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UPLC-ESI-MS/MS phytochemical profile, in vitro, in vivo, and in silico anti-Alzheimer's activity assessment of *Pithecellobium dulce* (Roxb.) Benth. leaves

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Abstract

Background Alzheimer's (AD) is a neurological disorder that worsens the quality of life and raises the need for care-takers with no available curative medication for the illness. Therefore, there is a growing concern about the use of herbal medicine, as it is cost-effective, has minimal side effects, and could slow AD progression and enhance patients' quality of life, making it a viable adjuvant therapy. *Pithecellobium dulce* (Roxb.) Benth F. Leguminosae is widely consumed in several countries to treat various illnesses. This study used in vitro, in vivo, and in silico studies to investigate the potential use of *P. dulce* leaves' methanolic extract in treating and preventing Alzheimer's disease.

Results The in vitro study showed that the extract inhibited 77% of the acetylcholinesterase (AChE) activity, and the IC₅₀ value was 19.23 ± 1.02 µg/ml. The in vivo study of scopolamine-induced Alzheimer's confirmed the result by significantly inhibiting AChE, dopamine, noradrenaline, and malondialdehyde levels and increasing acetylcholine, and glutathione levels. A phytochemical analysis of the leaves methanolic extract using UPLC-ESI-MS/MS revealed 67 compounds of different chemical classes, 22 flavonoids, 17 phenolic and organic acids, 8 fatty acids, 3 sterols, 6 amino acids and alkaloids, 5 coumarins, and 2 anthraquinones, 1 sugar, 1 lignin, 1 terpene, and 1 hydrocarbon. It was found that 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone had the strongest binding affinity for AChE (− 18.8 kcal/mol). Different computational modeling methods were employed, including principal component analysis, ligand-residue interaction, dynamics cross-correlation matrices analysis, and thermodynamics calculation. The binding of 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone to ACh protein decreased the fluctuation and influenced the ligand optimum orientation on the AChE protein conformational space. Additionally, the drug binding energy of AChE and the residue correlation in the 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone-AChE system was increased.

Conclusion The *P. dulce* extract contains secondary metabolites that could promisingly be a safe and effective natural treatment for Alzheimer's complications through the antioxidant activity, acetylcholinesterase, dopamine, and noradrenaline inhibition activities and also by increasing the acetylcholine level in the brain.

Keyword Leguminosae, *P. dulce*, UPLC-ESI-MS/MS, In vitro, In vivo Alzheimer, Molecular dynamics

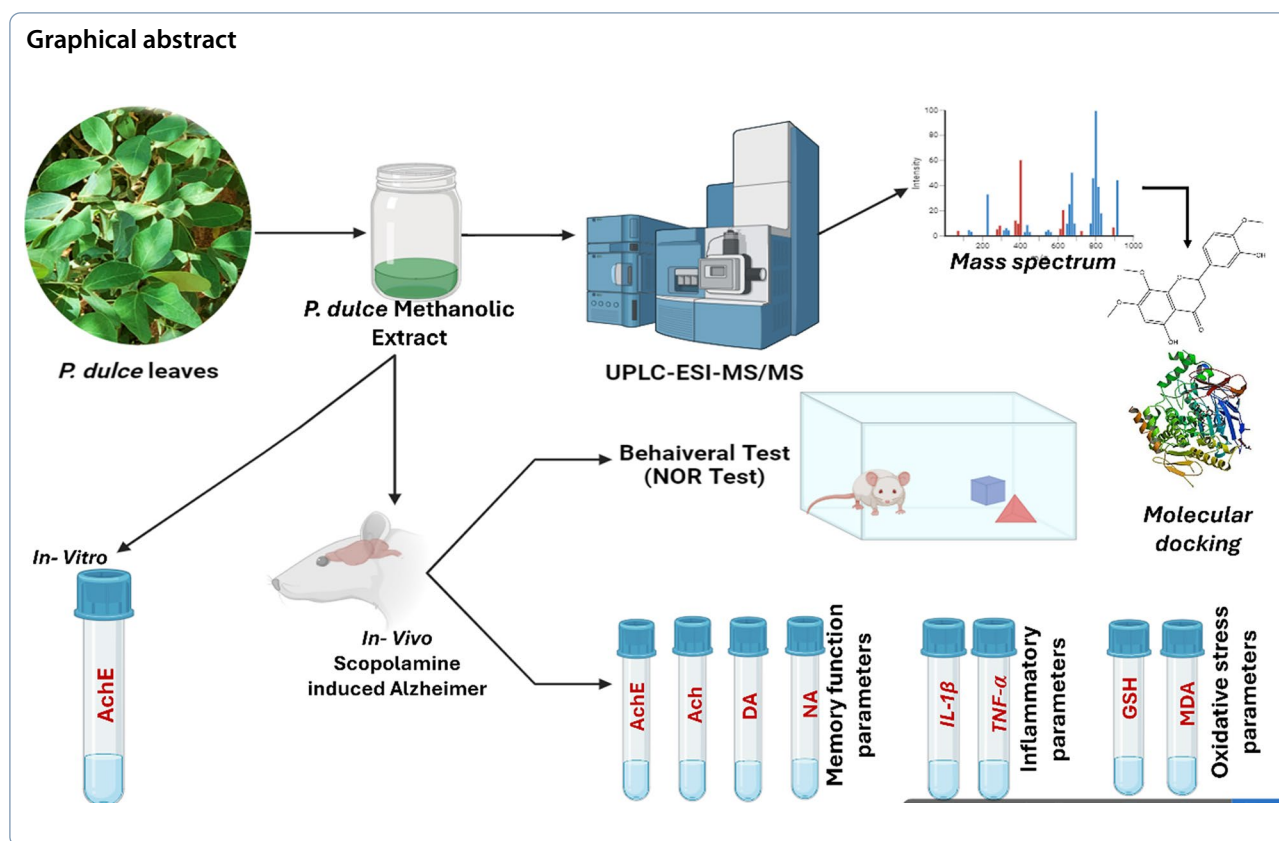
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Background

Pithecellobium dulce (Roxb.) Benth. (family Leguminosae) is a widespread evergreen tree, commonly known as Manila tamarind. It is the only *Pithecellobium* species that expanded outside its origin. The species' Latin name "*dulce*" denotes the edible pulp sweetness. In contrast, the genus name is a mix of the Greek words "Pithekos" (ape) and "Lobos" (pod), as a reference to the shape of its pod itself [1].

Several nations have traditionally used the different extracts of *P. dulce* to treat various ailments. Many active phytoconstituents in *P. dulce* are involved in its various biological activities, including antioxidant, antimicrobial, anti-diarrheal, anti-inflammatory, anticancer, anticonvulsant, hepatoprotective, neuroprotective, and cardioprotective effects [1].

P. dulce fruits are consumed due to their sweet flavor and nutritional value as a decoction, roasted, or mixed with agua fresca (a cold tea) or atole (a cornstarch-based hot beverage) in various parts of India [2]. The roasted seeds are used as a condiment or coffee substitute or cooked for treating diabetes mellitus and gastric ulcers [3]. Vitamins such as ascorbic acid, thiamine, and

riboflavin, as well as several necessary amino acids such as phenylalanine, valine, tryptophan, and lysine, and some vital minerals like Ca, Na, Fe, K, and P, are present in *P. dulce* seeds and fruits [4]. The leaves have been traditionally used for various medicinal purposes. They are employed as a remedy for indigestion, gall bladder ailments, and preventing miscarriage. Additionally, the leaves are used to treat both open and closed wounds due to their anti-inflammatory and antimicrobial properties [1].

Alzheimer's (AD) was first identified by the German psychiatrist Alois Alzheimer in 1906. It is a progressive and irreversible neurodegenerative disorder defined by short- and long-term memory impairment, disorientation, abnormal behavior, sleeping disturbance, motor skills deterioration, and cognitive function impairment, leading to the inability to perform simple daily tasks and eventual total reliance on caregivers. It is the 6th leading cause of death, with approximately 50 million cases globally in 2022 and expected to reach 152 million in 2050. AD burdens patients, their relatives, and the economy, with an estimated yearly global expense of \$1 trillion [5, 6]. AChE breaks down the neurotransmitter ACh,

which is vital in neurotransmission regulation at synapses throughout the nervous system [7]. Consequently, researchers have thoroughly studied cholinesterase inhibitors such as donepezil, galantamine, huperzine A, and rivastigmine as symptomatic therapies for AD. Cholinergic neurotransmission defects are significant for the progression of AD [8]. Oxidative stress and inflammation play crucial roles in the progression of Alzheimer's disease (AD) [9]. Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify these reactive intermediates. This imbalance leads to cellular damage, particularly in the brain, contributing to neurodegeneration. Inflammation is another key factor, with activated microglia and astrocytes responding to amyloid-beta ($A\beta$) deposits, leading to the release of inflammatory cytokines and further oxidative stress [10].

Phytochemical classes such as flavonoids, tannins, alkaloids, and phenolic acids, as well as their bioactive phytoconstituents such as quercetin, epigallocatechin-3-gallate, luteolin, and resveratrol have demonstrated neuroprotective attributes for the control and treatment of Alzheimer's by targeting acetylcholinesterase, inflammatory pathways, amyloid senile plaques, oxidation products, and certain brain receptors while causing little adverse effects [11].

This study intended to define the phytochemical content of the *P. dulce* leaves methanolic extract using UPLC-ESI-MS/MS analysis and assess the in vitro anti-AChE activity, in vivo anti-Alzheimer's activity through the scopolamine-induced Alzheimer in rat model. Also, utilizing a molecular docking and molecular dynamic simulations to correlate the identified compounds with the anti-Alzheimer's activity.

Methods

Chemicals, reagents, and materials

Chemicals and solvents included distilled water, 0.1% formic acid, methanol, scopolamine hydrobromide, donepezil hydrochloride, Abcam acetylcholine (ACh) rat ELIZA assay kit (ab65345), acetylcholinesterase (AChE), assay kit (ab138871), My BioSource dopamine (DA) rat ELIZA assay kit (MBS725908), nor-Adrenalin (NA) assay kit (MBS269993), tumor necrosis factor- α ($TNF-\alpha$) assay kit (MBS2507393), interleukin- 1β ($IL-1\beta$) assay kit (MBS825017), Lifespan Biosciences glutathione (GSH) assay kit (LS-F4612), and malonadehyde (MDA) assay kit (LS-F4236).

Plant material

Pithecellobium dulce (Roxb.) Benth. leaves were collected in September 2020 from a private garden located in El-Rabwa City, 6 October City, Giza, Egypt. Prof. Dr. Abdel-Halim Abdel-Magley, Head of Flora, and Phytotaxonomy Research Unit—Horticulture Research Institute—Giza, Egypt, recognized and verified the plant. A reference sample was preserved in the Flora and Phytotaxonomy Research Unit—Horticulture Research Institute—Giza, Egypt, under reference No. (CAIM – 218) and another one was kept in the herbarium of "Pharmacognosy department, Faculty of Pharmacy, Cairo University, Cairo, Egypt" under reference number 3-1-25-F.

Extraction

The leaves (100 gm) were collected and air-dried for a few days in the shade. Then, the leaves were ground into a coarse powder and macerated in 1 L of methanol for 24 h. The methanol was filtered, and the leaves powder was progressively macerated until exhaustion. This process was repeated 3 times. Following full extraction, the extract was evaporated using a rotary evaporator at low temperatures (less than 40 °C) and decreasing pressure till complete dryness. The prepared dry extract (20 gm) was kept at –20 °C until it was used for UPLC-MS/MS analyses and biological activity studies.

UPLC-ESI-MS/MS analysis

P. dulce leaves methanolic extract chemical profiling was carried out through UPLC-ESI-MS/MS analysis using an Acquity UPLC system coupled with (ACQUITY UPLC-BEH, C18, 50 2.1 mm, 1.7 mm particle size) a reversed-phase column at the Drug Discovery Research and Development Centre, Faculty of Pharmacy, Ain Shams University, Egypt. The analytical conditions were set as previously reported in [12]. In the range of 100–1000 m/z, mass spectra were collected for both positive and negative ion modes. Chromatographic peaks and mass spectra were obtained by Maslynx 4.1 software.

In vitro acetylcholinesterase activity

The inhibitory effect of *P. dulce* extract on AChE was assessed utilizing an acetylcholinesterase inhibitor screening kit (Quanti Chrom™, USA) in compliance with the manufacturer's guidelines. This assay was

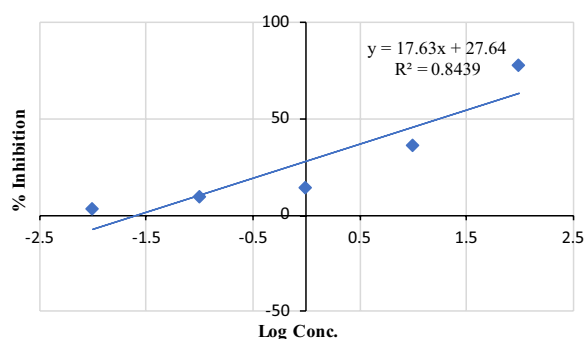


Fig. 1 AChE inhibition curve of *P. dulce* leaves methanolic extract

performed based on the method mentioned in [13]. Briefly, 50 μ L of *P. dulce* extract was mixed at pH 8.0 with 125 μ L of 5, 5'-dithio-bis-2-nitrobenzoate (DTNB, 99% purity) and 25 μ L of AChE solution in Tris-HCl buffer, and then for 15 min it was incubated at 25 $^{\circ}$ C. Donepezil was used as a standard drug. (0, 0.1, 1, and 10 μ g/mL) different concentrations of the extract were utilized, and each concentration was assessed three times [14]. The % of inhibition of AChE was determined using the subsequent equation:

$$\frac{[(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}] \times 100}{}$$

Plotting the percent of inhibition against the log concentration of *P. dulce* extract yielded an inhibition curve from which the IC_{50} was determined (Fig. 1).

