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*Original Article*

# *In silico* and *in vitro* Approach to Identify Memory Enhancers from *Sida rhombifolia* L.

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## ABSTRACT

**Background:** *Sida rhombifolia* L. is a well documented Ayurvedic medicine for the management of neurodegenerative diseases and to enhance cognitive function. Researchers demonstrated its activities under various animal model/s, but lack the probable molecular mechanism in the treatment of Alzheimer's disease. Current study was aimed to identify the acetylcholinesterase (AChE) inhibitory potency of phytocompounds and enriched fractions from *S.rhombifolia* using *in vitro* and network pharmacology approaches. **Methods:** Phytocompounds were retrieved from phytochemical databases, scientific reports and quired for druggability. Protein targets were predicted using BindingDB ($p \geq 0.7$). STRING database and KEGG pathway were utilized to perform gene set enrichment analysis and to identify the probable pathways modulated by the phytocompounds. Cytoscape v3.6.1 was used to construct a target-compound-pathway network. Docking was performed by PyRx 0.8v. Enriched fractions of *S. rhombifolia* were tested for *in vitro* AChE inhibitory potency using the AChE enzyme. **Results:** Among 35 compounds, 26 compounds showed positive drug likeness property. Out of 26 compounds, 9 compounds i.e. 2D-hydroxyecdysone, ecdysone, pterosterone-3-O-β-D-glucopyranoside, acacetin, kaempferol, sanguinine, vascicine, vasicinol, vasicinone were predicted to target AChE and other 9 therapeutic targets involved in Alzheimer's disease (AD). Acacetin scored lowest binding energy with AChE (-8.9kcal/mol). Among the selected enriched fractions, hexane fraction pertains highest AChE inhibition ($IC_{50}$ 12.87µg/ml) compared to clinical approved drug Donepezil ($IC_{50}$ 2.92µg/ml). **Conclusion:** The role of *S.rhombifolia* for the management of AD could be attributed due to the major effect of 2D-hydroxyecdysone, ecdysone, pterosterone-3-O-β-D-glucopyranoside, acacetin, kaempferol, sanguinine, vascicine, vasicinol, vasicinone on AChEand their action on multiple protein molecules associated with AD pathogenesis.

**Key words:** Alzheimer's disease, Acacetin, Acetylcholinesterase, *In silico* docking, Network pharmacology, *Sida rhombifolia*, Sangunine.

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## INTRODUCTION

The most commonly known neurodegenerative disorder with the age-related risk factor is Alzheimer's disease (AD). The specific regions of brain parts demonstrate loss of synaptic connections, including accumulation of abnormal proteins such as extracellular amyloid plaque, intracellular neurofibrillary tangles.[1] Another important characterized feature is the abnormal alteration of Acetylcholine (ACh) neurotransmission; due to lower amount of choline acetyltransferase and decreased ACh released leads to loss of cholinergic functions in neocortex and hippocampus regions of the brain and this phenomenon is common in AD patients.[2] As per Delphi study "24 million people suffering from dementia in 2001 worldwide and this Figure was estimated to double in 2020 and quadruple in 2040".[3] AD generates difficulty in the management of effective treatment due to its multiple mechanisms involved in pathogenesis.

Currently, US-FDA-approved drugs are cholinesterase inhibitors (Donepezil, Rivastigmine, Galantamine) and NMDA receptor modulator (Memantine) as first-line treatments but, these present limitations for the long term treatment due to unavoidable side effects mainly caused by peripheral cholinergic system activation.[4,5] Considerably, it is essential to explore new, effective, and safe drugs. Mankind has advantages from ancient days by the plants, which are an abundant source of phytoconstituents considered as a novel in the therapy of many ailments.[6] Because there is "eighty percent of the world population relies on medicinal plants to meet their primary health care" as per World Health Organization (WHO) reports.[7]

The genus *Sida*. L belongs to the family Malvaceae is well documented in Ayurveda; ancient Indian system of medicine and 17 species are reported to occur in India; used traditionally in *Rasayana* to treat various ailments including degenerative and musculoskeletal disease. Perennial plant *S. rhombifolia* L.is known as 'Mahabala' reported as an anti-inflammatory, anti-arthritic and hepatoprotective effect.[8] The hot aqueous extracts of dried leaf and root of the *S. rhombifolia* are used to treat nervous diseases, heart diseases, burning sensation of the body and as an aphrodisiac and tonic.[9] The recent report claim the effect *of S. rhombifolia* extract to decrease the beta-amyloid accumulation in rat brain.[10]

