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PHYTOCHEMICAL PROFILING OF PHENOLIC COMPOUNDS IN *ACACIA SALIGNA* AND ASSESSMENT OF THEIR CHOLINESTERASE INHIBITORY ACTIVITY THROUGH BOTH *IN VITRO* AND *IN SILICO* APPROACHES

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Abstract

Acacia saligna is a medicinal plant rich in phenolic compounds, emerging as a valuable reservoir of neuroprotective compounds. Cholinergic dysfunction plays a crucial role in neurodegenerative disorders, especially in Alzheimer's disease, highlighting the need for safe and effective natural therapies. Among the possible treatments, plant-derived polyphenols are notable for their capacity to influence key enzymes that contribute to the advancement of these diseases. Therefore, this study aimed to investigate its chemical constituents and its cholinesterase inhibitory activities. LC-ESI-MS/MS analysis was conducted for phenolic characterization of the ethanolic extract obtained from the aerial parts. The cholinesterase inhibitory potentials on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were determined using Ellman's. Molecular docking was performed to examine ligand-enzyme interactions. *Trans*-ferulic acid (5144.375 µg/g DM) and catechin (4045.991 µg/g DM) were the main constituents among fifteen phenolic compounds identified. The results revealed that *A. saligna* extract exhibited marked inhibitory effects toward both enzymes, with IC_{50} values of 44.74 ± 1.19 µg/mL and 22.93 ± 0.57 µg/mL, respectively. Molecular docking also revealed strong binding interactions for several constituents toward both enzymes, particularly rutin and naringenin with AChE and BChE enzymes, engaging both catalytic and peripheral regions in their active sites. Overall, these findings proposed that *A. saligna* is a promising source of bioactive compounds with cholinesterase inhibitory potential. However, further mechanistic investigations and *In vivo* research are needed to validate its therapeutic potential.

Key words: *Acacia saligna*; LC-ESI-MS/MS; Molecular docking; Enzyme inhibition; Bioactive metabolites

Introduction

Alzheimer's disease is the most common cause of dementia across the world and represents a progressive, multifactorial neurodegenerative disorder. AD is distinguished by persistent impairment of memory and behavioral disturbance, which eventually result in a significant impairment in regular daily activities (Lakhera *et al*., 2023; Cabreira *et al*., 2025). This disease is related to patients' several interconnected pathogenic processes, such as amyloid-β (Aβ) plaque formation, synaptic impairment, oxidative stress, and neuroinflammation. Among these mechanisms, the cholinergic deficit persists as one of the earliest and most reliable neurochemical alterations (Hassan *et al.,* 2023; Saggu et *al.,* 2025).

According to the cholinergic theory, the fundamental causes of dementia in Alzheimer's disease are cholinergic neuron degeneration in the basal forebrain and the resulting decrease in acetylcholine concentrations (Richardson *et al*., 2023). Acetylcholinesterase and butyrylcholinesterase are crucial enzymes that hydrolyze acetylcholine as well as associated choline esters to control synaptic neurotransmission. While AChE is more prevalent in healthy brain tissue, BChE production has been

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demonstrated to rise as AD progresses, indicating a complementary and possibly compensatory role in the disease's advanced phases. Therefore, combined inhibition of AChE and BChE is recognized as an effective therapeutic approach to restore cholinergic balance and improve cognitive abilities (Asmara *et al*., 2023; Ung and Asmara 2023; Begines *et al*., 2026).

Pharmacological inhibition of cholinesterases partially improves cognitive symptoms by increasing the concentration of acetylcholine available at synaptic clefts. Further connecting cholinesterase activity to AD pathogenesis beyond neurotransmitter hydrolysis, AChE has been linked to non-classical activities such as promoting Aβ aggregation and interacting with nicotinic cholinergic receptors (Kokturk *et al*., 2022; Yusnaini *et al*., 2023). Such observations reinforce the idea that cholinesterases should be targeted as both potential contributors to disease-modifying methods and symptomatic modulators (Kokturk *et al*., 2022).

The majority of currently regulated anti-Alzheimer medications, including galantamine, rivastigmine, and donepezil, are cholinesterase inhibitors that very modestly relieve symptoms. Nevertheless, their therapeutic effectiveness remains limited, and prolonged usage is frequently related to adverse side effects, such as hepatotoxicity, bradycardia, gastrointestinal problems, and other systemic issues (Mostafa & Asaad, 2026). Furthermore, there is an urgent need for safer, more effective, and multitarget-directed treatment alternatives due to these drugs not being able to halt or prevent the disease's progression (Hossain & Hussain 2025).

In this regard, natural products have attracted attention as a prospective source for structurally diverse compounds with potent neuroprotective properties (Soufi *et al*., 2023). Phenolic compounds derived from medicinal plants exhibit numerous pharmacological activities, such as anti-amyloidogenic, anti-inflammatory, antioxidant, and enzymes inhibitory capacities that are particularly significant in the multifactorial pathogenesis of AD. Consequently, the investigation of medicinal plants, long recognized for their traditional use in managing various disorders, offers a promising strategy to facilitate the discovery of new cholinesterase inhibitors exhibiting improved safety profiles (Ishola *et al*., 2021).

Acacia saligna is one of several perennial species widespread in the dry and semi-dry regions across northwestern Africa (Richardson *et al*., 2023). Traditional healing has long been practiced to treat various health conditions. Several secondary metabolites, including polyphenols, flavonoids, and tannins, known for their substantial biological activities, have been found in *A. sligna* according to phytochemical evaluations. Furthermore, significant antioxidant, antibacterial, anti-inflammatory, cytotoxic, and hypoglycemic effects have been demonstrated for extracts prepared from different plant organs, especially those obtained from the leaves and flowers (Kumari & Swer, 2025; Msimango *et al*., 2025).

Despite encouraging findings, research focusing on the neuroprotective properties and cholinesterase inhibitory potential of *A. saligna* remains scarce, as climatic and edaphic conditions may significantly influence phytochemical composition. Moreover, the relationship between *A. saligna* extracts' phenolic profile and their biological activities, particularly enzyme inhibition, has not been completely clarified.

The present research is the first report to analyze the phenolic content of *A. saligna* and to investigate its neuroprotective potential using an integrated approach combining targeted LC-ESI-MS/MS phytochemical profiling, *In vitro* acetylcholinesterase and butyrylcholinesterase inhibition assays, and molecular docking analyses. While previous investigations have primarily focused on the phytochemical profiling and general biological properties of *A. saligna*, its neuroprotective activity and the molecular basis underlying its cholinesterase inhibitory activity remain largely unexplored. Therefore, this work aimed to characterize major active constituents associated with *A. saligna* anticholinesterase activity and to provide molecular-level insights into their interactions and binding mode within the catalytic pockets of both enzymes.

Materials and methods

Reagents and chemicals: Galantamine, AChE, BChE, and LC-ESI-MS/MS standards were obtained from Sigma Aldrich (Germany). Every solvent was of analytical grade.

Plant sample: The aerial parts of *A. saligna* were gathered from the Djelfa region in Algeria (El Massaad, ALT 838 m, 34° 08′ 48″ North, 3° 30′ 29″ East). The authenticity of this plant material was confirmed through specialists from the High Directive for Steppe Development, located in Djelfa. The plant parts were cleaned, dried, and milled into a fine powder with a 0.5 mm particle size for subsequent analysis.

Preparation of plant extract: The maceration technique outlined by Yusnaini *et al*., (2023) was slightly modified in order to obtain the *A. saligna* extract. In brief, ethanol/water (96:4, v/v) was combined with 40 g of the sample. The mixture was continuously stirred in a darkened room. This procedure was carried out three times in every 24 hours using renewal solvent. Once completed, the solution underwent vacuum filtration using Whatman paper No. 1 to separate the solid residue from the liquid extract. The resultant filtrate was then concentrated with a rotary evaporator under controlled temperature at 35°C, leading to a crude extract with an extraction yield of 19.5%, calculated using the plant material's initial dry weight. To ensure longevity and efficacy, the resulting dry extract was kept at -20°C, protected from light and moisture until it was necessary for further analysis. For subsequent bioassays, the extract was prepared in methanol at a final concentration of 10 mg/mL, with further serial dilutions prepared as needed for each specific analysis.