In vivo anti-Alzheimer activity

Animals and experimental design

Forty-eight Wistar albino rat adult males (200–250 g) were kept in polypropylene cages at the National Research Centre, Giza, Egypt. The animals were kept under the same environmental conditions at 25 ± 2 $^{\circ}$ C. To help them adapt, the animals were given unrestricted access to a commercially available rat pellet diet and water for seven days before the start of the study. Six groups of eight rats were created. Rats were orally administered 3 different doses of *P. dulce* alcoholic extract (100, 250, or 500 mg/kg) for 9 days to investigate the possible preventative effect on Alzheimer's [11, 12]. Scopolamine (5 mg/kg/day) was prepared in saline and given intraperitoneally (IP) to the rats for 9 consecutive days.

Group 1 (normal) received saline. **Group 2** (disease) was given IP scopolamine (5 mg/kg). **Group**

3 (standard) was given donepezil (2.5 mg/kg), **and Groups 4, 5, and 6** (extract groups) were given 3 doses of *P. dulce* extract (100, 250, and 500 mg/kg), respectively. Scopolamine (5 mg/kg) was administered to each treatment group 30 min after the treatment.

Acute toxicity study

The lethality (LD_{50}) of the rats was evaluated using guideline no. 420 of OECD (Organization for Economic Co-operation and Development). Six groups of six rats were formed from overnight fasted rats. Alcoholic *P. dulce* extract suspended in saline was administered to the rats at escalating dosages (1000, 2000, 3000, 4000, and 5000 mg/kg body weight). The normal group was kept under the same conditions and given only a vehicle (saline). The animals were observed for signs of toxicity or death for 24 h. Food and drink were provided ad libitum.

NOR test

The NOR test is largely based on rats' natural curiosity to investigate new items. The rats' memory and cognitive function were evaluated on the final 2 days of the trial [17]. NOR test consists of habituation, familiarization, and test phases. Throughout the 3-min test, rats were allowed to explore if they touched the object or pointed their noses 2 cm away from it. A timer was used to track how long the rats spent investigating the items throughout the three-minute test. T_1 is the amount of time used to investigate familiar objects, and T_2 is the novel object exploration time. The difference was calculated as $T_2 - T_1$.

Memory function, oxidative stress, and inflammatory parameters

The rats were euthanized on the ninth day following the behavioral NOR test. Hippocampal tissue was extracted from six rats per group ($n=6$), and the brain was dissected for further biochemical mixing with 10% phosphate-buffered saline (PBS) for ELISA assessment of the memory function parameters AChE activity, ACh, DA, and NA; the antioxidant parameters GSH and MDA; and the inflammatory parameters $TNF-\alpha$ and $IL-1\beta$ [16].

ACh content and AChE activity were measured using a rat ELIZA kit from Abcam (Assay kit ab65345) (nmol/mg tissue) and (Assay kit ab138871) (U/mg tissue), respectively [7]. Additionally, DA (Catalog Number: MBS725908) (ng/mg tissue) and NA (Catalog Number:

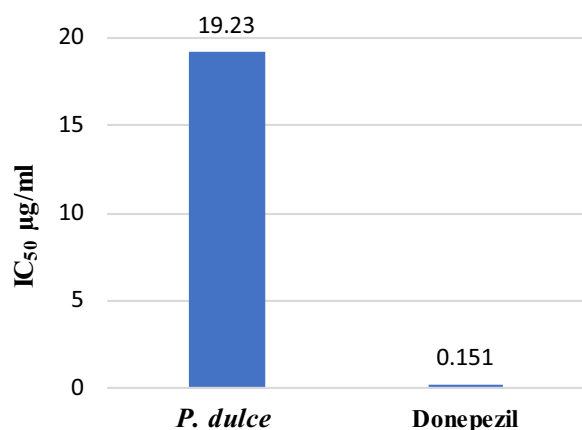


Fig. 2 AChE inhibitory effect of the *P. dulce* leaves methanolic extract

MBS269993) (Pg/mg tissue) were measured using a rat ELIZA kit from My BioSource.

GSH and MDA levels were assessed using a rat ELIZA kit from Lifespan Biosciences (Catalog Number: LS-F4612) (µg/mg tissue) and (Catalog Number: LS-F4236) (ng/mg tissue), respectively.

TNF-α and *IL-1β* levels were analyzed using My BioSource rat ELIZA kits (Catalog Number: MBS2507393) (Pg/mg tissue) and (Catalog Number: MBS825017) (Pg/mg tissue), respectively.

Statistical methods and data analysis

The statistical analyses included a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests ($p < 0.05$), which revealed (a) significant differences from the normal group and (b) significant differences from the scopolamine induction group. A t-test was carried out specifically for (Fig. 2A) to compare the timing of familiar and novel objects, (a) significant differences from the familiar object.

In silico study

System preparation

AChE crystal structure (4EY7) was acquired from the protein data bank [18], and UCSF Chimera [19] was utilized to construct the crystal structure and eliminate the hydrogen atoms for docking preparation. The pH was adjusted to 7.5 using PROPKA [20]. The 2D structure was obtained by ChemBioDraw Ultra 12.1 [21]. Using the MMFF94 force field and the steepest descent approach, Avogadro software [22] optimized the 2D structure for energy minimization.

Molecular docking

The docking calculations were made by AutoDock Vina [23], and Gasteiger partial charges [24] were assigned during docking. The MGL tools' AutoDock graphical user interface was utilized to outline AutoDock atom types [25]. The AutoDock Vina grid center coordinates were x (−6.57), y (−40.40), and z (30.303), and the search space size was adjusted to $20 \text{ Å} \times 20 \text{ Å} \times 20 \text{ Å}$ in the 3 cases and exhaustiveness=8. The docked conformations in declining order of docking energy were generated by the Lamarckian genetic method [26].

Molecular dynamic (MD) simulations

The atoms and molecules' physical motion assessment is facilitated by utilizing MD simulations to investigate biological systems, which is not readily accessible by other techniques [27]. This simulation offers a comprehensive understanding of the biological systems' dynamical evolution, including molecular affiliation and conformational changes [27]. AMBER 18 package GPU of the PMEMD engine was used to run the MD simulations for every system [28].

The GAFF (general amber force field) approach made by ANTECHAMBER [29] was utilized to compute every chemical partial atomic charge. Within the TIP3P water molecules' orthorhombic box, each system was implicitly solvated within 10 Å of any box edge by the AMBER 18 package Leap module. To neutralize the Leap module, the counter ions Cl^- and Na^+ were introduced to each system. A 2000-step initial minimization with an imposed restraint potential of 500 kcal/mol was applied to each system, and then a full minimization was performed for 1000-step with the conjugate gradient algorithm without restrictions.

Each system was heated incrementally over 500 ps, from 0 to 300 K, to guarantee that every system had a fixed volume and number of atoms throughout the MD simulation. The system's solutes experienced a potential harmonic limitation of 10 kcal/mol and a collision frequency of 1 ps. A constant temperature of 300 K was used to heat every system and then allowed to equilibrate for 500 ps. The pressure and the number of atoms in every system were maintained constant for every production simulation to replicate the NPT (isobaric-isothermal ensemble). Berendsen barostat was utilized to keep the pressure of the system at 1 bar [30]. Every system was MD simulated for 20 ns. The SHAKE approach was utilized for every simulation, to build the hydrogen bond atoms. Every simulation integrated an SPFP precision model and used a 2 fs step size. The NPT with randomized seeding, a 300 K temperature, 1 bar constant pressure, 2 ps pressure-coupling constant, and a Langevin thermostat

with 1 ps collision frequency were used for simulation conduction.

Post-MD analysis

Upon the MD simulations completion, the trajectories were stored at 1-ps intervals and examined utilizing the AMBER18 suite's CPPTRAJ [31] module. All the visualizations and graphs were constructed utilizing Chimera [19] and Origin data analysis software [32].

Thermodynamic calculation

Ligand-binding affinities calculation was made utilizing the MM/PBSA (Poisson–Boltzmann) [33] approach or the MM/GBSA (generalized Born and surface area continuum solvation) [34]. Within a specified force field, the MM/GBSA and MM/PBSA employ the molecular simulations of the protein–ligand complex to yield precise statistical-mechanical binding free energy [35].

The average of the binding free energy was estimated from over 200 snapshots produced from the 20 ns trajectory. The ΔG which is the change in binding free energy was estimated for each molecular species (complex, ligand, and receptor) as follows [36]:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{receptor}} - G_{\text{ligand}} \quad (1)$$

$$\Delta G_{\text{bind}} = E_{\text{gas}} + G_{\text{sol}} - TS \quad (2)$$

$$E_{\text{gas}} = E_{\text{int}} + E_{\text{vdw}} + E_{\text{ele}} \quad (3)$$

$$G_{\text{sol}} = G_{\text{GB}} + G_{\text{SA}} \quad (4)$$

$$G_{\text{SA}} = \gamma \text{ SASA} \quad (5)$$

where the gas-phase energy is represented by (E_{gas}), the internal energy by (E_{int}), the Coulomb energy by (E_{ele}), the van der Waals energy by (E_{vdw}), the solute total entropy by (S), the temperature by (T), the solution-free energy by (G_{sol}), and the polar states and the non-polar states energy by (G_{GB}) and (G_{SA}), respectively. The E_{gas} was assessed using the FF14SB force field words. To compute G_{sol} , the G_{GB} and (G_{SA}) were used. The G_{SA} was computed utilizing a 1.4 Å water probe radius from the SASA [37, 38]. Conversely, the GB equation was solved to assess the G_{GB} contribution. Amber18's MM/GBSA-binding free energy technique was utilized to estimate the impact of every residue on the overall binding free energy.

Results

UPLC-ESI-MS/MS analysis

UPLC-ESI-MS/MS was utilized to profile the *P. dulce* leaves methanolic extract metabolites. Retention time

(R_t), mass, and MS² (in both positive and negative ionization modes) were employed to identify the compounds by comparing them with the reported literature and online public databases. This led to the identification of 67 compounds of different chemical classes, 22 flavonoids, 17 phenolic and organic acids, 8 fatty acids, 6 amino acids and alkaloids, 5 coumarins, 3 sterols, 2 anthraquinones, 1 sugar, 1 lignin, 1 terpene, and 1 hydrocarbon (Table 1).