Presently drug discovery emerges advancement in the treatment of complex diseases like AD with the concept of multi-compound drug therapy using herbs targeting cluster of disease-associated proteins and pathways. Understanding of network pharmacology approach in a per view of the "Lock and Key" model to design ligand that acts on specific targets provides new insights to elucidate the multi-scale mechanisms of action of herbs. Moreover, it is well explained that the master key could "open multiple locks" by targeting many proteins instead of preferring a single target.[11]

However, there are no scientific reports to show the mechanism of *S. rhombifolia* fractions for the management of cognitive dysfunction

and AD. Hence, this work demonstrates to decipher the pathogenic processes, functions, and pathways modulated by the key bioactive phytocompounds against the progression of cognitive dysfunction and AD via compound-gene pathway enrichment, network pharmacology, and *in silico* docking study. Futher interlink the potent fraction from *S. rhombifolia* responsible for the acetylcholinesterase inhibition by an *in vitro* method.

## MATERIALS AND METHODS

### *Mining and drug-likeness property of phytocompounds*

Phytoconstituents from *Sida rhombifolia* (SR) were retrieved from herbs databases i.e. ChEBI, Dr. Duke's database, and earlier scientific reports[12-15] using a keyword "*Sida rhombifolia*". The information such as molecular weight, molecular formula, canonical SMILES, number of hydrogen bond donors, and acceptors of each compound was retrieved from the PubChem chemical database (https://pubchem.ncbi.nlm.nih.gov/). Based on "Lipinski's Rule of Five" model in MolSoft (https://molsoft.com/) the drug-likeness score for each compound was predicted.[16]

### *Target identification*

TTD (Therapeutic Target Database, https://db.idrblab.org/ttd/) was utilized to collect AD-related targets; alongside each protein, gene ID was collected from UniProt (https://www.uniprot.org/) protein database with standard reference to "Homo sapiens" species. Initially, canonical SMILES of phytocompounds having druggable characteristics were submitted into "Find My Compound Targets" of Binding DB web server for target identification at the percentile score of ≥70%. After the target prediction, compounds targeting only approved therapeutic targets involved in the AD which are deposited in the TTD were retrieved.[17] The probable targets modulated by the phytocompounds were shown in Supplementary Table 1.

### *Gene set enrichment and network analysis*

The target/protein(s) responsible for the development of pathogenesis in AD which is modulated by the compounds was speculated by using STRING (https://string-db.org/). The enriched pathways data were collected from the Kyoto Encyclopedia of Genes (KEGG) pathway. The compound-gene-pathway network was constructed using Cytoscape version 3.6.1. The network was analyzed using the command "Network Analyzer" and the network was treated as direct. Interaction among the compound and gene was explicated by the edge count.[18]

### *Docking study*

A docking study was performed using AutoDock Vina. Each compound 3D structure were retrieved from the PubChem database in .sdf format. Compounds were converted into .pdb format using Discovery Studio 2019. Protein molecules x-ray crystallographic structures were retrieved from the RCSB PDB in.pdb format. Compounds with their respective proteins were imported into PyRx 0.8v software and converted into .pdbqt format. Grid box was default and exhaustiveness was kept for 8. The protein-ligand complex having the lowest BE was visualized in DSV v2019.[19]

### *Plant collection and authentication*

Whole plant *S. rhombifolia* L. was collected from Botanical Garden, Karnataka University, Dharwad, Karnataka, India. Authenticated by a botanist from ICMR- National Institute of Traditional Medicine, Belagavi. The herbarium voucher specimen No. RMRC-1399 was deposited at ICMR-NITM, Belagavi for future reference.

### *Extraction and Fractionation of S. rhombifolia*

Drugs and Chemicals used for the study were of analytical grade and procured from (Himedia, India). Dry powder of *S. rhombifolia* (500gms) was subjected to cold maceration to extract thermolabile constituents if any with 70% v/v ethanol for 24 hr. The extract was filtered, and the marc was further subjected to soxhlation (95% v/v ethanol). Filtrates of both maceration and soxhlation were combined and concentrated using a rotary evaporator (IKA RV 10) at 40°C under reduced pressure.

The total extract was collected. Later, fractionation of *S. rhombifolia* extract was carried out as per the method described by Cos P *et al.*[20] with minor modifications.

