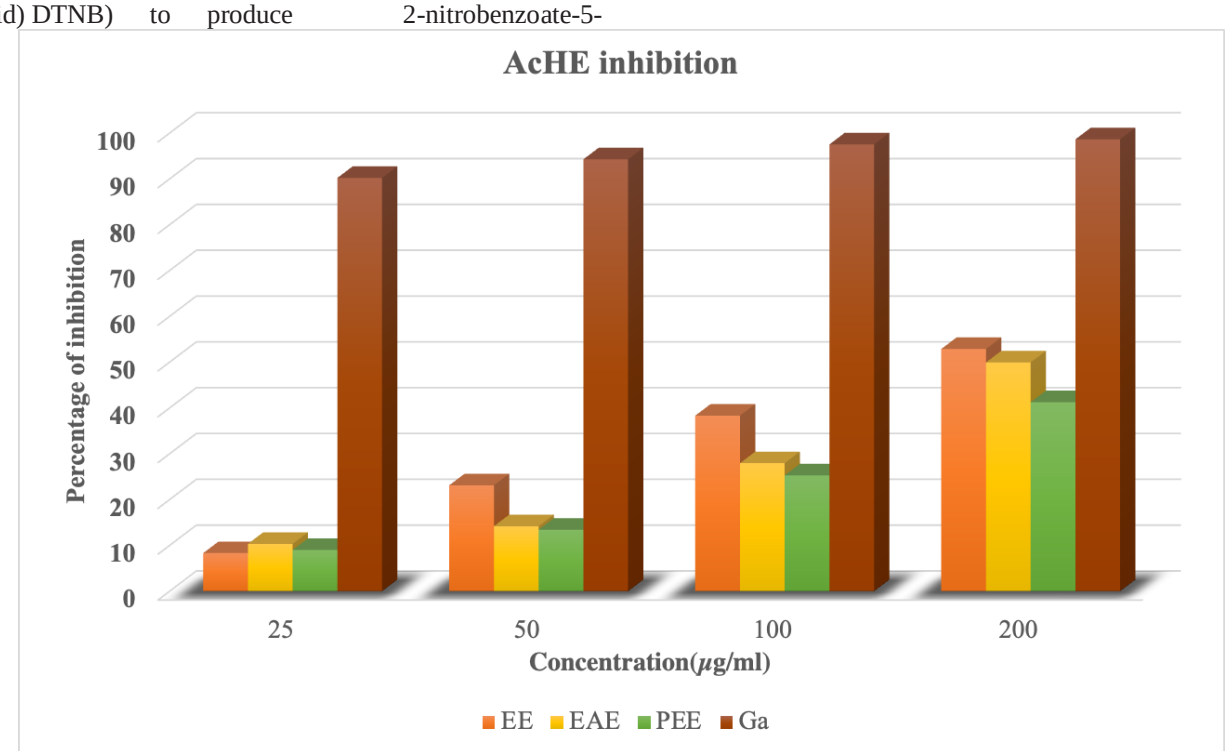

Figure 2: Inhibition of AChE activity of *Peperomia pellucida* extracts. The column represents the inhibition percentage obtained with Ethanolic extract (EE), Ethyl acetate extract (EAE), and Petroleum ether extract (PEE). Galantamine was used as a positive control.

(E) and optical density (OD), the percentage of anticholinesterase inhibition can be computed using the following formula.

$$\% \text{ inhibition} = \frac{E \times \Delta OD}{E} \times 100$$

***Insilico* Studies**

Software specifications

The docking analyses were conducted using AutoDock Vina, while the PASS software was utilised to analyse the activity predictions (http://way2drug.com/passonline/). The SWISS ADME used for the pharmacokinetic studies and ProTox-II used for the toxicological investigations.