Flavonoids and flavonoidal glycosides

Flavonoids and flavonoidal glycosides were the most prominent classes of tentatively identified metabolites representing 22 compounds. Compounds tentatively identified in the positive mode are described as follows quercetin-3-*O*-rhamnoside (quercitrin) (13) (Fig. S4) ($\text{C}_{21}\text{H}_{20}\text{O}_{11}$) was determined based on m/z 477/449 $[\text{M}-\text{H}]^- / [\text{M}+\text{H}]^+$ molecular ions and MS² at m/z 303 $[\text{M}+\text{H}-\text{C}_6\text{H}_{10}\text{O}_4]^+$ and 287 $[\text{M}+\text{H}-\text{C}_6\text{H}_{10}\text{O}_4-\text{OH}]^+$ [39]. Compound (47) detected with m/z 611 $[\text{M}+\text{H}]^+$ ($\text{C}_{27}\text{H}_{30}\text{O}_{16}$) and MS² at m/z 303 $[\text{M}+\text{H}-\text{C}_{12}\text{H}_{20}\text{O}_9]^+$ which was tentatively identified as rutin [39]. Compound (18) (Fig. S6) exhibited $[\text{M}+\text{H}]^+$ at m/z 287 and MS² of 241 $[\text{M}+\text{H}-\text{CHO}_2]^+$, 165 $[\text{M}+\text{H}-\text{C}_7\text{H}_5\text{O}_2]^+$, and 153 $[\text{M}+\text{H}-\text{C}_8\text{H}_5\text{O}_2]^+$ which tentatively identified as kaempferol [43]. Additionally, compounds (6) (Fig. S3) and (16) (Fig. S5) were tentatively identified as kaempferol-3-*O*-glucoside with $[\text{M}+\text{H}]^+$ at m/z 499 ($\text{C}_{21}\text{H}_{20}\text{O}_{11}$) and kaempferol-3-*O*-rhamnoside (afzelin) with $[\text{M}+\text{H}]^+$ at m/z 433 ($\text{C}_{21}\text{H}_{20}\text{O}_{10}$) with MS² at m/z 287 $[\text{M}+\text{H}-\text{C}_6\text{H}_{10}\text{O}_5]^+$ and $[\text{M}+\text{H}-\text{C}_6\text{H}_{10}\text{O}_4]^+$, respectively [39]. Isorhamnetin (32) showed $[\text{M}+\text{H}]^+$ at m/z 317 ($\text{C}_{16}\text{H}_{12}\text{O}_7$) and MS² at m/z 300 $[\text{M}+\text{H}-\text{CH}_3]^+$ [47]. Besides, compounds tentatively identified in the negative mode: Compound (8) was identified as 4''-*O*-methyl epigallocatechin 3-*O*-gallate with $[\text{M}-\text{H}]^-$ at m/z 471 ($\text{C}_{23}\text{H}_{20}\text{O}_{11}$) yielding product ions at m/z 319 $[\text{M}-\text{H}-\text{C}_7\text{H}_4\text{O}_7]^-$ and m/z 169 $[\text{M}-\text{H}-\text{C}_{16}\text{H}_{14}\text{O}_5]^-$ [41]. Kaempferol-di-*O*-rhamnoside (30) exhibited $[\text{M}-\text{H}]^-$ at m/z 577 ($\text{C}_{27}\text{H}_{30}\text{O}_{14}$) with MS² at m/z 431 $[\text{M}-\text{H}-\text{C}_6\text{H}_{10}\text{O}_4]^-$ and 285 $[\text{M}-\text{H}-\text{C}_{12}\text{H}_{20}\text{O}_8]^-$ [45]. Isorhamnetin glucuronide (7) was detected with $[\text{M}-\text{H}]^-$ at m/z 491 and MS² at m/z 315 $[\text{M}-\text{H}-\text{C}_6\text{H}_9\text{O}_6]^-$ [40]. One flavan-3-ol and two metabolites of proanthocyanidins were tentatively identified. Epicatechin or catechin (26) was detected with $[\text{M}-\text{H}]^-$ at m/z 289 ($\text{C}_{15}\text{H}_{14}\text{O}_6$), yielding the characteristic product ions at m/z 245 $[\text{M}-\text{H}-\text{CO}_2]^-$, m/z 205 $[\text{M}-\text{H}-\text{C}_4\text{H}_4\text{O}_2]^-$ and m/z 123 $[\text{M}-\text{H}-\text{C}_6\text{H}_6\text{O}_2]^-$ [41]. Likewise, $[\text{M}-\text{H}]^-$ at m/z 865 ($\text{C}_{45}\text{H}_{38}\text{O}_{18}$) showed a product ion at m/z 577 $[\text{M}-\text{H}-\text{C}_{15}\text{H}_{14}\text{O}_6]^-$ and m/z 289 $[\text{M}-\text{H}-\text{C}_{15}\text{H}_{14}\text{O}_6-\text{C}_{15}\text{H}_{14}\text{O}_6]^-$ and the $[\text{M}+\text{H}]^+$ at m/z 579 ($\text{C}_{30}\text{H}_{26}\text{O}_{12}$) showed a product ion at m/z 427 $[\text{M}+\text{H}-\text{C}_8\text{H}_8\text{O}_3]^+$ and m/z 291 $[\text{M}+\text{H}-\text{C}_{15}\text{H}_{14}\text{O}_6]^+$, ascribed for the existence of epicatechin or catechin and hence were identified as

Table 1 Metabolite profiling of *P. dulce* leaves extract via UPLC–ESI–MS/MS (positive and negative ion modes)

Peak no.	R_t (min)	Tentatively identified compounds	Molecular formula	$[M+H]^+ m/z$	$[M-H]^- m/z$	MS/MS fragmentation	References
Flavonoids and flavonoidal glycosides							
6	6.22	Kaempferol-3-O-glucoside (Astragalin)	$C_{21}H_{20}O_{11}$	449	–	287	[39]
7	6.22	Isorhamnetin glucuronide	$C_{22}H_{20}O_{13}$	–	491	315,301,285, 229, 175,151	[40]
8	6.22	4"-O-methyl epigallocatechin 3-O-gallate	$C_{23}H_{20}O_{11}$	–	471	319, 169	[41]
13	6.5	Quercetin-3-O-rhamnoside (quercitrin)	$C_{21}H_{20}O_{11}$	449	447	303, 287	[39]
16	6.95	Kaempferol-3-O- rhamnoside (afzelin)	$C_{21}H_{20}O_{10}$	433	431	287, 285	[39]
17	6.95	Procyanidin trimer	$C_{45}H_{38}O_{18}$	–	865	577, 289	[42]
18	6.95	Kaempferol	$C_{15}H_{10}O_6$	287	–	241, 165, 153	[43]
20	8.8	Luteolin	$C_{15}H_{10}O_6$	–	285	227, 135	[44]
26	10.66	Catechin/ Epicatechin	$C_{15}H_{14}O_6$	–	289	245, 205, 179	[41]
30	13.71	Kaempferol-di-O-rhamnoside	$C_{27}H_{30}O_{14}$	–	577	431, 285	[45]
31	14.28	Pentahydroxy tri-methoxy flavone	$C_{18}H_{16}O_{10}$	393	–	378,347,317, 206,193	[46]
32	14.28	Isorhamnetin	$C_{16}H_{12}O_7$	317	–	300	[47]
36	15.35	Myricetin	$C_{15}H_{10}O_8$	319	–	254, 153	[48]
40	15.6	Dihydroxy dimethoxy isoflavone	$C_{16}H_{12}O_5$	–	313	255	[49]
41	15.74	Rhamnosyl hexosyl methyl quercetin	$C_{28}H_{32}O_{16}$	–	623	608, 591, 519, 477, 459, 339, 315	[50]
42	16.6	Trihydroxy methoxy isoflavone (tectorigenin)	$C_{16}H_{12}O_6$	301	–	286, 269	[49]
43	16.6	Procyanidin dimer B2	$C_{30}H_{26}O_{12}$	579	–	427, 291	[51]
44	19.33	5,3'-Dihydroxy-7,8,4'-tri-methoxy flavanone	$C_{18}H_{18}O_7$	–	345	330, 315, 287	[52]
47	20.18	Rutin	$C_{27}H_{30}O_{16}$	611	–	609, 465, 303, 449	[39]
48	20.93	Isorhamnetin-O-pentosyl-hexoside	$C_{27}H_{30}O_{16}$	–	609	315	[53]
59	23.72	6"-O-acetyl daidzein	$C_{23}H_{22}O_{10}$	–	457	221	[41]
63	27.03	Naringin 4'-O-glucoside	$C_{21}H_{42}O_{19}$	–	741	433, 271	[41]
Phenolic and organic acids and their derivative							
2	0.77	Caffeic acid hexoside	$C_{15}H_{18}O_9$	–	341	179	[40]
11	6.5	Di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	–	515	353, 335, 191, 179	[41]
12	6.5	Ellagic acid	$C_{14}H_6O$	303	–	172, 158, 144, 127, 116	[46]
14	6.82	2-Hydroxy benzoic acid	$C_7H_6O_3$	–	137	93	[54]
15	6.82	Galloyl-coumaric acid pentoside	$C_{15}H_{18}O_8$	–	447	151	[49]
19	8.8	Caffeic acid derivative	$C_{16}H_{20}O_6$	–	327	179, 147	[55]
22	9.27	Dihydroxy benzoic acid methyl ester hexoside	$C_{14}H_{18}O_9$	–	329	270, 211	[49]
23	9.27	3-O-Methyl gallic acid	$C_8H_8O_5$	–	183	168, 140, 124	[54]
29	13.71	Cinnamic acid	$C_9H_8O_2$	–	147	103	[44]
33	15.35	Ferulic acid methyl ester	$C_{11}H_{12}O_4$	209	–	177, 149, 134	[56]
34	15.35	<i>p</i> -Coumaroyl tartaric acid	$C_{13}H_{18}O_{12}$	–	295	115	[54]
35	15.35	Cinnamoyl glucose	$C_{15}H_{18}O_7$	–	309	147, 131, 103	[54]
38	15.46	Caffeoyl tartaric acid	$C_{13}H_{18}O_9$	–	311	179, 161	[54]
54	22.63	Caffeic acid 3-O-glucuronide	$C_{15}H_{16}O_{10}$	–	355	179	[41]
56	22.96	Pantothenic acid	$C_9H_{17}NO_5$	220	–	202, 124, 90	[57]
64	27.32	Caffeic acid	$C_{21}H_{22}O_{10}$	–	179	151, 143, 133	[41]
66	31.27	4-O-Methylgallic acid	$C_8H_8O_5$	185	–	170, 142	[41]

Table 1 (continued)

Peak no.	<i>R_t</i> (min)	Tentatively identified compounds	Molecular formula	[M+H] ⁺ <i>m/z</i>	[M-H] ⁻ <i>m/z</i>	MS/MS fragmentation	References
Fatty acids, sterols, and terpenes							
27	10.66	9, 12-Octadecadienoic acid ethyl ester	C ₂₀ H ₃₆ O ₂	–	307	263, 95, 81, 67, 55	[54, 55]
45	19.33	Amyrin	C ₃₀ H ₅₀ O	–	425	421, 407	[60]
46	19.33	Linolenic acid	C ₁₈ H ₃₀ O ₂	279	277	259, 233, 205, 179, 223, 81, 95	[61, 62]
49	20.93	Linoleic acid	C ₁₈ H ₃₂ O ₂	–	279	261, 235, 54	[63]
50	20.93	Oleic acid	C ₁₈ H ₃₄ O ₂	–	281	263, 54	
53	22.63	Palmitic acid	C ₁₆ H ₃₂ O ₂	–	255	239, 229, 221, 213, 211, 209	[47]
55	22.96	9,17-Octadecadienal	C ₁₈ H ₃₂ O	–	265	81, 67, 48	[54, 55]
57	22.96	9,11,13-Octadecatrienoic acid methyl ester	C ₁₉ H ₃₂ O ₂	293	–	261, 243, 137	[64]
58	22.96	Oleic acid	C ₁₈ H ₃₄ O ₂	283	–	264, 125, 83, 69, 55	[65]
61	23.72	1-Stearoyl-glycerol	C ₂₁ H ₄₂ O ₄	359	–	341, 323, 267	[64]
62	25.44	Stigmasterol	C ₂₉ H ₄₈ O	413	–	395, 351, 273	[66]
65	31.06	α-Tocopherol (Vitamin E)	C ₂₉ H ₅₀ O ₂	430	–	205, 165, 43	[67]
Miscellaneous compounds							
Amino acids and alkaloids							
3	0.96	Pyroglutamic acid	C ₅ H ₇ NO ₃	130	–	102, 84, 74, 67, 56	[61]
4	0.96	Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	268	–	136	
5	0.96	2,2,6,6-tetramethyl-1-piperidinol	C ₉ H ₁₉ N	158	–	126, 70, 69, 41	[64]
24	9.71	Cytidine	C ₉ H ₁₃ N ₃ O ₅	244	–	112, 95	[57]
28	11.62	Cysteine	C ₃ H ₇ NO ₂ S	122	–	59, 76, 87	[57]
37	15.46	Hypoxanthine	C ₅ H ₄ N ₄ O	137	–	119, 110, 94	[57]
Coumarins							
21	8.8	Bergapten	C ₁₂ H ₈ O ₄	–	215	199, 185, 171	[68]
25	9.71	Hydroxy coumarin	C ₉ H ₆ O ₃	163	–	107	[69]
51	21.05	Bergamottin	C ₂₁ H ₂₂ O ₄	339	–	254	[70]
60	23.72	8-Acetyl-6,7-dimethoxy coumarin	C ₁₃ H ₁₂ O ₅	249	–	115	[69]
67	31.33	7-Hydroxy-4-methyl coumarin (Hymecromone)	C ₁₀ H ₈ O ₃	–	174	146, 130, 86	[71]
Anthraquinones							
9	6.22	Cassiamin B	C ₃₀ H ₁₈ O ₁₀	–	537	523	[72]
39	15.46	Aloe-emodin glucoside	C ₂₁ H ₂₀ O ₁₀	–	431	269, 240	[73]
Other compounds							
1	0.18	Dulcitol	C ₆ H ₁₄ O ₆	183	–	165, 129, 99	[74]
10	6.22	Alangilignoside D	C ₂₅ H ₂₄ O ₁₂	–	551	419, 404	[75]
52	21.05	3,7,11,15-tetramethyl 2-hexadecene	C ₂₀ H ₄₀	–	281	70, 69, 57	[54, 55]

their oligomers namely, procyanidin trimer (**17**) and dimer (**43**), respectively [42, 51].