### *In vitro* estimation of Acetylcholinesterase enzyme by Ellman's method

AChE activity was measured by using a spectrophotometer based on Ellman's method.[21] The enzyme hydrolyzes the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5 mercaptothiocholine and 5-thio-2- nitrobenzoate which can be detected at 412 nm.

Test tube containing 1710 µL of 50 mM Tris–HCl buffer pH 8.0 and 250 µL of crude fractions namely petroleum ether, hexane, chloroform, ethanol and aqueous fractions at the concentrations of 10– 160 µg/ mL, 10 µL 6.67UmL$^{-1}$ AChE and 20 µL of 10 mM of DTNB (5,5'-dithio-bis [2- nitrobenzoic acid]) in buffer was added. Positive control namely Donepezil hydrochloride (10-160µg/ml) (Sigma-Aldrich, USA) were prepared in serial concentration as same as test extract by dissolving in 50 mM Tris–HCl buffer pH 8.0. The mixture was incubated for 15 min at 37°C. Then, 10 µL of acetylthiocholine iodide (200 mM) in buffer was added to the mixture and the absorbance was measured at 412 nm every 10 sec for 3 min, for a blank with buffer instead of enzyme solution was used. The enzyme inhibition (%) was calculated from the rate of absorbance change with time (V= Abs/Δt).

### Statistical Analysis

Values were expressed as Mean±SEM, $p<0.001$ was considered as statistically significant.

## RESULTS

### *Mining and drug-likeness property of phytocompounds and targets prediction*

Thirty-five phytocompounds from the *S. rhombifolia* were identified from the phytochemical interaction database, Dr. Duke's database, Chemical Entities of Biological Interest (ChEBI), and scientific reports. Among 35 compounds, 26 compounds showed a positive drug-likeness score, and 2D-Hydroxyecdysone scored highest i.e. 1.39 (Table 1). Among 26 compounds, 23 compounds were predicted to target 167 protein molecules and 3 compounds failed to predict the targets due to no structural similarity in the database. Further, the peer-interpretation identified 9 compounds to target 10 therapeutic protein molecules associated with AD (Table 2).

### *Gene set enrichment and network analysis*

The gene set enrichment analysis showed 18 molecular pathways modulated by the 9 phytocompounds. Among them, 7 pathways were potentially involved in AD pathogenesis i.e. Serotonergic synapse, Inflammatory mediator regulation of TRP channels, Neuroactive ligand-receptor interaction, Calcium signaling pathway, Gap junction, Cholinergic synapse, and Alzheimer's disease (Table 3). The network analysis showed acacetin, 2D-hydroxyecdysone, ecdysone, pterosterone-3-O-β-D-glucopyranoside, and kaempferol, to score highest edge count and these compounds were identified as Flavonoids and Steroids. Sanguinine,

**Table 1: Druglikeness property of phytocompounds.**

| Compounds | PubChem CID | Molecular Formula | Molecular Weight(g/mol) | HBD | HBA | LogP | DLS |
|---|---|---|---|---|---|---|---|
| 2D-Hydroxyecdysone | 9912297 | $C_{27}H_{44}O_6$ | 464.60 | 5 | 6 | 1.81 | 1.39 |
| Acacetin | 5280442 | $C_{16}H_{12}O_5$ | 284.26 | 2 | 5 | 3.74 | 0.2 |
| Ecdysone | 19212 | $C_{27}H_{44}O_6$ | 464.6 | 5 | 6 | 0.92 | 1.06 |
| Kaempferol | 5280863 | $C_{15}H_{10}O_6$ | 286.24 | 4 | 6 | 1.61 | 0.5 |
| Pterosterone-3-O-β-D-Glucopyranoside | 441836 | $C_{27}H_{44}O_7$ | 480.6 | 6 | 7 | 0.74 | 1.1 |
| Sanguinine | 443722 | $C_{16}H_{19}NO_3$ | 273.33 | 2 | 4 | 1.09 | 0.77 |
| Vascicine | 72610 | $C_{11}H_{12}N_2O$ | 188.23 | 1 | 2 | 1.08 | 0.33 |
| Vasicinol | 442934 | $C_{11}H_{12}N_2O_2$ | 204.22 | 2 | 3 | 0.62 | 0.61 |
| Vasicinone | 442935 | $C_{11}H_{10}N_2O_2$ | 202.21 | 1 | 3 | 0.35 | 0.38 |

HBD Hydrogen Bond Donor; HBA Hydrogen Bond Acceptor; LogP; Partition Co-efficient; DLS Druglikeness score