Chemical profiling using LC-ESI-MS/MS: *A. saligna* extract underwent LC-ESI-MS/MS characterization, allowing the procedure by Kokturk *et al*. (2022). This analysis targeted 45 compounds using an Agilent 1260 Infinity HPLC system linked to a 6460 three-quadrupole mass spectrometer (Agilent Technologies, USA), utilizing both negative and positive ionization modes through electrospray ionization (ESI). Chromatography was conducted using a Poroshell 120 EC-C18 column at a controlled temperature of 25°C. For the mobile phase, 5 mM ammonium formate in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were employed, with varying flow rates and compositions at specified time

intervals. The solvent was delivered at 0.5 mL/min, whereas the injection volume was maintained at 5 µL. Key operational settings comprised a capillary voltage of 4000 V and gas temperatures at 350°C, with the dry and nebulizer gas flow rates were maintained at 15 L/min and 11 L/min, respectively. Method validation was based on Yilmaz, (2020), evaluating analytical performance metrics such as linearity and sensitivity. Phenolic compounds were quantified using external calibration curves, with the relationship between peak area and analyte concentration evaluated via correlation coefficient (R^2). Sensitivity metrics included the limits of detection (LOD) and limits of quantification (LOQ) derived from signal-to-noise ratios of 3 and 10, respectively.

Cholinesterase inhibitory capacity: The inhibitory activities of *A. saligna* extract against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were evaluated using the procedure described by Ellman *et al*., (1961) and Öztürk *et al*., (2011). Initially, *A. saligna* extract (10 µL), 100 mM sodium phosphate buffer (150 µL, pH 8.0), followed by the corresponding enzyme (AChE or BChE, 20 µL) were introduced into the reaction solution. The mixture was incubated for 15 minutes prior to adding 5,5′-dithiobis-2-nitrobenzoic acid (0.5 mM, 10 µL). Subsequently, the reaction was started by the addition of acetylthiocholine iodide (0.71 mM, 20 µL). Equation 1 was used to calculate the inhibition rates after absorbance at 412 nm:

$$\text{Inhibitory activity} = \frac{(E - S)}{(E)} \times 100 \qquad (1)$$

In this equation, E denotes enzyme activity measured without the extract (control), whereas S denotes enzyme activity measured in the presence of the extract. The *A. saligna* extract and galantamine were evaluated at concentrations between 12.5 and 400 µg/mL. Concentration-response curves were generated, and the corresponding IC_{50} (half-maximal inhibitory concentration) values were calculated.

Computational studies

Proteins retrieval and preparation: The 3D crystal structures of human acetylcholinesterase (PDB ID: 4EY7) and butyrylcholinesterase (PDB ID: 4TPK) were retrived from the RCSB Protein Data Bank (https://www.rcsb.org/pdb, visited March 29, 2025) at resolutions of 2.35 Å and 2.70 Å, respectively (Jang *et al*., 2018). Protein preparation was performed using Discovery Studio software (2021) by removing unbound water molecules, cofactors, and other heteromolecules, including co-crystallized ligands (Soufi *et al*., 2023).

Subsequently, Autodock Tools 1.5.6 was employed to prepare both enzymes for docking simulations. Polar hydrogen atoms were added, whereas non-polar hydrogen were merged to ensure proper intramolecular interactions. Before saving data in the pdbqt format, each protein received a Kollman charge, and the atoms were given AD4 parameters (Ishola *et al*., 2021). The centroids of the co-crystallized inhibitors (X, Y, and Z coordinates) were obtained from the PDB structures using the binding site tools in Discovery Studio software in order to construct the grid box around all enzymes' active sites.

Ligands preparation: The 3D structure of the studied compounds and the reference inhibitor were acquired from the PubChem databases (https://pubchem.ncbi.nlm.nih.gov/, accessed on March 28, 2025) in SDF format. Before docking, the molecular geometry of each compound was optimized using the MMFF94 force field in Avogadro software to achieve their minimum energy conformations (Fekadu *et al*., 2025). The optimized structure was then saved as a Mol2 version. Torsions were defined to allow ligand flexibility during simulations, after which the ligand structures were converted to pdbqt format using Autodock Tools 1.5.6 (Ishola *et al*., 2021).

Molecular docking: Binding affinity calculations were conducted using Autodock Vina software. Prior to docking, a configuration file defining the grid box parameters was generated for each target protein. The grid box was centered at x = -14.108464, y = -43.832714, and z = 27.669929 for AChE, whereas the corresponding cordinates for BChE were x = 4.022061, y = 10.766970, and z = 13.863333. A docking grid measuring 20×20×20 Å was used for all complexes, with an exhaustiveness level of 8. To validate the molecular docking protocol, each native co-crystallized ligand was obtained from its respective protein before being redocked into its initial binding pocket under identical docking parameters. Protocol performance was assessed by determining the root mean square deviation (RMSD) between the redocked pose and the experimentally determined crystallographic conformation (Derardja *et al*., 2025).

Statistical analysis

All experimental findings were expressed as mean ± standard deviation (SD). Each measurement was performed independently in triplicate. Statistical analyses were employed one-way analysis of variance (ANOVA), utilizing Tukey's honestly significant difference (HSD) test for post-hoc multiple comparisons. Statistical significance was defined as $p<0.05$. Data analysis was executed using the GraphPad Prism software (version 5.0) (Gurski & Dittel, 2026).

Results and Discussion

Phenolic compounds quantification: Figures 1 and 2 present the chromatographic profile and chemical structures of the compounds identified in the *A. saligna* extract, respectively, while their quantitative data are represented in Table 1. Targeted LC-ESI-MS/MS analysis identified fifteen phenolic compounds belonging to different phytochemical classes. Among these, *trans*-ferulic acid (5144.375 µg/g DM) and catechin (4045.991 µg/g DM) were the predominant constituents. Intermediate concentrations were recorded for isoquercitrin (1053.016 µg/g DM), salicylic acid (1012.388 µg/g DM), vanillin (776.003 µg/g DM), and naringenin (253.036 µg/g DM), whereas shikimic acid (164.429 µg/g DM), epigallocatechin (74.648 µg/g DM), *p*-hydroxybenzaldehyde (56.645 µg/g DM), syringic acid (22.678 µg/g DM), caffeine (20.765 µg/g DM), sinapic acid (5.761 µg/g DM), scutellarin (4.870 µg/g DM), fisetin (1.054 µg/g DM), and rutin (0.981 µg/g DM) were detected at comparatively lower concentrations.

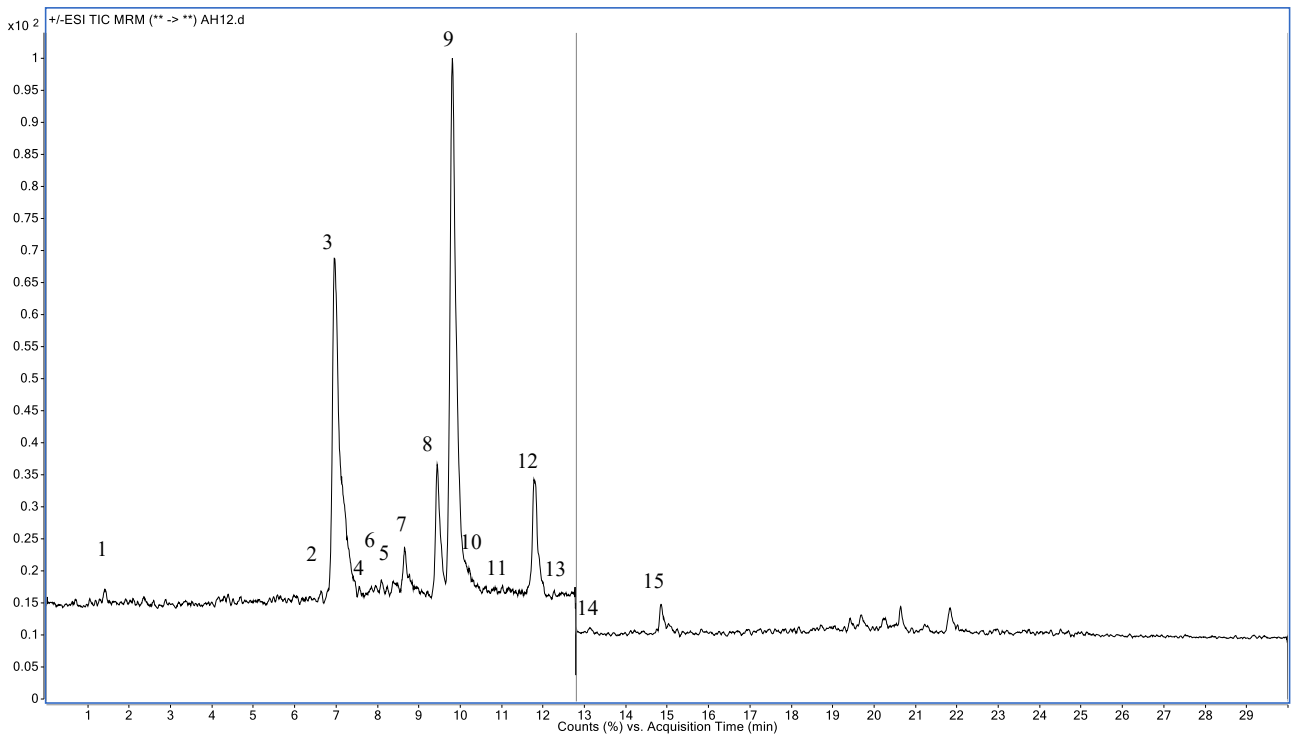


Fig. 1. LC-ESI-MS/MS chromatogram of the ethanolic extract of *A. saligna* with annotated peaks of the detected phytochemicals. 1. Shikimic acid 2. Epigallocatechin, 3. Catechin, 4. p-hydroxybenzaldehyde, 5. Syringic acid, 6. Caffeine, 7. Vanillin, 8. Salicylic acid, 9. *Trans*-ferulic acid, 10. Sinapic acid, 11. Scutellarin, 12. Isoquercitrin, 13. Rutin, 14. Fisetin, 15. Naringenin.