Phenolic and organic acids and their derivatives

Phenolic and organic acids were the second most prominent metabolic class with 17 compounds. Caffeic acid hexoside (**2**) was detected with [M-H]⁻ at *m/z* 341 (C₁₅H₁₈O₉), producing [M-H-C₆H₁₀O₅]⁻ product ions at *m/z* 179 [40]. Di-*O*-caffeoylquinic acid (**11**)

shows the parent ion [M-H]⁻ at *m/z* 515 (C₂₅H₂₄O₁₂) with MS² at *m/z* 353 [M-H-C₉H₆O₃]⁻, *m/z* 335 [M-H-C₉H₈O₄]⁻, and *m/z* 191 [M-H-C₁₈H₁₂O₆]⁻ [41]. Compound (**14**) was recognized as 2-hydroxy benzoic acid with [M-H]⁻ at *m/z* 137 (C₇H₆O₃) and [M-H-CO₂]⁻ at *m/z* 93 [54]. Compound (**19**) caffeic acid derivative gave a molecular ion peak [M-H]⁻ at *m/z* 327 (C₁₆H₂₀O₆), a 147 *m/z* base peak linked to cinnamic acid, and a peak at *m/z* 179 corresponding to caffeic acid [55]. Dihydroxy

benzoic acid methyl ester hexoside (**22**) was detected with $[M-H]^-$ at m/z 329 ($C_{14}H_{18}O_9$), producing product ion at m/z 270 $[M-H-COOCH_3]^-$ [49]. Whereas compound (**23**) showed $[M-H]^-$ at m/z 183 ($C_8H_8O_5$), yielding product ions at m/z 168 $[M-H-CH_3]^-$, m/z 140 $[M-H-CO_2]^-$, and m/z 124 $[M-H-COOCH_3]^-$ was annotated as 3-*O*-methyl gallic acid [54] while cinnamic acid (**29**) was detected with $[M-H]^-$ at m/z 147 ($C_9H_8O_2$), yielding m/z 103 $[M-H-COO]^-$ product ion [44]. Likewise, compound (**33**) revealed a molecular ion peak $[M+H]^+$ at m/z 209 ($C_{11}H_{12}O_4$), and product ions at m/z 177 $[M+H-CH_4O]^+$, m/z 149 $[M+H-C_2H_4O_2]^+$, and m/z 134 $[M+H-C_2H_4O-CH_3]^+$ were attributed to ferulic acid methyl ester [56]. Cinnamyl glucose (**35**) was recognized with $[M-H]^-$ at m/z 309 ($C_{11}H_{12}O_4$) that produced m/z 147 $[M-H-C_6H_{10}O_5]^-$, 131 $[M-H-C_6H_{10}O_6]^-$ and 103 $[M-H-C_7H_{10}O_7]^-$ fragment ions [54]. Caffeoyl tartaric acid (**38**) was detected with $[M-H]^-$ at m/z 311 ($C_{13}H_{18}O_9$) which generated fragment ions at m/z 179 $[M-H-C_4H_4O_5]^-$, and m/z 161 $[M-H-C_4H_4O_6]^-$ [54].

Fatty acids, sterols, and terpenes

One triterpene, 8 fatty acids, and 3 sterols were tentatively identified in both the positive and negative modes. Compound (**45**) was identified as amyirin with $[M-H]^-$ at m/z 425 ($C_{30}H_{50}O$) and m/z 407 $[M-H-H_2O]^-$ [60]. The identified fatty acids and their derivatives (**27**, **46**, **49**, **50**, **53**, **55**, **57**, **58**) were detected in both ESI modes. They shared the common loss of 18 m/z $[-H_2O]$ and 44 m/z $[-CO_2]$ and the characteristic presence of 54 m/z fragment resulting from the α -cleavage and double-bond transfer of the fatty acids MS/MS fragmentation [76]. The identified sterols (**60**, **61**, **64**) were tentatively identified in ESI-positive mode. 1-Stearoyl-glycerol (**61**) was

recognized at m/z 359 ($C_{21}H_{42}O_4$) and generated the fragment ions at m/z 341 $[M+H-H_2O]^+$ and m/z 267 $[M+H-C_3H_8O_3]^+$ [64] while stigmasterol (**62**) was recognized at m/z 413 ($C_{29}H_{48}O$), m/z 395 $[M+H-H_2O]^+$, m/z 351 $[M+H-H_2O-C_3H_8]^+$, and m/z 273 $[M+H-C_{10}H_{20}]^+$ [66]. α -Tocopherol (**65**) was detected at m/z 430 ($C_{29}H_{50}O_2$) and produced the fragment ions at m/z 205 $[C_{13}H_{17}O_2]^+$ and m/z 165 $[C_9H_{11}O_2]^+$ [67].

Miscellaneous compounds

Additional compounds of miscellaneous classes have been tentatively identified like the monosaccharide dulcitol (**1**) which was recognized by m/z 183 $[M+H]^+$ ($C_6H_{14}O_6$) and MS fragments at m/z 165, m/z 129, and m/z 99 [74]. Also, 3 amino acids (**3**, **24**, **28**) and 3 alkaloids (**4**, **5**, **37**) were detected in ESI-positive mode. Amino acids have a characteristic loss of 17 m/z $[-NH_3]$ followed by loss of 18 m/z $[-H_2O]$, 28 m/z $[-CO]$, 44 m/z $[-CO_2]$, and 42 m/z $[-CH_2CO]$ [61]. Adenosine (**4**) is presented by a molecular ion peak at m/z 268 ($C_{10}H_{13}N_5O_4$) and m/z 136 fragment ion [61]. Different coumarins (**21**, **25**, **51**, **60**, **67**) have been tentatively identified. Bergapten was identified with $[M-H]^-$ at m/z 215 and fragment ions $[M-CH_3]$ m/z 199, $[M-CO]$ m/z 171, and $[M-CO_2]$ m/z 145 [68]. Hydroxy coumarin (**25**) was detected with $[M+H]^+$ at m/z 163 and fragment ion at m/z 107 $[M+H-CO-CO]^+$, while bergamottin (**51**) was detected with $[M+H]^+$ at m/z 339 and fragment ion at m/z 245 $[M+H-C_5H_9O]^+$ [69]. 7-Hydroxy-4-methyl coumarin (**67**) was detected with $[M-H]^-$ at m/z 174 and produced the fragments at m/z 146 $[M-H-CO]^-$, m/z 130 $[M-H-CO_2]^-$, and m/z 86 $[M-H-C_3H_8]^-$ [71].

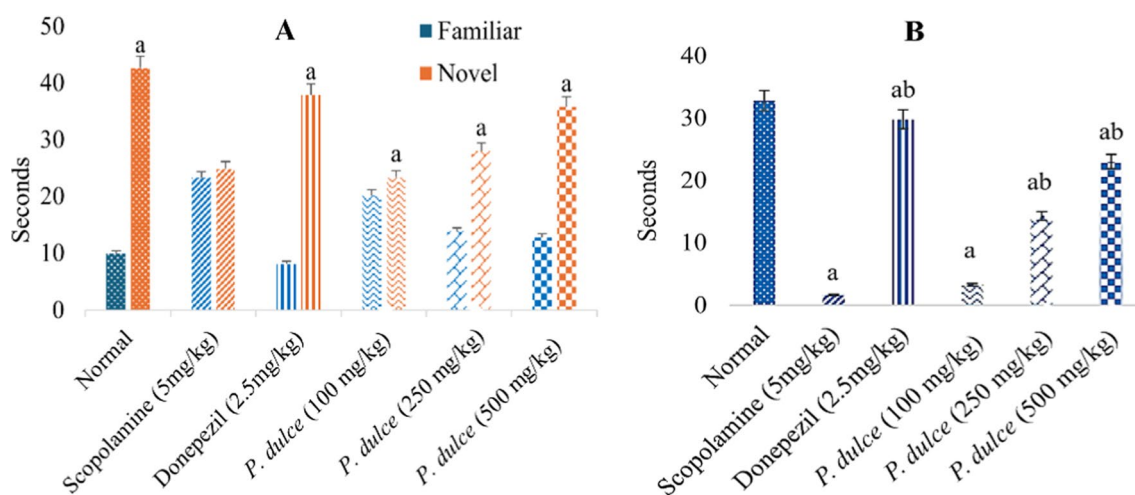


Fig. 3 **A** Phase 3 memory test. **B** NOR difference score memory test

In vitro acetylcholinesterase activity

The inhibitory effect of acetylcholinesterase (AChE) was determined using an enzyme-catalyzed kinetic reaction according to the method of [13]. The percent inhibition of AChE activity using 100 µg of the extract was 77%, and the IC_{50} was 19.23 ± 1.02 µg/ml. The experiment results are shown in (Fig. 2) and carried out using donepezil as a positive control (reference anti-Alzheimer's drug).

In vivo acute toxicity

Oral administration of *P. dulce* extract at doses of 1000, 2000, and 3000 mg/kg body weight resulted in 0% mortality, while doses of 4000 and 5000 mg/kg resulted in 16.67% and 33.3% mortality, respectively (one rat died with the 4000 mg/kg dose, and two rats died with the 5000 mg/kg dose).

Considering the minimal toxicity profile of the *P. dulce* extract, doses of 100, 250, and 500 mg/kg (less than 1/10 of the 5000 mg/kg dose) were approved for subsequent in vivo experiments.

In vivo anti-Alzheimer's activity

Novel object recognition (NOR) test

P. dulce extract administration enhanced memory function in a dose-dependent fashion. The actual duration of time the rats spent investigating the familiar and novel objects during phase 3 of the experiment is depicted in Fig. 3A. Moreover, the difference in the scores for the time spent investigating the familiar and novel objects ($T_2 - T_1$) is shown in Fig. 3B.

Effect of *P. dulce* extract on AChE activity and ACh concentration

The ACh concentration (Fig. 4) was substantially decreased in the disease group (1.3 ± 0.31 nmol/mg) when compared to the normal group (7.3 ± 0.47 nmol/mg) ($p < 0.05$). Meanwhile, in proportion to the dose, *P. dulce* extract increased the ACh concentration comparable to the disease group.

AChE activity (Fig. 4) was the lowest in the donepezil group (0.170 ± 0.016 U/mg) and normal group (0.180 ± 0.03 U/mg), while the disease group was the highest (0.870 ± 0.074 U/mg). The extract groups significantly inhibited the activity in a manner that was dependent on concentration (0.93 ± 0.13 U/mg) for the *P. dulce* 100 mg/kg dose and (0.22 ± 0.31 U/mg) for the *P. dulce* 500 mg/kg dose. Thus, the AChE activity was effectively inhibited by *P. dulce* extract administration.

Effect of *P. dulce* extract on DA and NA concentrations

The level of DA was significantly reduced using the extract (500 mg/kg) dose (19.7 ± 2.7 ng/mg) compared with the standard group (22.9 ± 2.8 ng/mg) ($p < 0.05$) (Fig. 4).

NA level (Fig. 4) was the lowest in the normal group (5.7 ± 0.9 Pg/mg). In the extract-treated group at the 500 mg/kg dose, the level of NA was significantly decreased (9.3 ± 1.6 Pg/mg), while for the *P. dulce* 100 mg/kg dose, it was ineffective (58.28 ± 2.15 Pg/mg), which was very close to the disease group. Thus, the higher extract dose (500 mg/kg) could effectively decrease the levels of DA and NA.

Effect of *P. dulce* extract on GSH and MDA concentrations

The potency of *P. dulce* extract was able to tip the balance of the antioxidant GSH over the oxidative stressor MDA. The GSH level was significantly increased in the groups treated with the extract in a concentration-dependent manner (Fig. 4), where it was (2.34 ± 0.29 µg/mg) in the *P. dulce* 100 mg/kg dose group and (7.23 ± 0.53 µg/mg) in the 500 mg/kg dose group, which was markedly greater than the standard group (4.982 ± 0.249 µg/mg).

MDA levels in the *P. dulce* 250 and 500 mg/kg dose groups (34.69 ± 3.04 ng/mg) and (22.05 ± 4.22 ng/mg), respectively, were lower than the donepezil-treated group (39.69 ± 2.83 ng/mg) (Fig. 4). These findings indicated that the extract markedly inhibited MDA production compared to the donepezil and normal groups.

Effect of *P. dulce* extract on TNF-α and IL-1β concentrations

The normal group and the donepezil group had significantly lower levels of *IFN-α* and *IL-1β* than the other groups (Fig. 4). The 100 mg/kg dose yielded a concentration of (156 ± 5.9 Pg/mg) for *IL-1β* and (153 ± 11.9 Pg/mg) for *IFN-α*, which were decreased with the 500 mg/kg dose to (123.3 ± 3.7 Pg/mg) and (109.2 ± 5.7 Pg/mg), respectively. As the concentration of the extract increased, levels of *IFN-α* and *IL-1β* diminished; however, they remained elevated compared to the standard group (81.34 ± 7.6 Pg/mg) and (87.48 ± 8.7 Pg/mg), respectively.