**Table 2: Probable targets involved in AD modulated by phytocompounds.**

| Compound | Compound type | Gene ID (compound – target probability score) |
|---|---|---|
| 2D-Hydroxyecdysone | Steroid | HTR2A (0.73), HTR2C (0.73), ACHE (0.7), BACE1 (0.76), BCHE (0.98) |
| Acacetin | Flavonoid | ACHE (0.7), ADORA2A (0.84), MAOA (0.84), MAOB (1.0), APP (0.79), BACE1 (0.7), BCHE (0.7) |
| Ecdysone | Steroid | HTR2A (0.71), HTR2C (0.71), ACHE (0.7), BACE1 (0.74), BCHE (0.7)1 |
| Kaempferol | Flavonoid | ACHE (0.7), ADORA2A (0.97), MAOA (0.97), MAOB (0.86), BACE1 (0.7) |
| Pterosterone-3-O-β-D-Glucopyranoside | Steroid | HTR2A (0.7), HTR2C (0.7), ACHE (0.7), BACE1 (0.73), BCHE (0.7) |
| Sanguinine | Alkaloid | ACHE (0.98), CHRM1 (0.7), BCHE (0.98) |
| Vascicine | Alkaloid | ACHE (0.7), BCHE (0.7) |
| Vasicinol | Alkaloid | ACHE (0.76), BCHE (0.76) |
| Vasicinone | Alkaloid | ACHE (0.7), BCHE (0.71) |

**Table 3: Enrichment analysis of protein targets involved in AD.**

| KEGG ID | Pathway description | Gene count | False Discovery Rate | Protein targets within the network |
|---|---|---|---|---|
| has04726 | Serotonergic synapse | 5 | 3.96E-08 | APP, HTR2A, HTR2C, MAOA, MAOB |
| hsa04750 | Inflammatory mediator regulation of TRP channels | 2 | 0.0016 | HTR2A, HTR2C |
| hsa04080 | Neuroactive ligand-receptor interaction | 3 | 0.00082 | CHRM1, HTR2A, HTR2C |
| hsa04020 | Calcium signaling pathway | 3 | 0.00054 | CHRM1, HTR2A, HTR2C |
| hsa04540 | Gap junction | 2 | 0.0016 | HTR2A, HTR2C |
| hsa04725 | Cholinergic synapse | 3 | 0.0022 | ACHE, BCHE, CHRM1 |
| hsa04728 | Dopaminergic synapse | 2 | 0.0027 | MAOA, MAOB |
| hsa05010 | Alzheimer's disease | 2 | 0.0041 | APP, BACE1 |

Vascicine, Vasicinol, and Vasicinone scored lowest edge count and these compounds were identified as Alkaloids, predicted to target only AChE and BChE (Figure 1 and Figure 2).

### *Docking study*

Acacetin scored lowest BE with ACHE (-8.9kcal/mol) by forming one hydrogen bond i.e. Thr124; with ADORA2A (-7.9kcal/mol) via one bond Glu169; with MAOA (-8.5kcal/mol) via two bonds i.e. Glu329, and Arg172; with MAOB (-10.4) via 5 bonds i.e. Met436, Arg42, Ser15, Gly16, and Arg36; with APP (-6.4) via 3 bonds i.e. Ala9, Glu7, and Phr23. Kaempferol scored lowest BE with BACE1 (-8.2kcal/mol) via 4 bonds i.e. Thr72, Thr72, Thr231, and Tyr198. Pterosterone-3-O-β-D-Glucopyranoside scored lowed BE with HTR2A (-8.1kcal/mol) via 4 bonds i.e. Arg173, Thr109, Thr109, and Asp172. Sanguinine OH group scored lowest BE with CHRM1 (-7.9kcal/mol) via one bond i.e. Tyr106. Pterosterone-3-O-β-D-Glucopyranosidescored with BCHE (-9.5kcal/mol) via 2 bonds i.e. Pro285 and Tyr440. However, Sanguinine, Vascicine, Vasicinol, and Vasicinone were also showed the best affinity with AChE and BChE via forming hydrogen bonds with amino acid residues. The probable score, binding energy, and hydrogen bond interaction of ligands with their respective targets were summarized (Table 4).