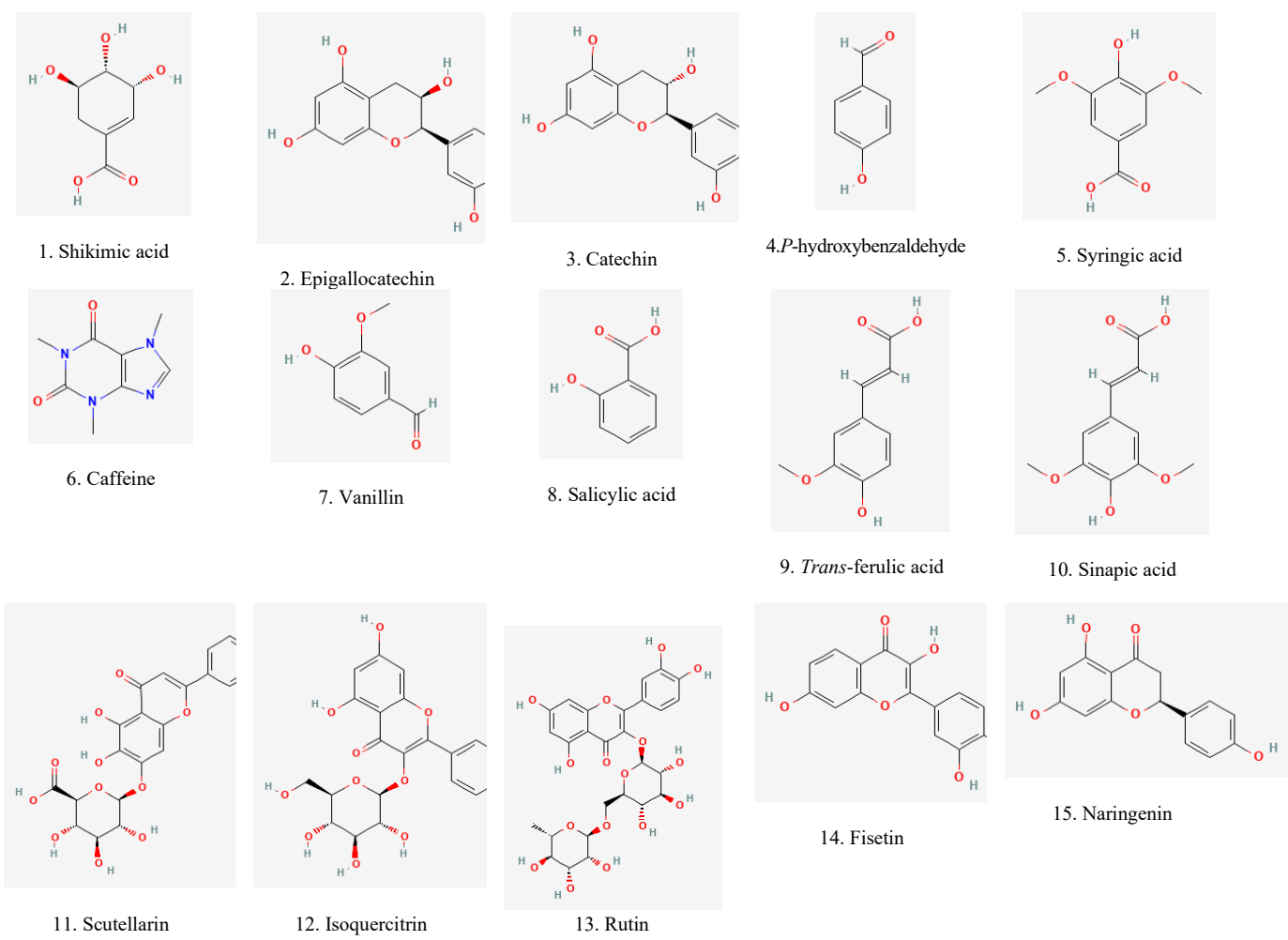


Fig. 2. Chemical structures of the phenolic constituents identified in the ethanolic extract of *A. saligna* by LC-ESI-MS/MSanalysis.

Table 1. Qualitative and quantitative (µg/g DM) characterization of phenolic compounds identified in *A. saligna* ethanolic extract.

S.No.	Phenolic compounds	RT (min)	*A. saligna* extract (µg/g DM)	Chemical class (subclass)	R^2	LOQ (µg/L)	LOD (µg/L)	Linearity range (µg/L)	Intra-day precision[a] (RSD %)	Intra-day Precision[b] (RSD %)	Recovery[c] (%)
01.	Shikimic acid	1.414	164.429	Organic acid (cyclohexane-carboxylic acid)	0.994	284.41	235.52	1-50	1.41	192.46	0.99 ± 2.66
02.	Epigallocatechin	6.838	74.648	Flavonoid (flavanol)	0.998	269.90	236.1	1-50	0.72	1.21	0.99 ± 3.56
03.	Catechin	6.958	4045.991	Flavonoid (flavanol)	0.993	78.65	55.22	0.2-10	1.38	2.44	0.99 ± 0.71
04.	*p*-hydroxybenzaldehyde	7.525	56.645	Phenolic aldehyde (hydroxybenzaldehyde)	0.999	12.86	4.9742	14.625–250	1.9	2.5	98.2 ± 1.76
05.	Syringic acid	8.398	22.678	Phenolic acid (hydroxybenzoic)	0.993	105.50	83.3	1-50	1.28	1.19	99.00 ± 1.12
06.	Caffeine	8.318	20.765	Alkaloid (methylxanthine)	0.999	25.34	6.9805	32.25–500	2.14	2.74	99.2 ± 2.21
07.	Vanillin	8.748	776.003	Phenolic aldehyde (hydroxybenzaldehyde)	0.995	41.54	13.588	62.52–1000	3.8	4.7	96.1 ± 2.72
08.	Salicylic acid	9.409	1012.388	Phenolic acid (hydroxybenzoic)	0.992	83.46	46.669	111.5–1800	4.64	5.25	97.3 ± 1.53
09.	*Trans*-ferulic acid	9.986	5144.375	Phenolic acid (hydroxycinnamic)	0.995	11.52	6.1184	31.25–1000	2.7	3.3	97.9 ± 2.65
10.	Sinapic acid	10.424	5.761	Phenolic acid (hydroxycinnamic)	0.999	248.35	218.2	0.05-2.5	2.21	1.64	99.7± 0.76
11.	Scutellarin	11.124	4.870	Flavonoid (flavone)	0.995	67.65	61.66	1-50	1.76	121.88	99.4 ± 2.23
12.	Isoquercitrin	11.687	1053.016	Flavonoid (flavonol)	0.998	14.5	9.7	0.1-5	2.23	0.88	97.9 ± 2.6
13.	Rutin	12.359	0.981	Flavonoid (flavonol)	0.999	22.7	15.7	0.1-5	1.38	1.09	99.4 ± 3.22
14.	Fisetin	13.36	1.054	Flavonoid (flavonol)	0.999	12.7	10.1	0.1-5	1.85	1.29	98.2 ± 4.91
15.	Naringenin	14.772	253.036	Flavonoid (flavanones)	0.999	3.96	2.62	0.1-5	2.42	1.71	98.3 ± 0.20

The identified metabolites comprised phenolic acids, flavonoids, phenolic aldehydes (hydroxybenzaldehydes), and alkaloids. Phenolic acids were represented by salicylic acid, syringic acid, *trans*-ferulic acid, and sinapic acid, corresponding to peaks 1, 8, 9, and 10, respectively. Shikimic acid, detected at peak 5, belongs to the cyclohexene-carboxylic acid class and represents a key intermediate of the shikimate biosynthetic pathway. The flavonoid fraction comprised flavanols, represented by epigallocatechin (peak 2) and catechin (peak 3); flavonols, including isoquercitrin (peak 12), rutin (peak 13), and fisetin (peak 14); the flavone scutellarin (peak 11); and the flavanone naringenin (peak 15). The phenolic aldehydes *p*-hydroxybenzaldehyde and vanillin, both belonging to the hydroxybenzaldehyde subclass, were detected at peaks 4 and 7, respectively. Finally, caffeine was detected at peak 6 as the only alkaloid identified in the extract, belonging to the methylxanthine subclass.