In silico Study

Molecular dynamics and system stability

The molecular dynamic simulation (MD) was established to forecast, through simulation, the interaction between the identified compounds and the protein's active site and its stability [72, 73]. System stability must be validated to avoid artifacts and spot the interrupted motions during the simulation. RMSD (root-mean-square deviation)

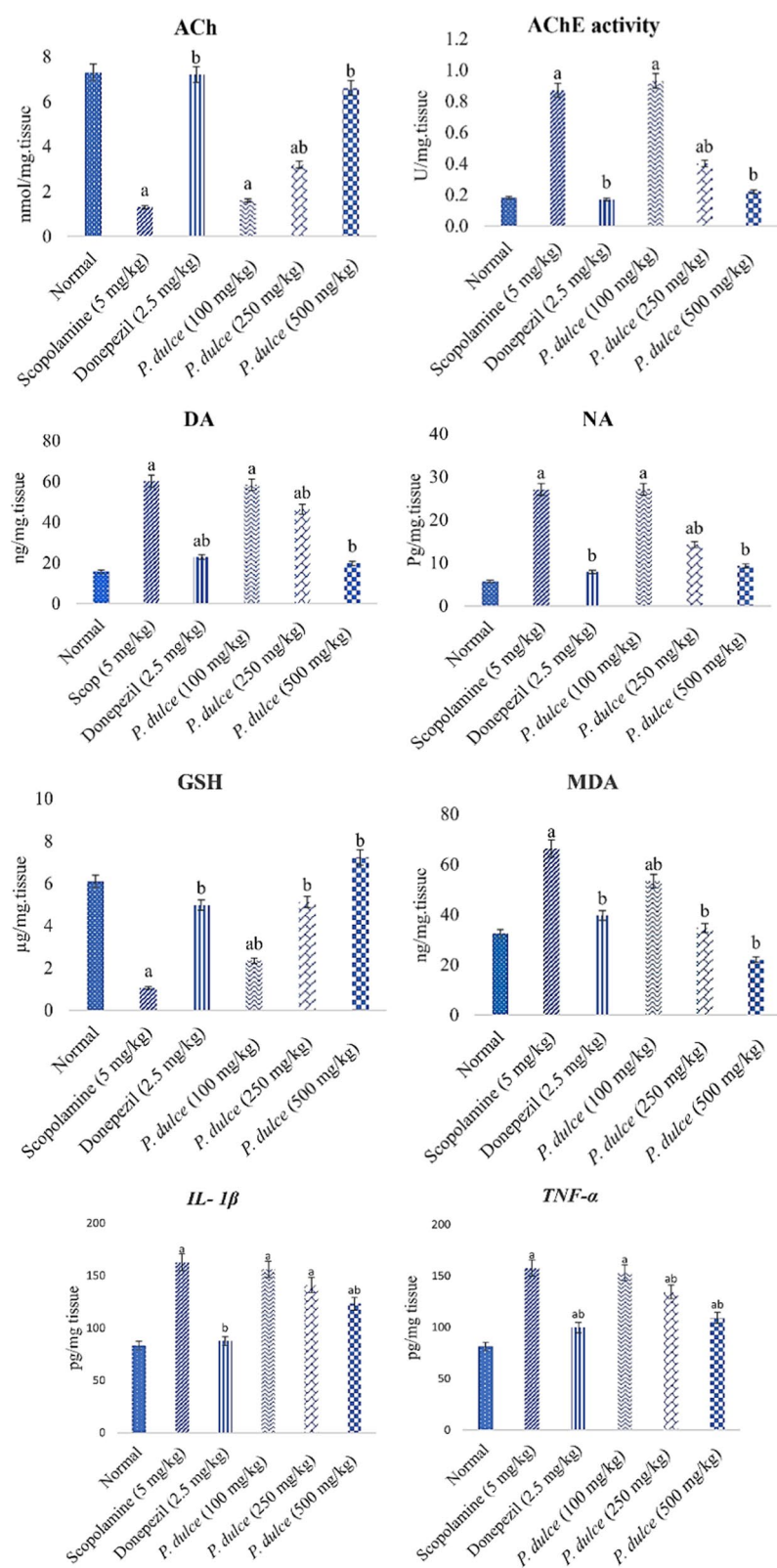


Fig. 4 Effect of different doses of *P. dulce* extract on the memory function parameters ACh, AChE activity, DA, and NA; the oxidative stress parameters MDA and GSH; and the anti-inflammatory parameters *TNF-α* and *IL-1β*

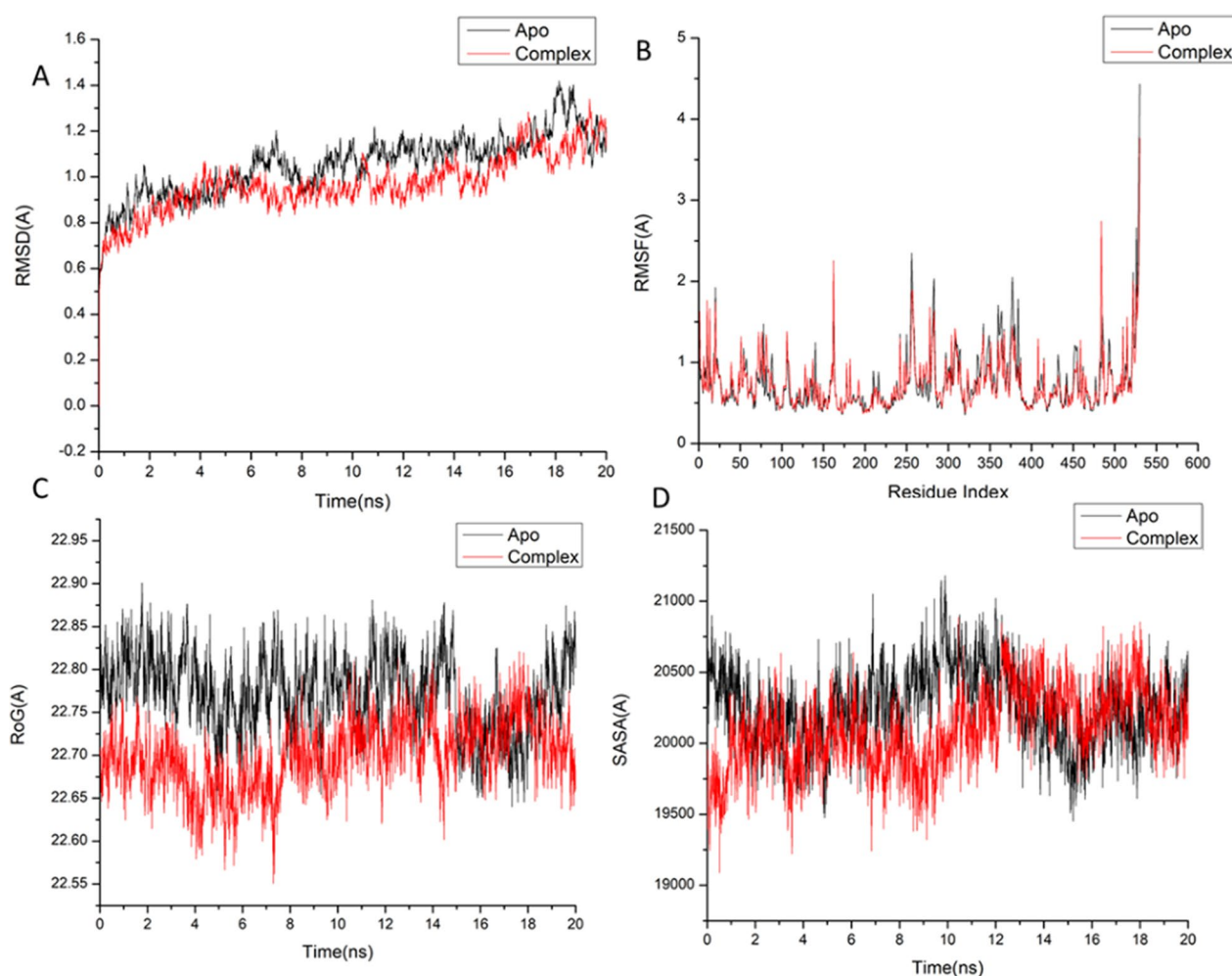


Fig. 5 **A** Ca-atoms RMSD of the protein backbone atoms. **B** Residues RMSF of the protein backbone Ca atoms. **C** ROG of Ca atoms of protein residues. **D** SASA of the Ca of the backbone atoms relative to the starting

was utilized in this study to assess the system stability throughout the 20 ns simulation. For the apo-protein and 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone-AChE systems, the average RMSD values were 1.05 ± 0.13 Å and 0.98 ± 0.13 Å, respectively (Fig. 5A). According to these findings, the 5, 7, 3', 4', 5' pentahydroxy-3,6,8-tri-methoxy flavone coupled to the protein complex system (flavone-protein complex) exhibited a more stable conformation in comparison with the other systems.

In MD simulation, analyzing residue connection to the ligand and its behavior requires assessing the flexibility of the protein structure following ligand binding [79]. The RMSF (Root-Mean-Square Fluctuation) method was utilized to evaluate the protein residue variations and assess the inhibitor binding impact on the appropriate targets during 20-ns simulations. For all apo-protein frames and 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy

flavone-AChE systems, the calculated RMSF average values were 0.75 ± 0.38 Å and 0.73 ± 0.33 Å, respectively (Fig. 5B). The flavone-protein complex showed residue fluctuation reduction compared to the other systems.

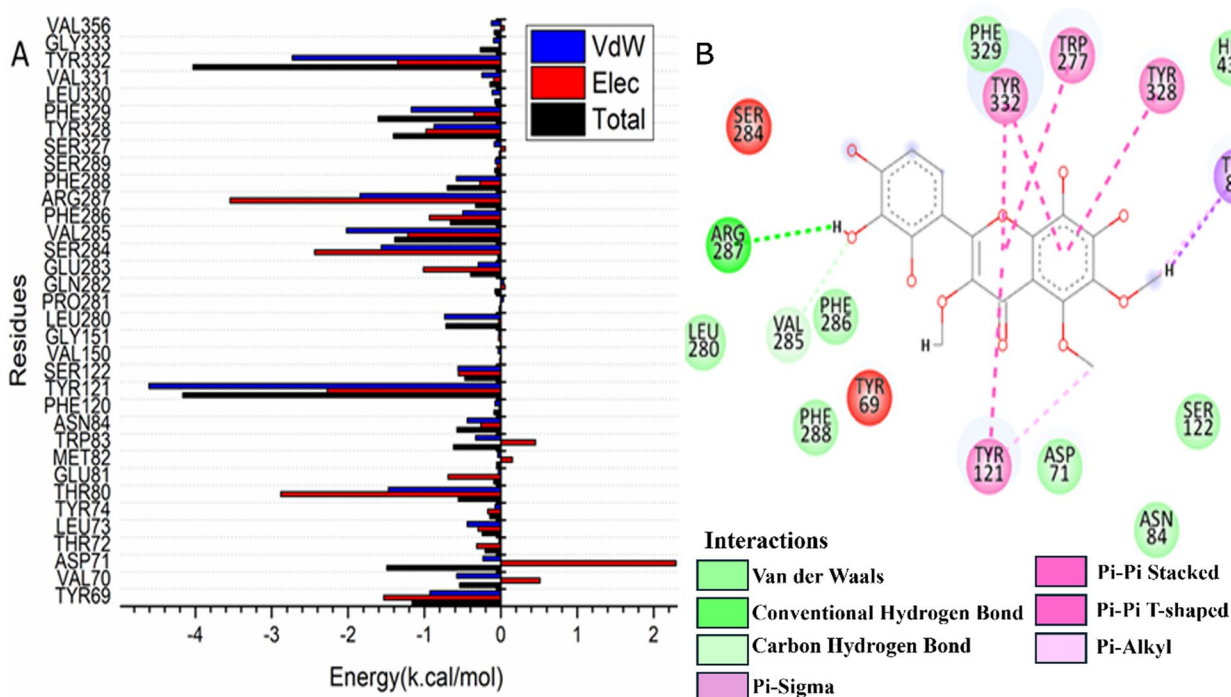
Throughout the MD simulation, ROG was used to analyze the overall system compactness and the ligand binding stability [80, 81]. The Rg averages for the apo-protein and 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone-AChE systems were 22.70 ± 0.04 °C and 22.00 ± 0.041 °C, respectively (Fig. 5C). Based on observed behavior, the flavone-protein complex possesses a very rigid structure against the target receptor's catalytic binding site.

The protein hydrophobic core compactness and the stability of the biomolecule were investigated using SASA (solvent-accessible surface area of protein) [82]. SASA averaged 20,264.44 Å for the apo-protein system and 20,099.98 Å for the 5, 7, 3', 4', 5'-pentahydroxy-3,

Table 2 Binding energy calculations for the 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone compound toward the catalytic binding site receptor. of AChE

Complex	Energy components (kcal/mol)				
	ΔE_{vdW}	ΔE_{elec}	ΔG_{gas}	ΔG_{solv}	ΔG_{bind}
5,7,3',4',5'-Penta- hydroxy-3,6,8-tri-methoxy flavone—AChE	-50.82 ± 0.99	-35.91 ± 1.57	-86.73 ± 1.38	44.22 ± 0.79	-42.51 ± 1.03

van der Waals energy (ΔE_{vdW}); electrostatic energy (ΔE_{elec}); solvation free energy (ΔG_{solv}); calculated binding free energy (ΔG_{bind})

**Fig. 6** **A** Per-residue decomposition graphs illustrating the energy contributions to 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone binding and stability inside the AChE receptor catalytic binding site, **B** corresponding inter-molecular interactions

6, 8-tri-methoxy flavone-AChE system (Fig. 5D). Combining the results from RMSD, ROG, RMSE, and SASA computations demonstrates the intactness of the flavone-protein complex within the target receptor's catalytic binding site.

Binding interaction mechanism based on binding free energy calculation

The molecular mechanics energy program (MM/GBSA) integrates the generalized Born solvation and surface area continuum, which is a common way to figure out the small compounds' free binding energies to biological macromolecules [83]. It may be much more precise than docking scores. Utilizing AMBER18's MM-GBSA program, snapshots of the systems' trajectories were used to calculate the binding free energies. Table 2 suggests

favorable interactions due to substantial negative values of all the computed energy components except the ΔG_{solv} .

The compound 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone and the target protein receptor residues interact due to the greater positive van der Waals energy component, which was demonstrated by a thorough analysis of each energy contribution that results in the observed binding free energies. (Table 2).