Ligand preparation

The flavonoids Acacetin, Isovitexin, and Apigenin the flavonoids that were selected *from Peperomia pellucida*. The ligands' structures are illustrated in Figure 1. The structure files of the ligands Acacetin (PubChem CID 5280442), Isovitexin (PubChem CID 162350), Apigenin (PubChem CID 5280443), Donepezil (PubChem CID 3152) and Rivastigmine (PubChem CID 77991) were procured from PubChem database.

Target Receptor

The target receptor protein for the docking analysis is human acetylcholinesterase in complex with dihydrotanshinone I (4M0E); the structure file is obtained from a protein data bank (http://www.rcsb.org). Acetylcholinesterase (AChE) is a target receptor protein responsible for hydrolyzing the acetylcholine neurotransmitter. It is one of the evaluating markers for Alzheimer's disease. The obtained ligand file was then energy minimised, removed water molecules, added charges, and converted into PDBQT file format prior to the docking analysis.

Process and Evaluation of Docking Parameters

The docking study for flavonoid compounds (Acacetin, Isovitexin, Apigenin) from *Peperomia pellucida* and standard Alzheimer's drugs such as Donepezil and Rivastigmine were carried out using Auto Dock Vina 1.1.2 software. The docking outcomes were assessed through the analysis of the binding affinity scores.

Evaluation of *Insilico* drug-likeness and ADME Characteristics

It was crucial to evaluate the pharmacokinetic parameters of drug candidates prior to the clinical trial in order to conserve time, money, and resources. The ADME program (http://www.swissadme.ch) was utilised to conduct an ADME computational analysis, which provides information on the drug profile including absorption, distribution, metabolism, and excretion. For the drug candidates, Smiles data obtained from Pubchem was utilised in the ADME property evaluations. This evaluation clarifies its BBB penetration, distribution, metabolism, absorption, and distribution, among other properties. Thus, this tool helps in the identification of drug-likeness properties[14]. Every potential pharmaceutical drug has to adhere to Lipinski's Rule of Five.

***Insilico* Toxicity studies**

It is essential to conduct toxicology studies on a potential drug to reduce adverse and undesirable effects on animals, plants, humans, and the environment. Organ toxicity, lethal dose, and target toxicity data were obtained by computational *insilico* toxicity studies. The selected flavonoids of *Peperomia pellucida* toxicity profile was assessed using the ProTox-II online program (https://tox-new.charite.de/protox II/). When the drug candidate name, structure, and canonical smiles obtained from PubChem are inputted into Pro-Tox-II, the software generates the LD50 and toxicity profile of the selected drug molecule. When the drug candidate name, structure, and canonical smiles obtained from PubChem are inserted into Pro-Tox-II, the software generates the LD50 and toxicity profile of selected drug molecule.

PASS Parameter Evaluation

PASS is an online platform for predicting biological activity (http://way2drug.com/passonline/). It provides insights into the pharmacology, toxicology, and adverse effects of a substance. The flavonoid smiles of *Peperomia pellucida* were acquired from the PubChem software and were inputted into the prediction portal of the PASS online application. PASS uses input files in MOL format and the results of prediction can be obtained in text format. Pa and Pi are the two probabilities of active or inactive respectively included in the PASS result.

Table 1: Extractive yield of various extracts of *Peperomia pellucida*

Plant part used	Solvents	Colour of extract	Average Extractive value (% w/w)
Whole plant of *Peperomia Pellucida*	Petroleum ether	Dark green	1.97
	Ethyl acetate	Green	1.53
	Ethanol	Brownish green	2.41

Table 2: Anticholinesterase inhibition (IC50, µg/mL) of *Peperomia pellucida* plant extracts and Galantamine(Ga - positive control).

Sample	AChE inhibition assay IC50(µg/mL)
EE	175.12±14.6
EAE	279.67±32.4
PEE	313.54±30.7
Ga	130.79±7.84

RESULTS AND DISCUSSION

Successive solvent extraction and Phytochemical screening of flavonoids of *Peperomia pellucida*

The powder material was successively extracted with petroleum ether, ethyl acetate, and ethanol. Table 1 shows details on the mean extraction yield. Recent studies demonstrated that flavonoids have the capacity to show neuroprotective effects. An initial screening for flavonoids in the plant extract was performed by employing both the Shinoda and alkaline reagent tests. The test results substantiated the identification of flavonoids in the plant extract of *Peperomia pellucida*.