It should be noted that the identification of fifteen (15) compounds is attributed to the targeted LC-ESI-MS/MS approach, which relied on a predefined panel of authentic reference standards. Consequently, metabolites not included in this analytical panel or present below the detection limits could not be identified. In addition, extraction conditions, plant matrix composition, developmental stage, and environmental factors are known to influence phytochemical profiles. Therefore, although the present analysis reliably identified the major targeted constituents, the presence of additional metabolites remains possible.

The phytochemical profile obtained here agrees well with earlier investigations on *Acacia* species, reflecting the richness of the genus in bioactive compounds associated with a broad spectrum of biological properties (Subhan *et al*., 2018; Hamdellou *et al*., 2023). Several studies have documented the occurrence of phenolic acids, flavonoids, and related phytochemicals in different organs of *Acacia* species. Analysis of a water-soluble flower extract of *A. saligna* identified abundant amounts of kaempferol, benzoic acid, salicylic acid, quercetin, catechol, naringenin, caffeine, caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, syringic acid, *o*-coumaric acid, *p*-coumaric acid, and ellagic acid (Al-Huqail *et al*., 2019). Likewise, phytochemical analyses of leaf extracts identified gallic acid, vanillin, *p*-hydroxybenzoic acid, protocatechuic acid, and syringic acid among the major constituents (Gumgumjee *et al*., 2015; Elansary *et al*., 2020; Guneidy *et al*., 2021). In bark extracts, chlorogenic acid, rutin, gallic acid, benzoic acid, caffeic acid, kaempferol, naringenin, rosmarinic acid, vanillic acid, ferulic acid, and caffeine have also been reported (Salem *et al*., 2021). Taken together, the available evidence indicates that the phytochemical composition of *A. saligna* varies among plant organs while consistently revealing a predominance of phenolic compounds.

In the present investigation, *trans*-ferulic acid and catechin emerged as the dominant constituents, corroborating observations described in multiple *Acacia* species (Batiha *et al*., 2022; Pedro *et al*., 2023). The consistent detection of these compounds across different members of the genus supports their classification as characteristic phenolic constituents of *Acacia* species. Given their well-documented antioxidant and pharmacological activities, their predominance may

explain, at least in part, the biological properties attributed to *A. saligna* extracts. However, while the qualitative composition was largely consistent with previous reports, quantitative differences were evident. Such variation is expected because the accumulation of secondary metabolites is strongly influenced by genetic background, the plant part examined, growth stage, the time of harvest, the extraction procedure, and the analytical methodology.

Furthermore, the relatively high abundance of phenolic compounds identified here could also results from the ecological conditions of the Djelfa region, which is characterized by an arid to semi-arid climate. Environmental stresses such as prolonged drought, high solar irradiance, and marked temperature fluctuations have been shown to stimulate the biosynthesis of secondary metabolites, particularly phenolic acids and flavonoids, as adaptive mechanisms against oxidative stress (Zahedi *et al*., 2021). Therefore, their accumulation in *A. saligna* may represent an adaptive strategy that supports its survival under arid and semi-arid conditions. While this investigation did not specifically examine the effects of environmental factors, differences in geographic origin, climatic conditions, and soil characteristics likely contributed to the observed phytochemical profile and may partly explain discrepancies with previous reports (Urbonaviciene *et al*., 2022).

Overall, the present findings reinforce the growing evidence that *Acacia* species represent valuable reservoirs of polyphenolic compounds with considerable biological potential. Comparative phytochemical studies have demonstrated substantial interspecific variation in the levels of flavonoids, tannins, phenolic derivatives, alkaloids, terpenoids, along with other secondary metabolites across the genus (Zheng *et al*., 2019; Akapo *et al*., 2025; Panigrahy *et al*., 2025; Kumari and Swer, 2026). Such variability highlights the importance of characterizing individual *Acacia* species, as their phytochemical composition largely determines their pharmacological, nutraceutical, and industrial value.

Cholinesterase activity: Alzheimer's disease (AD) represents one of the most common neurodegenerative disorders globally and is marked by a gradual decline in cognitive function associated with cholinergic dysfunction and neuronal degeneration (Tahami Monfared *et al*., 2022). A major therapeutic approach in the symptomatic management of AD involves inhibiting cholinesterase enzymes, which enhances acetylcholine levels in the synaptic cleft, thereby improving cholinergic neurotransmission (Phan *et al*., 2019). Consequently, considerable research has focused on identifying natural products as potential sources of cholinesterase inhibitors for AD therapy (Li *et al*., 2025; Nour *et al*., 2025). Besides their enzyme inhibitory activity, many plant-derived phytochemicals possess antioxidant properties that may protect neuronal cells against oxidative damage, an important contributor to AD pathogenesis. Among these natural resources, Acacia species are recognized for their rich bioactive phytochemicals, which exhibit a variety of pharmacological activities including anti-inflammatory, antioxidant, and neuroprotective effects (Tahami Monfared *et al*., 2022). Nevertheless, investigations evaluating the anticholinesterase potential of *A. saligna* remain limited. Therefore, our research was undertaken to evaluate the inhibitory effects of *A. saligna* ethanolic extract shows potential inhibitory effects against AChE and BChE, which are promising targets in Alzheimer's disease treatment.

As summarized in Table 2, *A. saligna* ethanolic extract demonstrated significant inhibitory activity toward both cholinesterase enzymes, with IC_{50} values of 44.74 ± 1.19 µg/mL for AChE and 22.93 ± 0.57 µg/mL for BChE. In comparison, galantamine, the reference drug, displayed IC_{50} values in order of 21.44 ± 0.45 µg/mL for AChE and 21.71 ± 2.30 µg/mL for BChE. Notably, the extract displayed a BChE inhibitory activity that was statistically comparable to that of the reference drug ($p \geq 0.05$). These findings indicate that *A. saligna* represents a promising natural source of cholinesterase inhibitors, particularly considering the increasing recognition of BChE as an important therapeutic target during the advanced stages of AD, where its activity progressively increases while AChE activity declines (Zhao *et al*., 2025).

Table 2. *In vitro* inhibitory activity of *A. saligna* ethanolic extract against AChE and BChE.

Samples	IC_{50} values (µg/mL)	
	AChE	BChE
A. saligna extract	44.74 ± 1.19[a]	22.93 ± 0.57 [a]
Galantamine	21.44 ± 0.45[b]	21.71 ± 2.30 [a]

The same subscript letters are not statistically different at $p<0.05$

Currently approved cholinesterase inhibitors, including donepezil, rivastigmine, and galantamine, remain the cornerstone of symptomatic AD treatment; however, they neither halt nor reverse disease progression. Furthermore, their long-term use may be associated with gastrointestinal disturbances, cardiovascular adverse effects, and variable therapeutic responses among patients (Haake *et al*., 2020). Consequently, increasing efforts have focused on identifying plant-derived compounds capable of providing cholinesterase inhibition while simultaneously targeting additional pathological processes involved in AD. In this context, the activity observed for *A. saligna* may be a valuable source of multifunctional neuroprotective phytochemicals.

The remarkable anticholinesterase activity observed in the present study is likely related to the rich phytochemical composition of the extract. Several researchs have established the ability of polyphenol-rich plant extracts, particularly those abundant in flavonoid, to inhibit cholinesterase enzymes (Nazir *et al*., 2021; Kamli *et al*., 2022). Considering the high levels of flavonoids identified in *A. saligna*, together with previous reports describing anticholinesterase activity of other *Acacia* species (Ghribia *et al*., 2014; Shah *et al*., 2022; Rauf *et al*., 2024), these findings further support the contribution of these phytochemicals to the observed enzyme inhibition. In the present study, LC-ESI-MS/MS analysis revealed that *trans*-ferulic acid and catechin were the predominant constituents, accompanied by several other biologically active compounds, which have individually been reported to exhibit cholinesterase inhibitory activity together with complementary neuroprotective properties.