Identification of the critical residues responsible for ligand binding

The total energy associated with the compound's binding to these enzymes was further decomposed into the contributions of each site residue, hence enhancing the knowledge of the critical residues implicated in inhibiting the catalytic binding site receptor. Figure 6

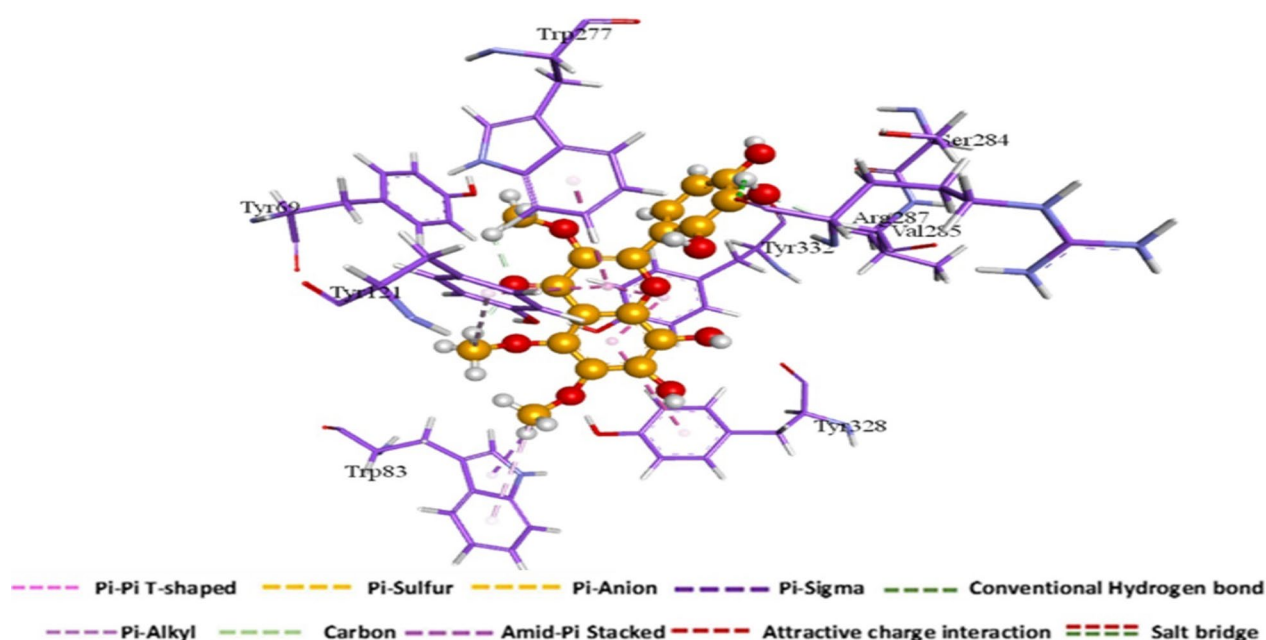


Fig. 7 The interaction residue of 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone into the catalytic binding site of AChE receptor

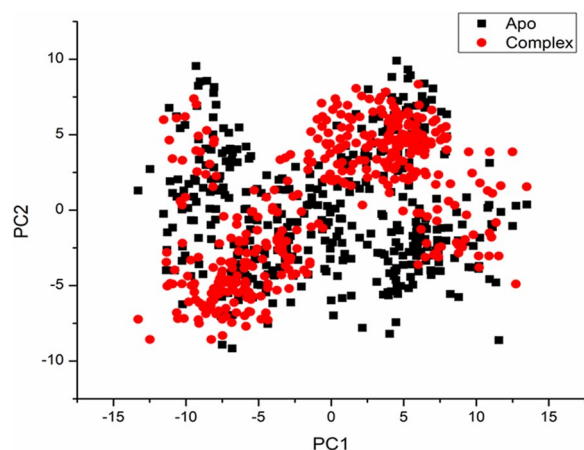


Fig. 8 PCA projection of Ca atoms movement created by plotting the PC1 against PC2 in conformational space

demonstrates that the most favorable contribution of 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone compound to the AChE catalytic binding site of the receptor is mostly noted from Tyr 69 (−0.93 kcal/mol), Val 70 (−0.577 kcal/mol), Asp 71 (−0.233 kcal/mol), Leu 73 (−0.442 kcal/mol), Thr 80 (−1.472 kcal/mol), Trp 83 (−0.329 kcal/mol), Asn 84 (−0.439 kcal/mol), Tyr 121 (−4.606 kcal/mol), Ser122 (−0.56 kcal/mol), Leu 280 (−0.736 kcal/mol), Glu283 (−0.296 kcal/mol), Ser 284 (−1.566 kcal/mol), Val 285 (−2.021 kcal/mol), Phe286 (−0.5 kcal/mol), Arg 287 (−1.842 kcal/mol), Phe 288 (−0.58 kcal/mol), Tyr 328 (−0.873 kcal/mol),

Phe 329 (−1.174 kcal/mol), Val 331 (−0.249 kcal/mol), and Tyr 332 (−2.731 kcal/mol) residues.

Ligand–residue interaction network profiles (5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone—AChE complex)

The 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone compound exhibits electrostatic and hydrophobic structural interactions in the AChE receptor catalytic binding site.

The hydroxyl group of 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone extended into the catalytic active site and formed hydrogen bonds with Arg 287, as shown in Fig. 7. The chromen-ring was allowed to develop Pi-pi stacking with Trp 277, Tyr 328, and Tyr 332 due to the size of 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone. Finally, the Pi-sigma interaction has been formed between Trp 83 and the hydroxyl group.

Principal component analysis (PCA)

The PCA plot (Fig. 8) illustrated complex and clear conformation waves along the two main components in significant subspace. There was an obvious movement difference in the apo-protein and flavone–protein complex systems. The calculated eigenvector from the 20-ns MD trajectories for the three systems exhibited significant variability, reflecting the variation in protein movement between the systems.

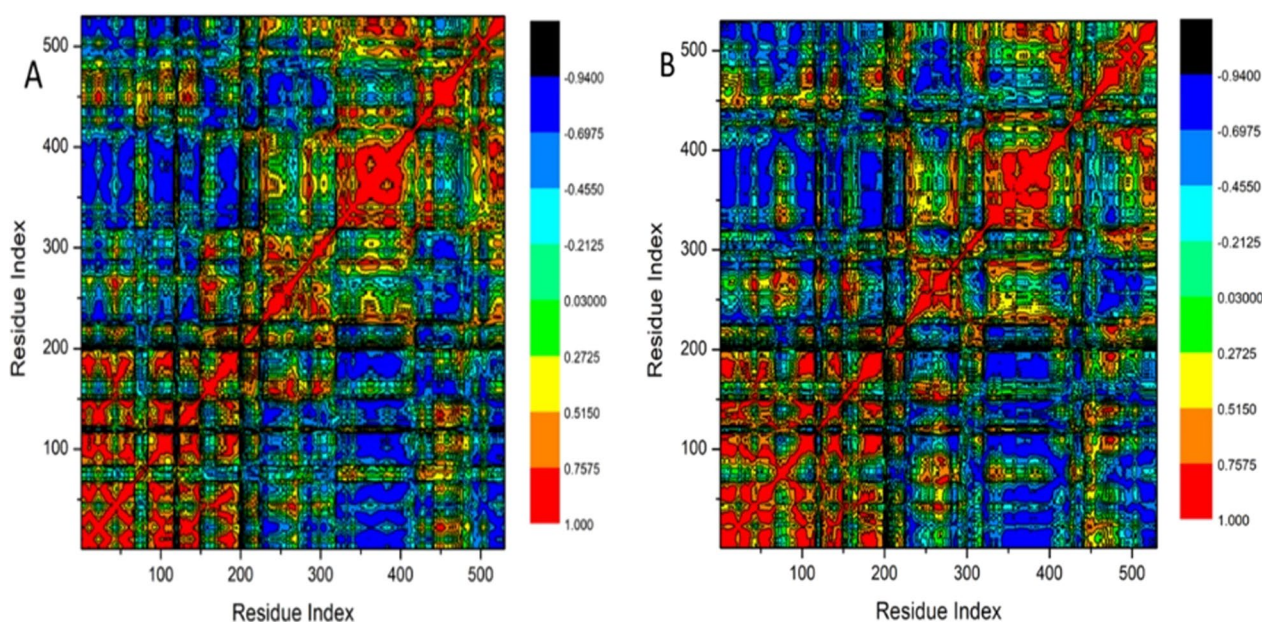


Fig. 9 A Free enzyme DCCM matrix analyses, B 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone—complex systems

Since the apo system had more atomic fluctuations than the flavone–protein complex, likely, the ligand binding to the flavone–protein complex active sites caused conformational dynamics, this was then reflected as PCs wave of motion. The flavone–protein complex was more densely packed than the other systems; hence, when 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone bound to the protein, conformational flexibility is reduced, and ligand binding to the active site increased.

Dynamics cross-correlation matrices (DCCM) analysis

The DCCM analysis was done on the α location throughout the simulations to evaluate the AChE conformational changes after 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone ligand interaction and to analyze the kinetics and existence of the associated motions (Fig. 9). Blue-black (color) regions show extremely negative-correlated motions of certain residues, while yellow and red-colored regions show significantly positive-correlated motions of some residues.

The total residue-correlated motions were compared to anti-correlated motions in the systems under investigation for this study. The binding of 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone to AChE proteins (Fig. 9) provides the protein's structural dynamics, resulting in conformational alterations that are represented by associated motion variation according to an examination of DCCM. The AChE protein's residues 0–100 and 300–400 contain the significantly correlated region. Being the

most dynamic parts of the receptor, the majority of the hydrophobic active site residues got taken in by these regions.

Discussion

The present study was made to discover a new medicinal use of *P. dulce* through in vitro, in vivo, and in silico studies since it contains many phytoconstituents that have been proven to possess a neuroprotective effect. Different phytochemical classes such as flavonoids, phenolic acids, tannins, amino acids, and alkaloids have been identified in *P. dulce* as previously reported [1]. Many of these phytoconstituents were proven to possess a neuroprotective effect through different mechanisms. Flavonoids promote the neurons' growth, regeneration, and function and prevent neural abnormalities. Furthermore, they enhance memory and learning abilities and have antioxidant, anti-inflammatory, and anti-AChE properties [84]. Phenolic acids protect the brain by reducing oxidative stress, increasing antioxidant and anti-inflammatory production, preventing protein aggregation, and inhibiting cell death signaling pathways [85]. Linolenic acid [86] and α -amyryn [87] could be promising treatment options for Alzheimer's by inhibiting oxidative stress and inflammatory parameters and reducing AChE levels. Alkaloids are the most promising natural products for AD treatment, because of their nitrogen-containing structures. The prototype inhibitor of AChE, physostigmine, was isolated from a plant and used to synthesize its analog

rivastigmine. Also, galantamine is an alkaloid that is presently marketed to alleviate symptoms of AD [88].

The in vitro study demonstrated that *P. dulce* extract was able to inhibit 77% of AChE activity, with an IC_{50} value higher than the positive control.

The in vivo study used scopolamine to induce Alzheimer's, as many neurobehavioral studies have demonstrated that scopolamine can lead to synaptic dysfunction, oxidative stress, amyloid deposition, and memory/learning deficits in both humans and animals [16], which has been related to a disturbance in ACh, AChE activity, oxidative markers like MDA, and GSH neuro-inflammatory markers like *IL-1 β* , and (*TNF- α*) levels in the brain [89], and increased hippocampal DA and NA levels [90]. The NOR test was used to assess the recovery of cognitive memory deficit, which revealed that *P. dulce* was able to enhance the memory function by decreasing the duration of time spent investigating the familiar and novel in a dose-dependent manner but to a lesser extent than donepezil group. *P. dulce* 500 mg/kg dose showed ACh, DA, NA, and AChE activity levels closer to normal and standard groups. Also, the lowest MDA and the highest GSH levels were detected with the same extract dose. The levels of *IL-1 β* and *TNF- α* were reduced; however, they were higher than the standard group. When all these results were compared to the normal, disease, and standard groups, it was found that *P. dulce* extract possesses a significant effect on the memory function parameters (ACh, AChE, DA & NA) and the oxidative stress parameters (GSH & MDA) with a lesser extent on the anti-inflammatory parameters (*IL-1 β* & *TNF*).

In the molecular docking study, AChE was chosen based on its ability to break down the neurotransmitter ACh, which is vital in neurotransmission regulation at synapses throughout the nervous system [7]. Consequently, researchers have thoroughly studied cholinesterase inhibitors such as donepezil, galantamine, huperzine A, and rivastigmine as symptomatic therapies for AD. Cholinergic neurotransmission defects are significant for the progression of AD [8]. Sixteen of the identified compounds were tested in this study for possible interaction with the AChE enzyme (Table S1) based on the supposed neuroprotective activity reported for their phytochemical classes [84–86]. 5, 7, 3', 4', 5'-Pentahydroxy-3, 6, 8-trimethoxyflavone, 4''-O-methyl epigallocatechin-3-O-gallate, kaempferol-di-O-rhamnoside, di-O-caffeoylquinic acid, dihydroxy benzoic acid methyl ester hexoside, catchine, rhamnosyl hexosyl methyl quercetin, and kaempferol-3-O-rhamnoside gave the higher binding energy S-scores, respectively. Different computational modeling methods were employed on 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxyflavone as it achieved the highest binding energy s-score, including principal

component analysis, ligand–residue interaction, dynamics cross-correlation matrices analysis, and thermodynamics calculation. This revealed that the most active flavone exhibited electrostatic and hydrophobic structural interactions in the AChE receptor catalytic binding site. The hydroxyl group of the flavone extended into the catalytic active site and formed hydrogen bonds with Arg 287, and due to its size, the chromen-ring was allowed to develop Pi-pi stacking with Trp 277, Tyr 328, and Tyr 332. Finally, the Pi-sigma interaction has been formed between Trp 83 and the hydroxyl group. The binding of the flavone to the ACh protein decreased the fluctuation and influenced the ligand optimum orientation on the AChE protein conformational space. Additionally, the drug binding energy of AChE and the residue correlation in the 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-tri-methoxy flavone-ACh system was increased.