Acetylcholinesterase Inhibitory Activity

The destruction of acetylcholine, an essential

Table 3: Physiochemical properties of ligand.

Ligand	Molecular weight	Hydrogen bond Donor	Hydrogen Bond Acceptor	XLogP3-AA	Binding affinity
Acacetin	284.26	2	5	2.1	-7.7
Isovitexin	432.4	7	10	0.2	-8.7
Apigenin	270.24	3	5	1.7	-7.8
Donepezil	379.5	0	4	4.3	-8.2
Rivastigmine	250.34	0	3	2.3	-6.0

Table 4: Drug likeness and ADME properties of flavonoids of *Peperomia pellucida* and Standard Alzheimer's drugs

Drug Molecule	solubility	GI absorption	Bioavailability Score	Lead likeness violations
Acacetin	Moderately Soluble	High	0.55	0
Isovitexin	Soluble	Low	0.55	1
Apigenin	Moderately Soluble	High	0.55	0
Donepezil	Poorly soluble	High	0.55	2
Galantamine	Soluble	High	0.55	0
Rivastigmine	Soluble	High	0.55	0

neurotransmitter in the cholinergic system, occurs via acetylcholinesterase. An optimal degree of Ach is required for better cognitive performance. Recent research studies have focussed on finding a potential drug candidate that has enhanced anti-cholinesterase action and may prove to be a viable option for the treatment of AD. To determine the neuroprotective potential of *Peperomia pellucida*, the acetylcholinesterase inhibitory activities of various extracts were examined in the present investigation. The investigation of acetylcholinesterase inhibitory activity was conducted utilising Ellmann's approach. The results are listed in Table 2, which summarizes the IC50 values of different extracts of *Peperomia pellucida*, including ethanolic extract (EE), ethyl acetate extract (EAE), and petroleum ether extract (PEE). The ethanolic extract of *Peperomia pellucida* showed the lowest IC50 value, at 175.12 µg/mL, followed by the ethyl acetate extract at 279.67 µg/mL and the petroleum ether extract at 313.54 µg/mL, respectively. In comparison to other extracts, the ethanolic extract of *Peperomia pellucida* exhibited superior acetylcholinesterase inhibitory activity. The AChE inhibition of extracts at different doses rises in a dose-dependent fashion, as illustrated in Figure 2. The IC50 value of galantamine, which was employed as a standard AChE inhibitor, was 130.79µg/mL.

***Insilico* Docking Study**

Table 3 provides a summary of the physicochemical parameters and binding affinity. Figure 3 illustrates a molecular docking view of three flavonoids (Acacetin, Isovitexin, Apigenin) in the presence of acetylcholinesterase. According to the docking studies, Isovitexin binds with a significantly higher affinity (-8.7) than Acacetin, Apigenin, and standard drugs. However, Isovitexin exhibits non-compliance with Lipinski's rule of five, whereas Acacetin and Apigenin do not demonstrate such violations. The binding affinity of all these three flavonoids observed in *Peperomia pellucida* is superior to that of conventional drugs. Therefore, these flavonoids may exhibit protective effects against Alzheimer's disease.