Trans-ferulic acid has been explored for its neuroprotective potentials due to its notable antioxidant and anti-inflammatory activities, with several studies highlighting its possible role in mitigating AD pathology (Di Giacomo *et al*., 2022). Experimental investigations by Subhan *et al*. (2018) showed that catechin, major constituent identified in the present study, has demonstrated significant anticholinesterase activity and anti-amnesic effects in several experimental models. For instance, treatment with

catechin obtained from *A. catechu* (10, 20, and 40 mg/kg) significantly improved learning and memory performance in mice, further supporting its relevance in the management of cognitive impairment (Subhan *et al*., 2018).

Importantly, the possible neuroprotective effects of *A. saligna* cannot be attributed solely to its two major constituents. Isoquercitrin has been shown to attenuate oxidative damage together with neuroinflammation, limit amyloid-β accumulation, improve cognitive performance, and regulate neuroprotective signaling pathways in preclinical models of Alzheimer's disease (Yang *et al*., 2021; Li *et al*., 2023). Similarly, naringenin exerts multitarget neuroprotective effects by inhibiting AChE, BChE, and β-secretase (BACE1), while simultaneously attenuating oxidative stress as well as suppressing neuroinflammation (Nouri *et al*., 2019). Epigallocatechin has likewise demonstrated broad neuroprotective activity by reducing oxidative damage, preventing amyloid-β aggregation, modulating inflammatory responses, promoting autophagy, improving mitochondrial function, and supporting neuronal survival (Islam *et al*., 2025). Moreover, vanillin showed the ability to protect neuronal tissues against oxidative brain injury through its antioxidant properties (Salau *et al*., 2020). Taken together, these evidences suggest that several constituents identified within *A. saligna* target multiple pathological mechanisms involved in neurodegeneration beyond cholinesterase inhibition alone. This multitarget profile is particularly relevant because AD involves multiple pathological processes, including cholinergic dysfunction, oxidative stress, chronic neuroinflammation, amyloid-β deposition, mitochondrial dysfunction, and progressive neuronal loss. Unlike currently available cholinesterase inhibitors, which primarily provide symptomatic improvement through enhancement of cholinergic neurotransmission, naturally occurring polyphenols frequently exert complementary antioxidant, anti-inflammatory, anti-amyloidogenic, and neuroprotective activities (Cichon *et al*., 2025). Therefore, the phytochemical composition of *A. saligna* may provide therapeutic advantages by simultaneously modulating several interconnected pathological pathways involved in AD.

An additional consideration for developing neuroprotective agents is whether they can access the brain. Previous studies have investigated the pharmacokinetic behavior and neuroprotective mechanisms of several phytochemicals identified in *A. saligna*.

Among the major constituents, *trans*-ferulic acid, catechin, and naringenin are capable of penetrating the blood-brain barrier (BBB), allowing them to exhibit antioxidant, anti-inflammatory, and neuroprotective effects in brain tissue. Trans-ferulic acid was reported to cross the BB barrier in murine models. Once inside the brain, it is converted into an active form that possess significant antioxidant properties, suggesting a protective role for neural tissues against oxidative stress. Likewise, catechin was shown to penetrate into the brain parenchyma through the BBB, where it may enhance neuritogenesis and improve cognitive function in experimental models (Pervin *et al*., 2019; Stompor-gor, 2021). Naringenin has similarly demonstrated BBB permeability together with cholinesterase inhibitory, antioxidant and anti-inflammatory effects, supporting its possible application in neurodegenerative disorders (Zhu *et al*., 2024; Yang *et al*., 2025). In contrast, isoquercitrin and epigallocatechin display relatively limited BBB penetration; nevertheless, numerous experimental studies have demonstrated that both compounds retain significant neuroprotective activity through the attenuation of oxidative stress, neuroinflammation, as well as amyloid-related pathology (Pervin *et al*., 2017; Zhang *et al*., 2018). The cumulative observations suggest that the neuroprotective potential of *A. saligna* may result from the complementary actions of multiple phytochemicals with distinct pharmacokinetic properties and mechanisms of action. However, because BBB permeability and bioavailability were not evaluated in the present study, these observations support the possibility that at least some of the major metabolites identified in *A. saligna* may reach pharmacologically relevant concentrations in the brain. Nevertheless, further pharmacokinetic investigations are required to clarify their absorption, metabolism, brain distribution, whereas additional preclinical studies remain necessary to validate the therapeutic effects of the extract.

Comparison with previous reports further highlights the promising anticholinesterase potential of *A. saligna*. The ethanolic extract exhibited substantially stronger inhibitory activity than extracts of *Acacia nilotica* (IC_{50} = 637.01 and 491.98 µg/mL against AChE and BChE, respectively) and *Acacia catechu* (IC_{50} = 204.38 µg/mL for AChE) (Thangavelu and Ramasamy, 2015; Rauf *et al*., 2024). Differences among species are likely explained by variations in phytochemical composition, extraction procedures, environmental conditions, and metabolite abundance (Martins-Gomes *et al*., 2023). Moreover, previous investigations have likewise demonstrated cholinesterase inhibitory activity in several members of the genus, namely *A. seyal*, *A. farnesiana*, *A. sieberiana*, *A. catechu*, *A. modesta*, *A. saligna*, and *A. stenophylla* (Khan *et al*., 2014; Ghribia *et al*., 2014; Thangavelu and Ramasamy, 2015; Subhan *et al*., 2018; Shah *et al*., 2022). The pronounced inhibitory effects observed in the present study further emphasizes the therapeutic promise and the unique properties of *A. saligna* compared to other species.

Overall, the present findings demonstrate that *A. saligna* possesses significant cholinesterase inhibitory activity, surpassing numerous other *Acacia* species, supported by an interesting phytochemical profile with established neuroprotective properties. The combined cholinesterase inhibitory, antioxidant, anti-inflammatory, and anti-amyloidogenic activities suggests that *A. saligna* may represent a promising source of multitarget neuroprotective agents. Nevertheless, further investigations are needed to confirm the current outcomes and to clarify its translational relevance as a therapeutic candidate in Alzheimer's disease.

Molecular docking-based binding mode: The reliability of the molecular docking workflow was first verified through redocking of the native bound ligands into the AChE and BChE binding sites. The predicted poses closely overlapped with their experimental counterparts, producing RMSD values of 1.99 Å (Fig. S1) and 1.04 Å (Fig. S2), respectively. As both values satisfied the widely accepted validation criterion (<2.0 Å), the docking protocol was considered sufficiently reliable.

Following validation, molecular docking analysis examined how the phenolic compounds identified in *A. saligna* extract interact with the cholinesterase enzymes. Binding affinities, together with key protein-ligand interactions, were analyzed to characterize ligand-binding patterns and estimate the affinity of each compound relative to galantamine (Table 3).

Validation of the molecular docking protocol by performing a redocking procedure for both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

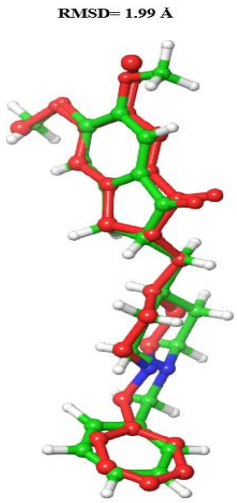


Fig. S1. Superposition of the crystallographic and redocked binding poses of the co-crystallized ligand within the acetylcholinesterase (AChE) active site. The crystallographic ligand is shown in green and the redocked pose is shown in red.

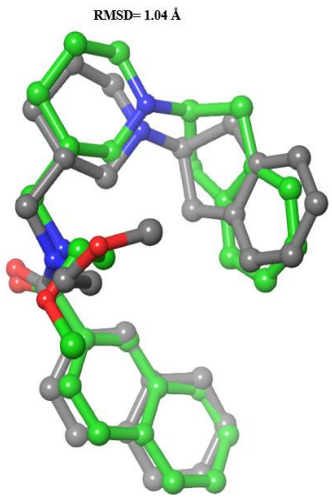


Fig. S2. Superposition of the crystallographic and redocked binding poses of the co-crystallized ligand within the butyrylcholinesterase (BChE) active site. The crystallographic ligand is shown in green, whereas the redocked pose is shown in gray.

For AChE, the reference ligand galantamine exhibited a docking score of -10.4 kcal/mol, whereas the studied compounds displayed docking scores comprised between -10.3 and -5.9 kcal/mol. Among them, naringenin and fisetin demonstrated the strongest binding affinities, with docking scores of -10.3 and -10.1 kcal/mol, respectively. These results suggest they are potent AChE inhibitors, almost comparable to galantamine.