Conclusion

In this study, the *P. dulce* leaves methanolic extract exhibited neuroprotective and anti-amnesic properties, which were evaluated in vitro and in vivo against scopolamine-induced cognitive decline and cerebral damage, as well as in silico experiments. This activity may result from the extract's ability to inhibit AChE activity, reduce DA, and NA levels, and elevate ACh level, also for its antioxidant properties by decreasing MDA and increasing GSH levels, and the anti-inflammatory properties by lowering *TNF- α* and *IL-1 β* levels. UPLC-ESI-MS/MS in positive and negative modes was employed to identify 66 metabolites belonging to numerous chemical classes such as flavonoids, phenolic acids, fatty acids, sterols, amino acids, and alkaloids that might be responsible for this activity. The in silico study was carried out to indicate the most active compounds and their mechanisms of action. The compound identified as 5, 7, 3', 4', 5'-pentahydroxy-3, 6, 8-trimethoxy flavone was the most effective AChE inhibitor, indicating that it is a safe and effective natural treatment for Alzheimer's complications. In addition to clinical trials, further studies may be required to confirm this postulation and introduce this drug as an approved therapy.

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer disease
DA	Dopamine
ELISA	Enzyme-linked immunoassay
GSH	Glutathione
<i>IL-1β</i>	Interleukin-1 β
MDA	Malondialdehyde
NA	Noradrenaline
NOR	Novel object recognition
<i>TNF-α</i>	Tumor necrotic Factor- α

Xevo TQD Triple quadrupole mass spectrometry
 OECD Organization for economic co-operation and development
 UPLC-ESI-MS/MS Liquid chromatography-electrospray ionization- mass spectrometry

Supplementary Information

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Additional file 1.

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Author contributions

A.A.H. contributed to phytochemical study, data curation, formal analysis, writing original draft and editing. A.M.F. contributed to supervision, conceptualization, reviewing and editing. A.A.R. contributed to in silico study and software. A.M.F. contributed to in vivo study. M.A.A.F. and E.M. contributed to methodology, supervision, conceptualization, formal analysis, writing original draft, reviewing and editing. All authors approved the final manuscript.

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Availability of data and materials

This article and its supplementary information file include all data generated and analyzed during this study.

Declarations

Ethics approval and consent to participate

All experiments and methodologies followed the applicable national and international regulations and standards of the Faculty of Pharmacy, University of Sadat City, Sadat City, Egypt. The *in vivo* protocol was accepted by the Ethical Committee of the Faculty of Pharmacy Cairo University (FOPCU), Egypt, with ethical approval no. MP (4149) and in compliance with the National Institutes of Health's publication of the National Academy of Sciences Handbook for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. El-hewehy A, Mohsen E, El-fishawy MA, Fayed MAA (2024) Traditional, phytochemical, nutritional and biological importance of *Pithecellobium dulce* (Roxb.) Benth. *Yüzüncü Yıl Üniv Tarım Bilimleri Dergisi* 34:354–380. <https://doi.org/10.29133/yyutbd.1329407>
2. Reddy K, Kumar KVP, Baig MS (2022) Nutritional, health and therapeutic benefits of *Pithecellobium dulce*. *Agriculture and od* 04:231–233
3. Narsing Rao G (2013) Physico-chemical, mineral, amino acid composition, in vitro antioxidant activity and sorption isotherm of *Pithecellobium dulce* L. Seed Protein Flour. *J Food PharmSci* 1:74–80. <https://doi.org/10.14499/jfps>
4. Murugesan S, Lakshmanan DK, Arumugam V, Alexander RA (2019) Nutritional and therapeutic benefits of medicinal plant *Pithecellobium dulce* (Fabaceae): a review. *J Appl Pharm Sci* 9:130–139. <https://doi.org/10.7324/JAPS.2019.90718>
5. Kumar A, Sidhu J, Goyal A, Tsao JW (2022) Alzheimer Disease. StatPearls Publishing, Treasure Island (FL)
6. Breyjeh Z, Karaman R (2020) Comprehensive review on Alzheimer's disease: causes and treatment. *Molecules* 25:5789
7. Choi JH, Lee EB, Jang HH et al (2021) *Allium hookeri* extracts improve scopolamine-induced cognitive impairment via activation of the cholinergic system and anti-neuroinflammation in mice. *Nutrients*. <https://doi.org/10.3390/nu13082890>
8. Mehta M, Adem A, Sabbagh M (2012) New acetylcholinesterase inhibitors for Alzheimer's disease. *Int J Alzheimers Dis* 2012:728983. <https://doi.org/10.1155/2012/728983>
9. Fanlo-Ucar H, Picón-Pagès P, Herrera-Fernández V et al (2024) The Dual role of amyloid beta-peptide in oxidative stress and inflammation: unveiling their connections in Alzheimer's disease etiology. *Antioxidants* 13:1208
10. Firdous SM, Khan SA, Maity A (2024) Oxidative stress-mediated neuroinflammation in Alzheimer's disease. *Naunyn Schmiedeberg's Arch Pharmacol* 397:8189–8209. <https://doi.org/10.1007/s00210-024-03188-3>
11. Bhat BA, Almilaibary A, Mir RA et al (2022) Natural therapeutics in aid of treating Alzheimer's disease: a green gateway toward ending quest for treating neurological disorders. *Front Neurosci*. <https://doi.org/10.3389/fnins.2022.884345>
12. Ayoub IM, George MY, Menze ET et al (2022) Insights into the neuroprotective effects of *Salvia officinalis* L. and *Salvia microphylla* Kunth in the memory impairment rat model. *Food Funct* 13:2253–2268. <https://doi.org/10.1039/D1FO02988F>
13. Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95. [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9)
14. Ahammed S, Afrin R, Uddin N et al (2021) Acetylcholinesterase inhibitory and antioxidant activity of the compounds isolated from *Vanda roxburghii*. *Adv Pharmacol Pharm Sci*. <https://doi.org/10.1155/2021/5569054>
15. Bhuvanendran S, Kumari Y, Othman I, Shaikh MF (2018) Amelioration of cognitive deficit by embelin in a scopolamine-induced Alzheimer's disease-like condition in a rat model. *Front Pharmacol*. <https://doi.org/10.3389/fphar.2018.00665>
16. Chen C, Li X-H, Zhang S et al (2014) 7,8-Dihydroxyflavone ameliorates scopolamine-induced Alzheimer-like pathologic dysfunction. *Rejuvenation Res* 17:249–254. <https://doi.org/10.1089/rej.2013.1519>
17. Lueptow LM (2017) Novel object recognition test for the investigation of learning and memory in mice. *JoVE* 126:e55718. <https://doi.org/10.3791/55718>
18. Cheung J, Rudolph MJ, Burshteyn F et al (2012) Structures of human acetylcholinesterase in complex with pharmacologically important ligands. *J Med Chem* 55:10282–10286. <https://doi.org/10.1021/jm300871x>
19. Pettersen EF, Goddard TD, Huang CC et al (2004) UCSF Chimera—A visualization system for exploratory research and analysis. *J Comput Chem* 25:1605–1612. <https://doi.org/10.1002/jcc.20084>
20. Navo CD, Jiménez-Osés G (2021) Computer prediction of pKa values in small molecules and proteins. *ACS Med Chem Lett* 12:1624–1628
21. Halford B (2015) Reflections on chemdraw. *Chem Eng News Arch* 92:26–27. <https://doi.org/10.1021/cen-09233-scitech1>
22. Hanwell M, Curtis D, Lonie D et al (2012) Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *J Chem Inf* 4:17. <https://doi.org/10.1186/1758-2946-4-17>

23. Trott O, Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31:455–461. <https://doi.org/10.1002/jcc.21334>
24. Bikadi Z, Hazai E (2009) Application of the PM6 semi-empirical method to modeling proteins enhances docking accuracy of AutoDock. *J Cheminform* 1:1–16. <https://doi.org/10.1186/1758-2946-1-15/FIGURES/3>
25. Huey R, Molecular GM-TSRI, (2006) Undefined using autodock with autodocktools: a tutorial. Researchgate.net
26. Morris GM, Goodsell DS, Halliday RS et al (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem* 19(14):1639–1662
27. Hospital A, Goñi JR, Orozco M, Gelpi JL (2015) Molecular dynamics simulations: advances and applications. *Adv Appl Bioinforma Chem* 8:37–47
28. Lee TS, Cerutti DS, Mermelstein D et al (2018) GPU-accelerated molecular dynamics and free energy methods in Amber18: Performance enhancements and new features. *J Chem Inf Model* 58:2043–2050. <https://doi.org/10.1021/acs.jcim.8b00462>
29. Watanabe C, Watanabe H, Okiyama Y, Takaya D (2021) Development of an automated FMO calculation protocol to construction of FMO database. In: Mochizuki Y, Tanaka S, Fukuzawa K (eds) Recent advances of the fragment molecular orbital method: Enhanced performance and applicability. Springer, Singapore, pp 183–203
30. Stroet M, Setz M, Lee T et al (2024) On the validation of protein force fields based on structural criteria. *J Phys Chem B* 128:4602–4620. <https://doi.org/10.1021/acs.jpcc.3c08469>
31. Roe D, Cheatham T (2013) PTRAJ and CPPTRAJ: software for processing and analysis of molecular dynamics trajectory data. *J Chem Theory Comput* 9:3084–3095. <https://doi.org/10.1021/ct400341p>
32. Seifert E (2014) OriginPro 9.1: scientific data analysis and graphing software—software review. *J Chem Inf Model* 54:1552. <https://doi.org/10.1021/ci500161d>
33. Song LF, Lee TS, Zhu C et al (2019) Using AMBER18 for relative free energy calculations. *J Chem Inf Model* 59:3128–3135. <https://doi.org/10.1021/acs.jcim.9b00105>
34. Yllauri M, Pentikäinen OT (2013) MMGBSA as a tool to understand the binding affinities of Filamin–peptide interactions. *J Chem Inf Model* 53:2626–2633. <https://doi.org/10.1021/ci4002475>
35. Hayes JM, Archontis G (2012) MM-GB(PB)SA calculations of protein-ligand binding free energies. In: Wang L (ed) *Molecular Dynamics*. IntechOpen, Rijeka
36. Hou T, Wang J, Li Y, Wang W (2011) Assessing the performance of the MM/PBSA and MM/GBSA methods. 1. The accuracy of binding free energy calculations based on molecular dynamics simulations. *J Chem Inf Model* 51:69–82. <https://doi.org/10.1021/ci100275a>
37. Greenidge P, Kramer C, Mozziconacci J-C, Wolf R (2012) MM/GBSA binding energy prediction on the PDBbind data set: successes, failures, and directions for further improvement. *J Chem Inf Model*. <https://doi.org/10.1021/ci300425v>
38. Fossat MJ, Zeng X, Pappu RV (2021) Uncovering differences in hydration free energies and structures for model compound mimics of charged side chains of amino acids. *J Phys Chem B* 125:4148–4161. <https://doi.org/10.1021/acs.jpcc.1c01073>
39. Jang GH, Kim HW, Lee MK et al (2018) Characterization and quantification of flavonoid glycosides in the *Prunus* genus by UPLC-DAD-QTOF/MS. *Saudi J Biol Sci* 25:1622–1631. <https://doi.org/10.1016/j.sjbs.2016.08.001>
40. Ezzat MI, Issa MY, Sallam IE et al (2022) Impact of different processing methods on the phenolics and neuroprotective activity of *Fragaria ananassa* Duch. extracts in a α -galactose and aluminum chloride-induced rat model of aging. *Food Funct* 13:7794–7812. <https://doi.org/10.1039/d2fo00645f>
41. Leng Z, Zhong B, Wu H et al (2022) Identification of phenolic compounds in australian-grown bell peppers by liquid chromatography coupled with electrospray ionization-quadrupole-time-of-flight-mass spectrometry and estimation of their antioxidant potential. *ACS Omega* 7:4563–4576. <https://doi.org/10.1021/acsomega.1c06532>
42. Jakopic J, Petkovsek MM, Likozar A et al (2011) HPLC-MS identification of phenols in hazelnut (*Corylus avellana* L.) kernels. *Food Chem* 124:1100–1106. <https://doi.org/10.1016/j.foodchem.2010.06.011>
43. Jiang C, Gates PJ (2024) Systematic characterisation of the fragmentation of flavonoids using high-resolution accurate mass electrospray tandem mass spectrometry. *Molecules*. <https://doi.org/10.3390/molecules29225246>
44. Mekky RH, Abdel-Sattar E, Segura-Carretero A, Contreras MDM (2021) Metabolic profiling of the oil of sesame of the Egyptian cultivar ‘Giza 32’ employing LC-MS and tandem MS-based untargeted method. *Foods*. <https://doi.org/10.3390/foods10020298>
45. Lachowicz S, Oszmiński J, Rapak A, Ochmian I (2020) Profile and content of phenolic compounds in leaves, flowers, roots, and stalks of *Sanguisorba officinalis* L. Determined with the LC-DAD-ESI- QTOF-MS/MS analysis and their *in vitro* antioxidant, antidiabetic, antiproliferative potency. *Pharmaceuticals* 13:1–22. <https://doi.org/10.3390/ph13080191>
46. Razgonova M, Zakharenko A, Pikula K et al (2021) LC-MS/MS screening of phenolic compounds in wild and cultivated grapes *Vitis amurensis* Rupr. *Molecules*. <https://doi.org/10.3390/molecules26123650>
47. Hamdan DI, Fayed MAA, Adel R (2021) *Echinops taekholmiana* Amin: optimization of a tissue culture protocol, biological evaluation, and chemical profiling using GC and LC-MS. *ACS Omega* 6:13105–13115. <https://doi.org/10.1021/acsomega.1c00837>
48. Magdy M, Elosaily AH, Mohsen E, EL Hefnawy HM (2024) Chemical profile, antioxidant and anti-Alzheimer activity of leaves and flowers of *Markhamia lutea* cultivated in Egypt: *in vitro* and *in silico* studies. *Futur J Pharm Sci*. <https://doi.org/10.1186/s43094-024-00679-1>
49. Kotb SS, Ayoub IM, El-Moghazy SA, Singab AN (2022) Phytochemical analysis of *Pithecellobium dulce* (Roxb) Benth Bark via UPLC-ESI-MS/MS and evaluation of its biological activity. *Nat Prod Res*. <https://doi.org/10.1080/14786419.2022.2140153>
50. Ben SR, Hamed AI, Mahalel UA et al (2017) Tentative characterization of polyphenolic compounds in the male flowers of *Phoenix dactylifera* by liquid chromatography coupled with mass spectrometry and DFT. *Int J Mol Sci*. <https://doi.org/10.3390/ijms18030512>
51. Symma N, Hensel A (2022) Advanced analysis of oligomeric proanthocyanidins: latest approaches in liquid chromatography and mass spectrometry based analysis. *Phytochem Rev* 21:809–833
52. Nadeem M, Mumtaz MW, Danish M et al (2020) Antidiabetic functionality of vitex negundo L. leaves based on UHPLC-QTOF-MS/MS based bioactive profiling and molecular docking insights. *Ind Crops Prod*. <https://doi.org/10.1016/j.indcrop.2020.112445>
53. Sobral F, Calhelha RC, Barros L et al (2017) Flavonoid composition and antitumor activity of bee bread collected in Northeast Portugal. *Molecules*. <https://doi.org/10.3390/molecules22020248>
54. Ali A, Bashmil YM, Cottrell JJ et al (2021) LC-MS/MS-QTOF screening and identification of phenolic compounds from australian grown herbs and their antioxidant potential. *Antioxidants*. <https://doi.org/10.3390/antiox10111770>
55. El Sayed AM, Basam SM, El-Naggar EIBA et al (2020) LC-MS/MS and GC-MS profiling as well as the antimicrobial effect of leaves of selected *Yucca* species introduced to Egypt. *Sci Rep*. <https://doi.org/10.1038/s41598-020-74440-y>
56. Huang G, Liang J, Chen X et al (2020) Isolation and identification of chemical constituents from Zhideke Granules by ultra-performance liquid chromatography coupled with mass spectrometry. *J Anal Methods Chem*. <https://doi.org/10.1155/2020/8889607>
57. Lu W, Kimball E, Rabinowitz JD (2006) A high-performance liquid chromatography-tandem mass spectrometry method for quantitation of nitrogen-containing intracellular metabolites. *J Am Soc Mass Spectrom* 17:37–50. <https://doi.org/10.1016/j.jasms.2005.09.001>
58. P.J. Linstrom, W.G. Mallard Eds (2023) NIST Chemistry WebBook, nist standard reference database number 69, national institute of standards and technology, Gaithersburg MD, 20899, (retrieved May 9, 2024). National Institute of Standards and Technology. <https://doi.org/10.18434/T4D303>. Accessed 9 May 2024
59. Vanitha V, Manikandan K (2016) Bio-activity guided determination of active compounds in the leaves of *Pithecellobium dulce*. *RASAYAN J Chem (RJC)* 9:471–477
60. Hamed AR, El-Hawary SS, Ibrahim RM et al (2021) Identification of chemopreventive components from halophytes belonging to Aizoaceae and Cactaceae through LC/MS - Bioassay guided approach. *J Chromatogr Sci* 59:618–626. <https://doi.org/10.1093/chromsci/bmaa112>
61. Khalil AM, Sabry OM, El-Askary HI et al (2024) Identification of cyclooxygenase-II inhibitory saponins from fenugreek wastes: insights from liquid chromatography–tandem mass spectrometry metabolomics, molecular