### *In vitro AChE inhibition*

The preliminary phytochemical investigation identified the presence of flavonoids, saponins, alkaloids, tannins, steroids, and polyphenols in a hydroalcoholic extract of the whole plant of *S.rhombifolia*. Similarly, the phytoconstituents reported in fractions were summarized in Table 5 as per earlier reports.[22-25]

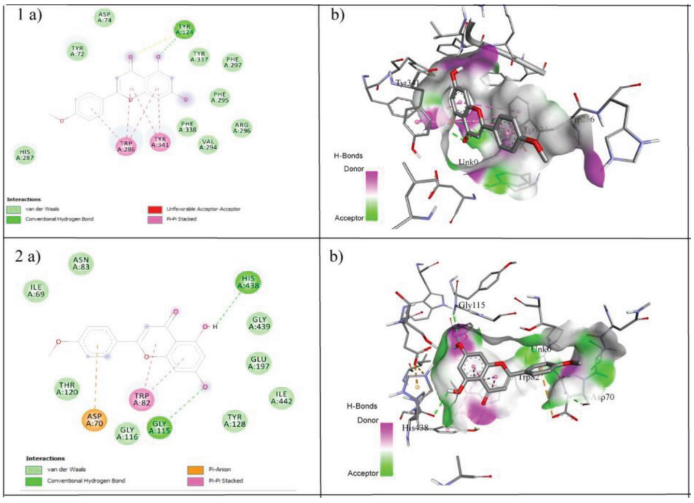


**Figure 1:** Network representation of the interaction of Phytocompounds-target proteins-pathways.

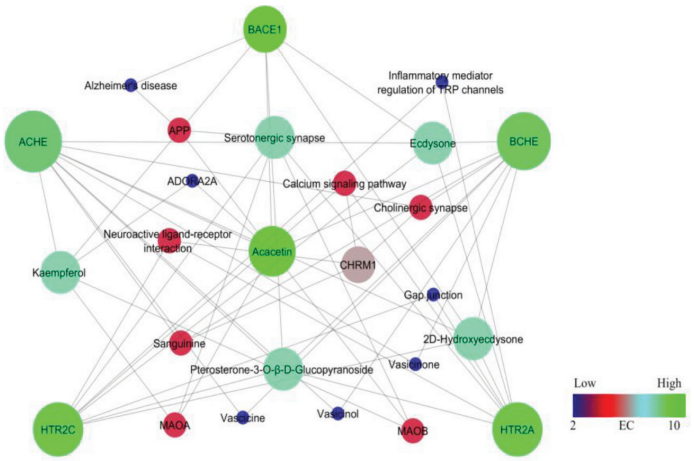


**Figure 2:** Binding analysis of Acacetin with AChE and BChE. **1)** Interaction of Acacetin with AChE a) 2D representation b) Acacetin within AChE binding pocket **2)** Interaction of Acacetin with BChE a) 2D representation b) Acacetin within BChE binding pocket

The hexane fraction reported the presence of terpenoids, steroids, phytosterols; Ethanol fraction showed the presence of alkaloids, terpenoids, flavonoids, saponins, steroids; Aqueous fraction showed the presene of alkaloids, flavonoids, saponins; Chloroform fractions reported the presence of alkaloids, phenols; Pet ether solvent extracted terpenoids, fixed oil and fats from *S. rhombifolia.*

potentially inhibited the Acetylcholinesterase enzyme; Hexane fraction> Ethanol fraction >Aqueous fraction >Chloroform fraction >Pet ether fraction with $IC_{50}$ value at 12.87<18.96<20.75<30.47<41.95 (µg/ml) respectively. The Hexane fraction was found to show prominent AChE inhibitory activity which was compared to clinically approved molecule Donepezil -2µg/ml (Table 5).

## DISCUSSION

The current study dealt to reveal the effect of *S. rhombifolia* in the treatment of AD by network pharmacology coupled with docking studies further, correlated with *in vitro* AChE inhibition assay to assure modulation of hallmark in AD by *S. rhombifolia* enriched fractions. The network pharmacology analysis was carried out to known the molecular mechanism of action and pharmacological basis of *S. rhombifolia* with specific proteins and their relationship with disease-modifying capability. Traditional herbs are the source of new drug discovery hence, utilization of multiple compounds containing herbs having a traditional claim against particular disease with minimal side effects via polypharmacology and bioinformatics approaches could be a new treatment strategy, supports the scientific validation of herbal medicines.[26] Polypharmacology and bioinformatics deal with the effect of drugs on multiple protein targets modulating disease pathways.[27]