The favorable binding affinities observed for several flavonoids may be attributed to their structural characteristics. Unlike galantamine, which possesses a relatively rigid alkaloid scaffold, flavonoids such as naringenin and fisetin contain multiple hydroxyl groups and conjugated aromatic rings that enable the formation of diverse non-covalent interactions within the enzyme active site. Their hydroxyl substituents facilitate hydrogen-bond formation with catalytically important residues, whereas their aromatic systems promote π-π stacking contacts with aromatic residues lining the catalytic gorge (Navarro-Hoyos *et al*., 2026). The combined contribution of hydrogen bonds, π-interactions, together with hydrophobic contacts likely enhance protein-ligand complex stability, explaining their favorable docking scores.

Moreover, results obtained for naringenin and fisetin are further supported by previous experimental studies demonstrating their cholinesterase inhibitory activity. Naringenin has been reported to inhibit AChE *In vitro* and to exert neuroprotective effects through its antioxidant and anti-inflammatory activities, highlighting its potential for managing neurodegenerative disorders (Lee *et al*., 2018). Likewise, fisetin has demonstrated AChE inhibitory activity while exhibiting multiple neuroprotective effects, such as reducing oxidative stress, regulating neuroinflammatory pathways, and maintaining neuronal function (Tang *et al*., 2023; Shi *et al*., 2024). The comparable docking scores and interaction profiles observed in the present study therefore provide a structural rationale for the previously reported biological activities of these flavonoids.

Consistent findings were obtained for BChE, with rutin exhibiting the highest predicted binding affinity. Its superior docking performance may be associated with its larger glycosylated flavonoid framework, which allows the establishment of numerous hydrogen bonds and van der Waals contacts throughout the wider and more flexible binding gorge of BChE. This observation is consistent with the known structural differences between cholinesterase enzymes, as BChE possesses a larger catalytic cavity than AChE (Sridhar & Gumpeny, 2024), enabling the accommodation of bulkier ligands and consequently favoring interactions with highly substituted flavonoids. The high predicted binding affinities of rutin and isoquercitrin toward BChE are consistent with earlier reports demonstrating the cholinesterase inhibitory potential of glycosylated flavonoids. Rutin has been shown to inhibit both AChE and BChE *In vitro* while also exhibiting antioxidant and neuroprotective properties (Qu *et al*., 2014; Qanash *et al*., 2023). Isoquercitrin has likewise demonstrated cholinesterase inhibitory activity and has attracted attention for its antioxidant and anti-inflammatory effects, which may contribute to its neuroprotective action (Liu *et al*., 2021; Song *et al*., 2025). Overall, the observed binding behavior suggests that these phytochemicals possess structural features that favor stable association with the cholinesterase binding pocket.

Detailed interaction analysis further supported these findings (Table 4 and Fig. 3). Naringenin was stabilized within the AChE binding site via a hydrogen bond with Tyr124, extensive van der Waals contacts involving Gly120, Tyr337, Phe295, Ser203, His447, Gly121, Ala204, Phe297, and Val294, along with interactions pi-pi interactions with Phe338, Tyr341, and Trp286. In contrast, fisetin formed two hydrogen bonds with Tyr133 and Gln71, numerous van der Waals interactions with Asp74, Tyr72, Val73, Pro88, Gly126, Gly121, Ile451, Tyr119, Gly120, Ser203, Glu202, Gly448, His447, Tyr337, and Tyr341, and one pi-pi interaction with Trp86. In contrast, galantamine's interaction with AChE was stabilized by single hydrogen bond with Ser203, van der Waals contacts with Tyr133, Tyr119, Gly120, Gly121, Ile451, Gly448, His447, Ala204, Phe295, Gly122, Phe297, and Tyr341, along with pi-pi interactions with Trp86 (Pi-Alkyl), Tyr337 (Pi-Sigma), and Phe338 (Pi-Pi shaped). Notably, naringenin, fisetin, and galantamine shared several key residues within the catalytic gorge, suggesting that the investigated flavonoids occupy binding orientations comparable to that of the reference inhibitor

Table 3. Binding energies of *A. saligna* constituents and galantamine towards AChE and BChE active sites.

Compound	PubChem CID	Molecular formula	Molecular weight	Docking score (kcal/mol)	
				AChE	BChE
Galantamine (Reference inhibitor)	9651	$C_{17}H_{21}NO_3$	287.35	-10.4	-8.6
Naringenin	439246	$C_{15}H_{12}O_5$	272.25	-10.3	-9.1
Fisetin	5281614	$C_{15}H_{10}O_6$	286.24	-10.1	-9.5
Rutin	5280805	$C_{27}H_{30}O_{16}$	610.5	-9.1	-10.4
Isoquercitrin	5280804	$C_{21}H_{20}O_{12}$	464.4	-9.2	-10.0
Scutellarin	185617	$C_{21}H_{18}O_{12}$	462.4	-9.8	-9.9
Sinapic acid	637775	$C_{11}H_{12}O_5$	224.21	-7.4	-6.7
Trans-ferulic acid	445858	$C_{10}H_{10}O_4$	194.18	-7.6	-6.6
Salicylic acid	338	$C_7H_6O_3$	138.12	-6.8	-5.9
Vanillin	1183	$C_8H_8O_3$	152.15	-6.3	-5.7
Caffeine	2519	$C_8H_{10}N_4O_2$	194.19	-6.9	-6.3
Syringic acid	10742	$C_9H_{10}O_5$	198.17	-6.6	-5.8
p-hydroxybenzaldehyde	126	$C_7H_6O_2$	122.12	-5.9	-5.4
Catechin	9064	$C_{15}H_{14}O_6$	290.27	-9.9	-9.2
Epigallocatechin	72277	$C_{15}H_{14}O_7$	306.27	-9.8	-8.5
Shikimic acid	8742	$C_7H_{10}O_5$	174.15	-6.3	-5.6

Previous crystallographic studies have demonstrated that the AChE active site comprises several functionally important regions, including the catalytic triad (Ser203, His447, and Glu334), the peripheral anionic site (Asp74, Trp286, Tyr124, Ser125, Tyr337, and Tyr341), the oxyanion hole (Gly120, Gly121, and Ala204), the anionic subsite (Trp86, Tyr133, Glu202, Gly448, and Ile451), and the acyl-binding pocket (Kuzu *et al*., 2019; Peitzika & Pontiki, 2023).

Therefore, the interaction profiles obtained in the present study suggest that naringenin and fisetin simultaneously engage several of these functional regions, particularly residues belonging to the catalytic triad, peripheral anionic site, oxyanion hole, and anionic subsite. Such multi-site interactions are considered advantageous because they can stabilize ligand binding while potentially interfering with substrate recognition and catalytic turnover.

Analysis of the interactions exhibited by the highest-ranked constituents against BChE further supported their favorable docking scores (Table 5 and Fig. 3). Rutin established multiple hydrogen bonds with Thr120, Glu197, Tyr128, and Ala328, numerous van der Waals contacts, alongside pi-pi interactions with Trp82, Trp430, and Ala328. Conversely, isoquercitin formed four hydrogen bonds with Asn83, Glu197, Ser198, and Ser287, along with van der Waals and Pi-Pi interactions with various residues. In comparison, galantamine formed two hydrogen bonds at the BChE active site, with Gly115 and Thr120, along with seven van der Waals interactions with Asp70, Asn83, Tyr128, Gly439, His438, Tyr332, and Ala328, and one pi-pi contact with Trp82.

It's noteworthy that the most effective compounds, along with galantamine, had the ability to attach with BChE's peripheral anionic site (PAS) (Przybyłowska *et al.,* 2021). This binding was facilitated by interactions with Asp70 and Tyr332, which play a role in positioning positively charged substrates. Additionally, rutin and isoquercitrin demonstrated a binding affinity for other critical regions of the enzyme's binding pocket (Karima et *al.,* 2025). Notably, the compounds established interactions within the acylation site, comprising Ser198, His438, and Glu325, by engaging with the catalytic residues Ser198 and His438. Furthermore, they interacted with the choline-binding site by engaging with Trp82, which, along with Phe329, is crucial for stabilizing the quaternary ammonium group of choline ester substrates (Yadav et *al.,* 2025). Moreover, only isoquercitrin was predicted to occupy the acyl-binding pocket of BChE through van der Waals interactions with Val288, Leu286, and Trp231.