- networking, and molecular docking. *Phytochem Anal.* <https://doi.org/10.1002/pca.3322>
62. Younis IY, Sedeek MS, Essa AF et al (2025) Exploring geographic variations in quinoa grains: unveiling anti-Alzheimer activity via GC–MS, LC–QTOF–MS/MS, molecular networking, and chemometric analysis. *Food Chem.* <https://doi.org/10.1016/j.foodchem.2024.141918>
 63. Darwish RS, Shawky E, Nassar KM et al (2021) Differential anti-inflammatory biomarkers of the desert truffles *Terfezia clavari* and *Tirmania nivea* revealed via UPLC–QqQ–MS-based metabolomics combined to chemometrics. *LWT.* <https://doi.org/10.1016/j.lwt.2021.111965>
 64. Wiyono T, Frediansyah A, Sholikah EN, Pratiwi WR (2022) UHPLC–ESI–MS analysis of Javanese *Tamarindus indica* leaves from various tropical zones and their beneficial properties in relation to antiobesity. *J Appl Pharm Sci* 12:137–147. <https://doi.org/10.7324/JAPS.2022.120814>
 65. Tulloch AP (1985) Mass Spectra of TMS esters of deuterated decanoic acids and of tms ethers of deuterated decanols. *Lipids* 20:404–411
 66. Lolok N, Sumiwi SA, Sahidin I, Levita J (2023) Stigmasterol isolated from the ethyl acetate fraction of *Morinda citrifolia* fruit (using the bioactivity-guided method) inhibits α -amylase activity: *In vitro* and *in vivo* analyses. *World Acad Sci J.* <https://doi.org/10.3892/wasj.2023.202>
 67. Fiorentino A, Mastellone C, D'Abrascia B et al (2009) δ -Tocomenol: a new vitamin E from kiwi (*Actinidia chinensis*) fruits. *Food Chem* 115:187–192. <https://doi.org/10.1016/j.foodchem.2008.11.094>
 68. Mohsen E, Ezzat MI, Sallam IE et al (2024) Impact of thermal processing on phytochemical profile and cardiovascular protection of Beta vulgaris L. in hyperlipidemic rats. *Sci Rep* 14:27539. <https://doi.org/10.1038/s41598-024-77860-2>
 69. Tine Y, Renucci F, Costa J et al (2017) A method for LC–MS/MS profiling of coumarins in *Zanthoxylum zanthoxyloides* (Lam.) B. Zepernich and Timler extracts and essential oils. *Molecules.* <https://doi.org/10.3390/molecules2010174>
 70. Xia C, Deng J, Tong W et al (2023) Evaluation of the antioxidant potential of *Citrus medica* from different geographical regions and characterization of phenolic constituents by LC–MS. *ACS Omega* 8:32526–32535. <https://doi.org/10.1021/acsomega.3c02861>
 71. Abdl Aziz F, Temraz A, Hassan M (2024) Metabolites profiling by LC–ESI–MS/MS technique and *in-vitro* antioxidant activity of *Bauhinia madagascariensis* Desv. and *Bauhinia purpurea* L. aerial parts cultivated in Egypt: a comparative study. *Azhar Int J Pharmaceut Med Sci* 4:169–188. <https://doi.org/10.21608/aijpm.2023.212409.1215>
 72. Franca MGA, Cavalheiro AJ, Silva MG (2021) A comprehensive LC–DAD–QTOF–MS method for dereplication of bioactive compounds in *Senna* extracts. *Rev Bras* 31:32–39. <https://doi.org/10.1007/s43450-021-00137-6>
 73. Zibae E, Akaberi M, Tayarani-Najaran Z et al (2023) Comparative LC–ESI–MS–Based metabolite profiling of *Senna italica* with *Senna alexandrina* and evaluating their hepatotoxicity. *Metabolites.* <https://doi.org/10.3390/metabo13040559>
 74. Sawada Y, Akiyama K, Sakata A et al (2009) Widely targeted metabolomics based on large-scale MS/MS data for elucidating metabolite accumulation patterns in plants. *Plant Cell Physiol* 50:37–47. <https://doi.org/10.1093/pcp/pcn183>
 75. Simayi J, Abulizi A, Nuermaimaiti M et al (2022) UHPLC–Q–TOF–MS/MS and network pharmacology analysis to reveal quality markers of Xinjiang *cydonia oblonga* Mill. for antiatherosclerosis. *Biomed Res Int.* <https://doi.org/10.1155/2022/4176235>
 76. Thomas MC, Dunn SR, Altwater J et al (2012) Rapid identification of long-chain polyunsaturated fatty acids in a marine extract by HPLC–MS using data-dependent acquisition. *Anal Chem* 84:5976–5983. <https://doi.org/10.1021/ac3006523>
 77. Hasanin M, Hashem A, El-Rashedy A, Kamel S (2021) Synthesis of novel heterocyclic compounds based on dialdehyde cellulose: characterization, antimicrobial, antitumor activity, molecular dynamics simulation and target identification. *Cellulose.* <https://doi.org/10.1007/s10570-021-04063-7>
 78. Mirzaei S, Eivsand F, Hadizadeh F et al (2020) Design, synthesis and biological evaluation of novel 5,6,7-trimethoxy-N-aryl-2-styrylquinolin-4-amines as potential anticancer agents and tubulin polymerization inhibitors. *Bioorg Chem* 98:103711. <https://doi.org/10.1016/j.bioorg.2020.103711>
 79. Machaba KE, Mhlongo NN, Soliman MES (2018) Induced mutation proves a potential target for TB therapy: a molecular dynamics study on LprG. *Cell Biochem Biophys* 76:345–356. <https://doi.org/10.1007/s12013-018-0852-7>
 80. Pan L, Patterson JC (2013) Molecular dynamics study of Zn(α) and Zn(β)2. *PLoS ONE* 8:70681
 81. Wijffels G, Dalrymple B, Kongsuwan K, Dixon NE (2005) Conservation of eubacterial replicases. *IUBMB Life* 57:413–419. <https://doi.org/10.1080/15216540500138246>
 82. Steinbach PJ (2018) Peptide and protein structure prediction with a simplified continuum solvent model. *J Phys Chem B* 122:11355–11362. <https://doi.org/10.1021/acs.jpcc.8b07264>
 83. Cournia Z, Allen B, Sherman W (2017) Relative binding free energy calculations in drug discovery: recent advances and practical considerations. *J Chem Inf Model* 57:2911–2937. <https://doi.org/10.1021/acs.jcim.7b00564>
 84. Uddin MdS, Kabir MdT, Niaz K et al (2020) Molecular insight into the therapeutic promise of flavonoids against Alzheimer's disease. *Molecules.* <https://doi.org/10.3390/molecules25061267>
 85. Caruso G, Godos J, Privitera A et al (2022) Phenolic acids and prevention of cognitive decline: polyphenols with a neuroprotective role in cognitive disorders and Alzheimer's disease. *Nutrients.* <https://doi.org/10.3390/nu14040819>
 86. Lee AY, Lee MH, Lee S, Cho EJ (2018) Neuroprotective effect of α -linolenic acid against β -mediated inflammatory responses in C6 glial cell. *J Agric Food Chem* 66:4853–4861. <https://doi.org/10.1021/acs.jafc.8b00836>
 87. BVDas RRR (2023) Investigation of the pro-active role of alpha amylin nanoemulsions in quashing neurodegeneration, excitotoxicity, and neuronal inflammation–A combined *in vivo* and *in silico* approach. *Indian J Pharmaceut Edu Res* 58:240–253. <https://doi.org/10.5530/ijper.58.1.26>
 88. Konrath EL, Dos PSC, Klein-Júnior LC, Henriques AT (2013) Alkaloids as a source of potential anticholinesterase inhibitors for the treatment of Alzheimer's disease. *J Pharm Pharmacol* 65:1701–1725. <https://doi.org/10.1111/jphp.12090>
 89. Afzal M, Alzarea SI, Alharbi KS et al (2022) Rosiridin attenuates scopalamine-induced cognitive impairments in rats via inhibition of oxidative and nitrate stress led caspase-3/9 and *TNF- α* signaling pathways. *Molecules.* <https://doi.org/10.3390/molecules27185888>
 90. Chi-Rei Wu, Lin H-C, Muh-Hwan Su (2014) Reversal by aqueous extracts of *Cistanche tubulosa* from behavioral deficits in Alzheimer's disease-like rat model: relevance for amyloid deposition and central neurotransmitter function. *BMC Compl Altern Med.* <https://doi.org/10.1186/1472-6882-14-202>

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