Initially, phytocompounds druggable characteristics were predicted and compounds having drug-like properties were utilized for further analysis. We predicted probable targets of each phytocompounds having a drug-like property using Binding DB and compounds only modulating therapeutic targets associated with AD at the probable score of ≥0.7 were separated, and gene set pathways enrichment and network analysis were performed. Target prediction phased with the known structures to modulate biological activity by its probable protein targets by ligand as agonists or antagonists,[28] considering pathological hallmarks of AD includes a variety of signaling pathways abnormalities along with dysfunction of G protein-mediated adenylate cyclase signaling unit.[29] Current finding reports that the phytocompounds of *S. rhombifolia* targets G-Protein belonging receptors such as CHRM1, CHRM3, and HTR2A as well as production of amyloid precursor protein (APP), beta-amyloid (Aβ) signaling cascade, and Aβ- degradation. This is supported by Liu *et al.*[30] reports suggesting that G-protein serves as a therapeutic key for AD. The probable target proteins modulated by the *S. rhombifolia* phytochemicals were subjected to gene set enrichment analysis to identify the pathways modulated by the compounds. We identified 9 compounds to modulate 10 therapeutic protein targets associate with AD i.e. Amyloid Precursor Protein (APP), 5-hydroxytryptamine receptor 2A (HTR2A), 5-hydroxytryptamine receptor 2C (HTR2C), Cholinergic Muscarinic 1 (CHRM1), Acetylcholinesterase (AChE), Butyrylcholinesterase (BChE), Amine oxidase [flavin-containing] A (MAOA), Amine oxidase [flavin-containing] B (MAOB), and Beta-secretase 1 (BACE1). These compounds were recognized as steroids, flavonoids, and alkaloids. Further, Serotonergic synapse, Inflammatory mediator regulation of TRP channels, Neuroactive ligand-receptor interaction, Calcium signaling pathway, Gap junction, Cholinergic synapse were identified as an enriched pathways in AD pathogenesis. Interestingly, these pathways were identified as G protein-mediated pathways.

Serotonin neuronal loss is involved in cognitive mechanisms such as short and long-term memory alongside this also regulates and co-localizes with cholinergic, GABAergic, glutamatergic neurons.[31] Transient Receptor Potential Ankyrin 1 (TRPA1) channel considered as a "promiscuous" receptor expressed in many regions of the hippocampus including in astrocytes which plays a major role in intracellular calcium level and activation of this channel by free radicals or inflammatory mediator leads to several disruptive changes in the brain.[32] In the current study, three-hit druggable steroidal molecules i.e. 2D-Hydroxyecdysone, Ecdysone, and Pterosterone-3-O-β-D-Glucopyranoside from *S. rhombifolia* were predicted to interact with the HTR2A and HTR2C protein targets, which are directly linked with TRPA1 and serotonergic synaptic pathways. Predominantly, these compounds are also predicted to interact with AChE, BChE, and BACE1.

Disruption of $Ca^{2+}$ and its homeostasis is ubiquitously involved to induce synaptic deficits which promotes accumulation of Aβ plaques and neurofibrillary tangles.[33] Another hallmark of AD is a phenotypic change in microglia and astrocytes occurring in gliosis is another hallmark in AD, this response is interlinked with alteration in function and protein expressions forming gap junction channels are