Overall, molecular docking results were generally consistent with the experimental cholinesterase inhibition assays, providing a structural basis for the observed biological activities. Several *A. saligna* constituents exhibited favorable docking scores, particularly naringenin and fisetin against AChE and rutin and isoquercitrin against BChE. The extensive interactions established with key residues within the catalytic gorge support their experimentally observed enzyme inhibition and suggest that these compounds may exert their activity through stable occupation of the active site. Nevertheless, minor discrepancies between docking predictions and experimental inhibition are expected, as enzyme activity is influenced not only by binding affinity but also by factors such as ligand solvation, protein flexibility, binding kinetics, and assay conditions, which are not fully captured by molecular docking.

In summary, this investigation provides significant findings, however, its limitations should also be taken into account. Although the integrated phytochemical, *In vitro*, and computational analyses support the cholinesterase inhibitory potential of *A. saligna* phenolic compounds, enzyme kinetic studies were not performed; therefore, the mode of inhibition remains to be established. In addition, while the identified compounds exhibited promising neuroprotective potential, their efficacy should be further validated in relevant cellular and animal models to confirm their neuroprotective effects and elucidate the underlying mechanisms. Furthermore, comprehensive toxicity and pharmacokinetic studies are required to evaluate their safety profile, blood-brain barrier permeability, and therapeutic potential before considering clinical applications. Finally, the predicted binding modes should be further confirmed using complementary approaches, such as molecular dynamics simulations.

Table 4. Two-Dimensional interaction profiles of the top compounds with AChE.

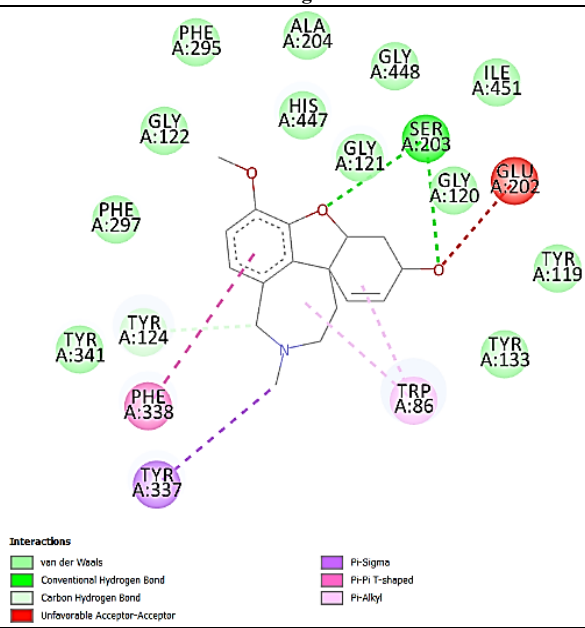
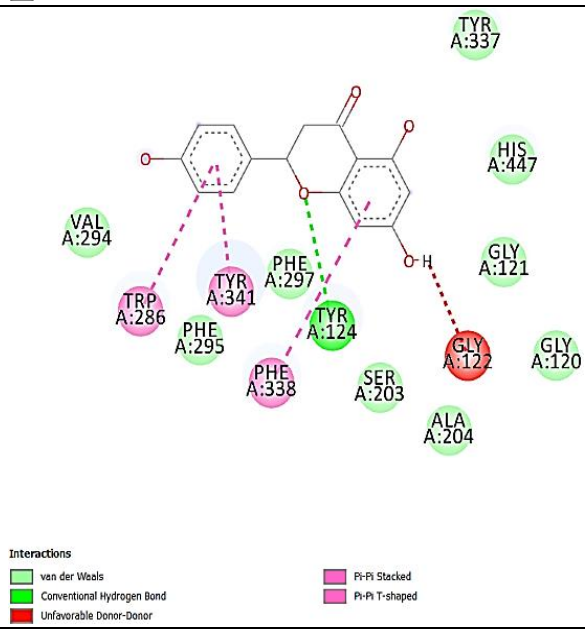
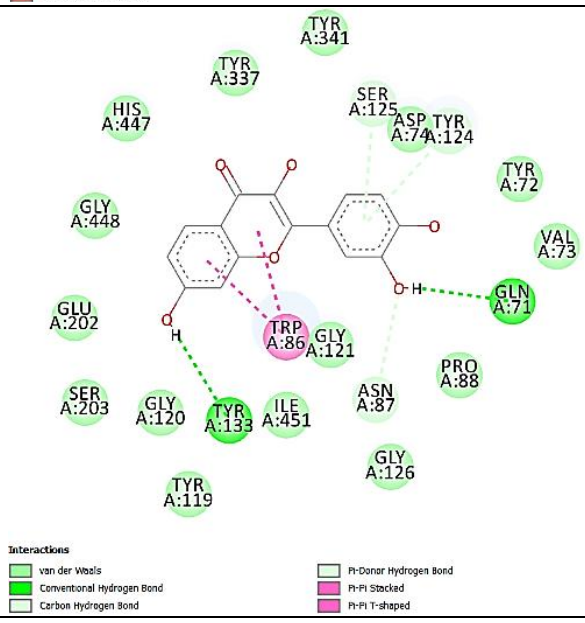
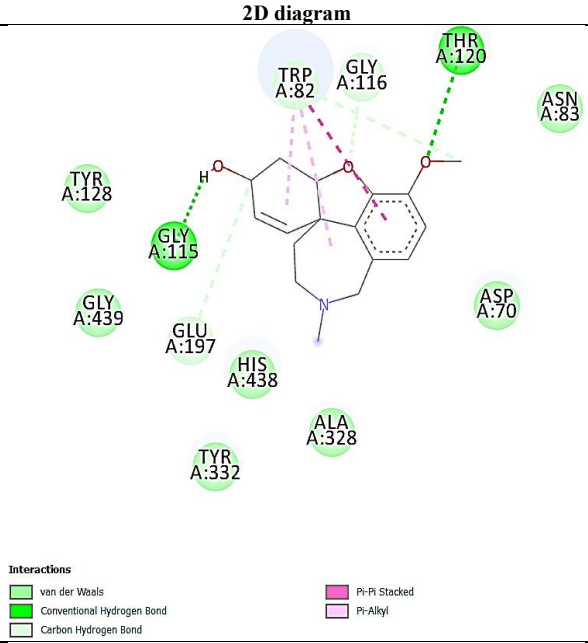
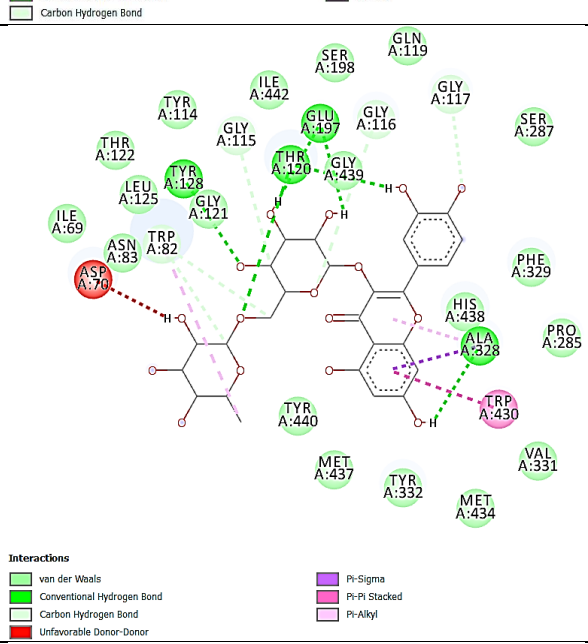
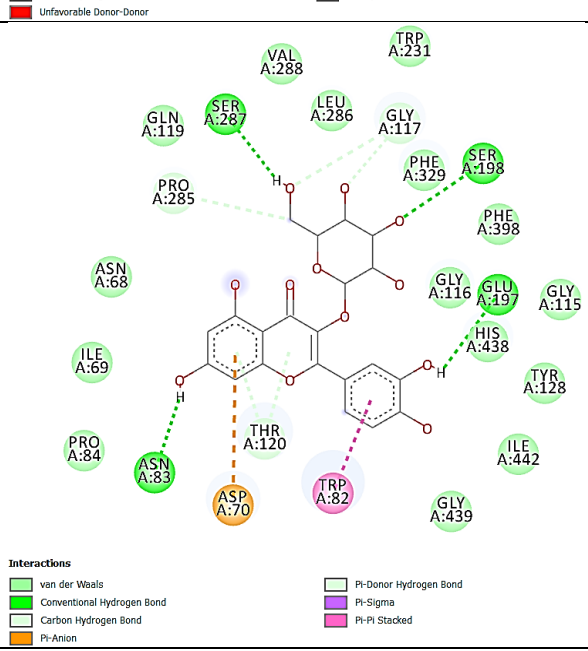
Protein-ligand complex	2D diagram	Interaction profile
Galantamine-AChE complex		H-bond: Ser203. Carbon hydrogen bond: Tyr124. Van der Waals contacts: Tyr133, Tyr119, Gly120, Gly121, Ile451, Gly448, His447, Ala204, Phe295, Gly122, Phe297, Tyr341. Pi-Pi interaction: Trp86, Tyr337, Phe338.
Naringenin-AChE complex		H-bond: Tyr124. Van der Waals contacts: Tyr337, His447, Gly121, Gly120, Ala204, Ser203, Phe297, Phe295, Val294. Pi-Pi interaction: Phe338, Tyr341, Trp286.
Fisetin-AChE complex		H-bond: Tyr133, Gln71. Carbon hydrogen bond: Asn87. Van der Waals contacts: Asp74, Tyr72, Val73, Pro88, Gly126, Gly121, Ile451, Tyr119, Gly120, Ser203, Glu202, Gly448, His447, Tyr337, Tyr341. Pi-Pi interaction: Trp86.