Drug-likeness and ADME properties

Table 5: Toxicity profile of selected flavonoids of *Peperomia pellucida*

Drug	Predicted LD50 mg/kg	Predicted toxicity class	Targeted pathways/target toxicity
Acacetin	4000	5	PPAR-γ, ERα, Nuclear receptor signalling pathways
Isovitexin	5000	5	Carcinogenicity
Apigenin	2500	5	PPAR-γ, ERα, Nuclear receptor signalling pathways

Prediction of the *insilico* drug-likeness characteristics were done via the web-based SWISS ADME program. The data obtained from the evaluation of flavonoids (Acacetin, Isovitexin, Apigenin) and standard Alzheimer's drugs (Donepezil, Galantamine, and Rivastigmine) are presented in Table 4. Apigenin and Acacetin have moderate solubility, high GI absorption, and no lead likeness violations. Regarding Isovitexin, it exhibits lead-likeness violations despite its superior solubility and low GI absorption. All drugs demonstrated the same bioavailability score. ADME data indicates that Acacetin and Apigenin have a more favourable pharmacological profile than Isovitexin.

***Insilico* Toxicity Studies**

The safety profile of flavonoids taken from *Peperomia pellucida* was examined for toxicity prediction studies using the ProTox-II online tool. The LD_{50}, hepatotoxicity, and toxicity endpoints (carcinogenicity, mutagenicity, and immunotoxicity) of each compound were predicted. An analysis was conducted on the estimated likelihood and toxicity class of each chemical. The acquired data are detailed in Table 5. Acacetin, Isovitexin, and Apigenin have respective LD_{50} values of 4000 mg/kg, 5000 mg/kg, 2500 mg/kg. Similar targeted effects are observed for Acacetin and Apigenin, however, Isovitexin is carcinogenic.

Table 6: PASS analysis of selected flavonoids of *Peperomia pellucida*

	Acacetin		Isovitexin		Apigenin	
	Pa	Pi	Pa	Pi	Pa	Pi
Vasoprotector	0.864	0.004	-	-	0.891	0.003
Free radical scavenger	0.754	0.003	-	-	0.719	0.004
Histamine release inhibitor	0.754	0.004	-	-	-	-
Cytoprotectant	0.694	0.005	-	-	-	-
5 Hydroxytry ptamine release inhibitor	0.685	0.005	-	-	-	-
Leukotriene-B4 20-monooxygenase inhibitor	0.684	0.005	-	-	-	-
Cardioprotectant	0.682	0.004	0.961	0.002	-	-
Antihyper cholesterolemic	0.678	0.009	-	-	-	-
Antioxidant	0.628	0.004	-	-	0.732	0.004
Hepatoprotectant	0.633	0.010	0.905	0.002	-	-

PASS analysis

A PASS analysis was conducted on Apigenin, Isovitexin, and Acacetin using the PASS software. The acquired findings are presented in Table 6. Both Acacetin and Apigenin exhibit significant free radical scavenging activity and possess substantial vasoprotective properties (Pa value greater than 7). Additionally, they have an antioxidant impact. Thus, these two medications will be beneficial in the treatment of AD. Furthermore, Acacetin exhibits antihypercholesterolemic, cardioprotective, and cytoprotective properties. Both Acacetin and Isovitexin exhibit hepatoprotective and cardioprotective properties. Isovitexin demonstrates more cardioprotection and hepatoprotection than Acacetin; nevertheless, its further effects remain unknown.

CONCLUSION

The present study is focused on the *Insilico - In-vitro* anti-cholinesterase activity of *Peperomia pellucida.* Among the different extracts under the study, the ethanolic extract of *Peperomia pellucida* confirmed better *invitro* anti-cholinesterase inhibition than petroleum ether extract or ethyl acetate extract. Acacetin, Apigenin and Isovitexin are reported flavonoids present in *Peperomia pellucida*. *Insilico* study suggests that these flavonoids may be responsible for Alzheimer's protective effect. Here, Acacetin and Apigenin had better docking binding activity and toxicity profiles. Both of them obey Lipski's rule of five and have similar values in PASS analysis. In the case of Isovitexin, it possesses carcinogenicity and violates Lipinski's rule of five but it has high cardiac protective action too. The present study confirmed that the flavonoids of *Peperomia pellucida* have a significant role in Alzheimer's protective action. Among these Acacetin and Apigenin will be good phytoconstituent for Alzheimer's drug development.

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