**Table 4: Affinity of phytocompounds with their respective targets.**

| Target name | PDB ID | Compound | BE (kcal/mol) | HBI (amino acid….ligand) |
|---|---|---|---|---|
| **5-hydroxytryptamine receptor 2A (HTR2A)** | 6A94 | 2D-Hydroxyecdysone | -7.7 | Asn107…O-, Asn107…OH, Asn110…OH, Asn384…OH |
| | | Ecdysone | -8.0 | Asn110…=O, Glu318…OH |
| | | Pterosterone-3-O-β-D-Glucopyranoside | -8.1 | Arg173…=O, Thr109…OH, Thr109…=O, Asp172…OH, His183…OH |
| **Acetylcholinesterase (AChE)** | 4PQE | 2D-Hydroxyecdysone | -7.4 | Gln413…OH, Glu313…OH, Thr238…=O, His405…OH, |
| | | Acacetin | -8.9 | Thr124…O- |
| | | Ecdysone | -7.4 | Asn233…OH |
| | | Kaempferol | -8.5 | Ser293…OH |
| | | Pterosterone-3-O-β-D-Glucopyranoside | -8.2 | Arg296…OH, Pro235…OH, Pro368…OH |
| | | Sanguinine | -7.8 | Ser293…OH |
| | | Vascicine | -7.9 | Thr83…OH |
| | | Vasicinol | -8.2 | Nil |
| | | Vasicinone | -8.5 | Ser125…=O, Thr83…OH |
| **Adenosine Receptors A2a (ADORA2A)** | 3UZA | Acacetin | -7.9 | Glu169…OH |
| | | Kaempferol | -7.7 | Thr227…OH, Asn39…=O |
| **Amine oxidase [flavin-containing] A (MAOA)** | 2Z5X | Acacetin | -8.5 | Glu329…OH, Arg172…O- |
| | | Kaempferol | -8.2 | Phe112…=O, Phe112…OH, Val115…O- |
| **Amine oxidase [flavin-containing] B (MAOB)** | 1OJD | Acacetin | -10.4 | Met436…O-, Arg42…O-, ser15…=O, Gly16…=O, Arg36…Oh, Tyr393…OH |
| | | Kaempferol | -9.0 | Pro102…OH, Gln206…O-, Leu164…OH |
| **Beta-secretase 1 (BACE1)** | 3UQU | 2D-Hydroxyecdysone | -6.6 | Gly11…OH, Glu310…O- |
| | | Acacetin | -8.0 | Thr231…=O, Thr72…=O |
| | | Ecdysone | -6.9 | Gly90…OH |
| | | Kaempferol | -8.2 | Thr72…=O, Thr72…O-, Thr231…=O, Tyr198…OH |
| | | Pterosterone-3-O-β-D-Glucopyranoside | -6.8 | Gly230…OH, Thr232…OH, Thr232…O- |
| **Butyrylcholinesterase (BChE)** | 4TPK | 2D-Hydroxyecdysone | -8.9 | Thr120…OH, Ser287…OH, Glu197…OH |
| | | Acacetin | -9.2 | His438…OH, Gly115…O- |
| | | Ecdysone | -8.7 | Pro285…OH |
| | | Pterosterone-3-O-β-D-Glucopyranoside | -9.5 | Pro285…Oh, Tyr440…OH |
| | | Sanguinine | -8.7 | His438…OH, Glu197…OH, Ser198…OH |
| | | Vascicine | -7.8 | His438…NH, Tyr440…OH |
| | | Vasicinol | -7.9 | His438…OH, Trp82…O- |
| | | Vasicinone | -8.1 | Trp82…O-, His438…NH |
| **Cholinergic, muscarinic 1 (CHRM1)** | 5CXV | Sanguinine | -7.9 | Tyr106…OH |

PDB Protein Data Bank; BE Binding Energy; HBI Hydrogen bond Interaction

majorly connexins(Cxs). Amyloid β having the most reactive astrocytes regions were found to have increased Cxs expression.[34,35] APP undergoes proteolysis through BACE1 which generates Aβ. Current research focuses on germline deletion of the BACE1 gene so that the formation of Aβ is blocked which is a future promising to treat AD.[36,37] The previous literature by Semwal *et al.*[38] supports that acacetin is a potent flavonoid that acts as neuroprotective, anti-inflammatory, antidiabetic, anticancer, and antimicrobial agent, also expresses inhibitory effects against acetyl-cholinesterase, cyclo-oxygenase, xanthine oxidase enzymes, glutathione reductase, and aldose reductase enzymes.[39] Considering Kaempferol, a natural flavonol, ameliorates disruption of the neuronal cell membrane and mitochondria caused by oxidative stress indicates anti-oxidant properties.[40] Presently, we identified acacetin, Kaempferol as potent inhibitors of APP, AChE, ADORA2A, MAOA, MAOB, APP, BACE1, and BChE; this is also linked by *S. rhombifolia* flavonoid-rich fraction inhibited AChE enzyme as indicated by *in vitro* study.

**Table 5: Major phytoconstituents and AChE inhibitory activity of *S. rhombifolia fractions*.**

| *S. rhombifolia* Fractions (%yeild) | Major phytoconstituents reported in fractions | AChE inhibition $IC_{50}$ ( µg/ml) |
|---|---|---|
| **Pet Ether (0.3)** | terpenoides, fixed oil and fats | 41.95±2.14* |
| **Hexane (0.56)** | terpenoids, steroids, phytosterols | 12.87±0.64* |
| **Chloroform (0.28)** | alkaloids, phenols | 30.47±1.10* |
| **Ethanol (0.69)** | alkaloids, terpenoids, flavonoids, saponins, steroids, | 18.96±0.96* |
| **Aqueous (0.46)** | alkaloids, flavonoids, saponins, | 20.75±0.55* |
| **Donepezil** | Standard drug | 2.92±0.39 |

Mean±SEM were expressed as * p<0.001 compared to Donepezil.