Table 5. Two-Dimensional interaction diagrams of the best compounds with BChE.

Protein-ligand complex	2D diagram	Interaction profile
Galantamine-BChE complex		H-bond: Gly115, Thr120. Carbon hydrogen bond: Trp82, Gly116, Glu197. Van Der Waals contacts: Asp70, Asn83, Tyr128, Gly439, His438, Tyr332, Ala328. Pi-Pi interaction: Trp82.
Rutin-BChE complex		H-bond: Thr120, Glu197, Tyr128, Ala328. Carbon hydrogen bond: Trp82, Gly115, Gly116, Gly117. Van Der Waals contacts: Gln119, Ser198, Gly439, Ser287, Ile442, Tyr114, Gly121, Thr122, Leu125, Ile69, Asn83, Phe329, Pro285, His438, Val331, Met434, Tyr332, Met437, Tyr440 Pi-Pi interaction: Trp82, Trp430, Ala328.
Isoquercitrin-BChE complex		H-bond: Asn83, Glu197, Ser198, Ser287. Van Der Waals contacts: Asn68, Ile69, Pro84, Gln119, Val288, Leu286, Trp231, Phe329, Phe398, Gly116, Gly115, His438, Tyr128, Ile442, Gly434 Pi-Pi interaction: Trp82, Asp70.

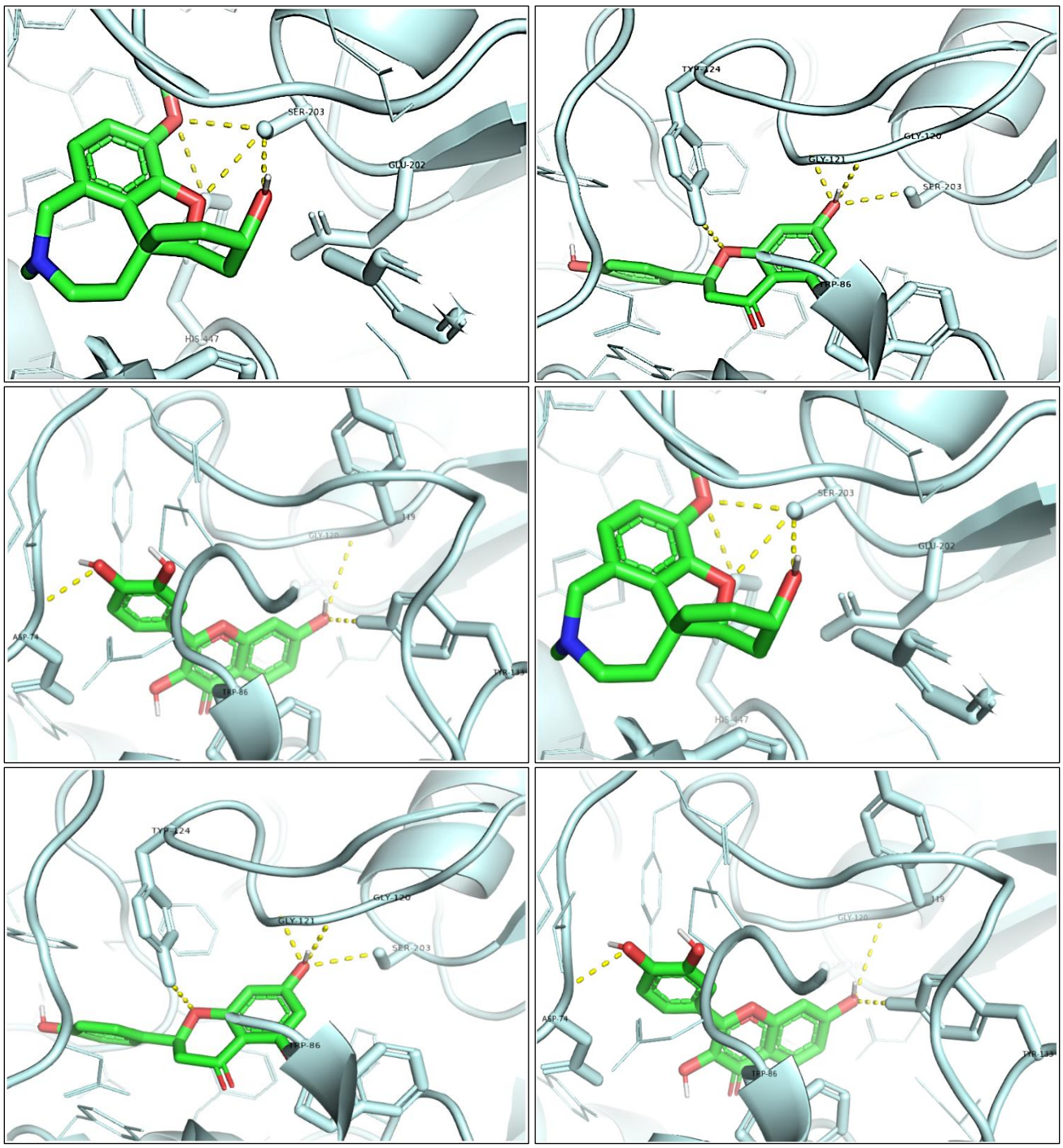


Fig. 3. Three-dimensional visualization of the interactions between the best compounds and the reference inhibitor within AChE and BChE. (A) Galantamine-AChE complex, (B) Naringenin-AChE complex, (C) Fisetin-AChE complex, (D) Galantamine-BChE complex, (E) Rutin-BChE complex, (F) Isoquercitin-BChE complex.

Conclusion

This investigation represents the first comprehensive assessment of the neuroprotective properties of *Acacia saligna* by combining targeted LC-ESI-MS/MS phytochemical profiling, *In vitro* cholinesterase inhibition assays, and molecular docking analyses. The phytochemical investigation identified a chemically diverse profile characterized predominately by phenolic acids and flavonoids, with *trans*-ferulic acid, catechin, isoquercitrin, naringenin, epigallocatechin, rutin, and scutellarin identified among the principal constituents. These compounds have been widely associated with cholinesterase inhibition and neuroprotective activities, suggesting that the biological effects of *A. saligna* are likely related to the combined action of multiple bioactive metabolites. Nevertheless, because the present analysis was based on a targeted LC-ESI-MS/MS approach, additional untargeted metabolomic investigations are warranted to further characterize the phytochemical diversity of this species.

The ethanolic extract exhibited significant inhibitory activity against both acetylcholinesterase and butyrylcholinesterase, supporting the relevance of *A. saligna* as a promising candidate of natural cholinesterase

inhibitors. Molecular docking further complemented the experimental findings by demonstrating favorable binding interactions between the major identified compounds and the catalytic sites of both enzymes, providing mechanistic support for the observed anticholinesterase activity. These findings propose that the neuroprotective activities of *A. saligna* may arise from the complementary and possibly synergistic actions of its phenolic constituents.

Although the combined phytochemical, biological, and computational approaches provide valuable evidence supporting the neuroprotective potential of *A. saligna*, the present findings should be interpreted with caution, as *In vitro* and *In silico* approaches do not fully capture physiological conditions. Therefore, further studies involving bioactivity-guided fractionation, enzyme kinetic analyses, pharmacokinetic and blood-brain barrier evaluation, toxicity assessment, and preclinical experiments are warranted to confirm the therapeutic relevance of the identified constituents and facilitate their future development as candidates for managing Alzheimer's disease and other neurodegenerative disorders.

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