BChE is closely associated with the regulation of AChE levels and in AD, this is predominantly increased which is denoted by the presence of neurofibrillary tangles and neuritic amyloid-rich plaque.[41] The earlier study reports that PCR and western blot analysis revealed that the acacetin interferes with Aβ production by activity on BACE-1 and APP synthesis as well as regulates the human BACE-1 and APP mRNA levels.[42]

Many of the flavonoids have been implicated in cognitive and neuroprotective functions through their mechanism of actions on AChE, BChE, and BACE-1 inhibitory properties by interacting with PI3-kinase/Akt and ERK signaling pathways. Alongside they also improve hippocampal neurogenesis and vascular blood flow hence; these are effective to manage AD.[43,44] Sanguinine showed inhibitory activity against CHRM1 receptor along with AChE and BChE indicating its potent capability to treat AD. As per the earlier reports, Sanguinine alkaloid obtained from many plants of the genus *Narcissus* belongs to the Amaryllidaceae family have Galantamine and Lycorine skeleton types and found to possess potent AChE inhibitory.[45,46] Interestingly, all nine compounds namely 2D-hydroxyecdysone, Acacetin, ecdysone, kaempferol, pterosterone-3-O-β-D-glucopyranoside, Sanguinine, vascicine, vasicinol, vasicinone belongs to steroidal, flavonoid, and alkaloid class showed potent inhibitory activity against AChE which idealized its importance in AD treatment.

Furthermore, ADORA2A (Adenosine Receptor Subtype A2a) plays a crucial role in the control of synaptic plasticity and neurogenesis in the CA3 region of the hippocampus. This is also involved in NMDA-dependent synaptic transmission in the hippocampus. ADORA2A receptor inhibition can prevent Aβ- induced synaptotoxicity and memory dysfunction through a p38 MAPK-dependent pathway; along with localization of microglial cells.[47,48] Further, *S.rhombifolia* contained flavonoids acacetin and kaempferol act on the ADORA2A protein target potentially gives important clues for the treatment of AD.

*S. rhombifolia* enriched fractions inhibited AChE enzyme especially hexane fraction major phytoconstituents: phytosterols, steroids, and terpenoids reduce the enzyme levels which was reflected by the previous study demonstrated by Mah SH *et al*.[49] Another recent study explored anticholinergic and antioxidant activities of *S. rhombifolia* hydro-ethanolic extract in cognitive deficit rats.[10]

## CONCLUSION

Compound-gene set enrichment, network pharmacology, and docking studies of *S.rhombifolia* modulated pathogenic pathways associated with AD via targeting major therapeutic targets such as ACHE, BCHE, APP, BACE1, ADORA2A, MAOA, MOAB by steroids (2D-hydroxyecdysone, ecdysone, pterosterone-3-O-β-D-glucopyranoside), alkaloids (sanguinine, vascicine, vasicinol, vasicinone), and flavonoids (kaempferol, acacetin) hence, reports multiple-protein targets with synergistic effects in the treatment of AD. The *in silico* study data was correlated with *in vitro* AChE enzyme inhibition which assured to modulate major pathological pathway of AD. Furtustically, a study can be extrapolated to well-designed wet-lab protocols that will prove the findings accordingly.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**AChE:** Acetylcholinesterase; **AD:** Alzheimer's disease; **ADORA2A:** Adenosine A2a Receptor; **APP:** amyloid precursor protein; **BChE:** Butylcholinesterase; **BE:** Binding Energy; **ChEBI:** Chemical Entities of Biological Interest; **CHRM1:** Cholinergic Receptor Muscarinic 1; **CHRM3:** Cholinergic Receptor Muscarinic 3; **DSV:** Discovery Studio Visualizer; **HTR2A:** 5:Hydroxytryptamine Receptor 2A; **$IC_{50}$:** Half:maximal inhibitory concentration; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **NMDA:** N:methyl:D:aspartate; **PCR:** Polymerase Chain Reaction; **RCSB:** Research Collaboratory for Structural Bioinformatics; **PDB:** Protein Data Bank; **SMILES:** Simplified molecular input line entry system; **STRING:** Search Tool for the Retrieval of Interacting Genes/Proteins; **TTD:** Therapeutic Data Base.

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