A combinatorial approach to screen structurally diverse acetylcholinesterase inhibitory plant secondary metabolites targeting Alzheimer’s disease

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ABSTRACT
Alzheimer’s disease (AD) is a form of Dementia known to diminish the brain’s function by perturbing its structural and functional components. Though cholinesterase inhibitors are widely used to treat AD, they are limited by numbers and side effects. Hence, present study aims to identify structurally diverse Acetylcholinesterase (AChE) inhibitory plant secondary metabolites (PSM) by employing high throughput screening and computational studies. AChE inhibitory activity was performed using 390 crude extracts from 63 plant parts belongs to 58 plants. The lowest IC50 value was recorded by acetone extract of Cyperus rotundus rhizome at 0.5 mg/ml, followed by methanol extract of Terminalia arjuna bark (0.95 mg/ml) and water extract Acacia catechu stem (0.95 mg/ml). A virtual library containing 487 PSM belongs to 18 plants found positive for AChE inhibition (IC50 ≤5 mg/ml) was prepared. Through ADMET analysis, 78 PSM fulfilling selected drug-likeness parameters were selected for further analysis. Molecular docking studies of selected PSM against AChE recorded a wide range of binding energy from −3.40 to −10.90 Kcal/mol. Further molecular dynamics simulation studies also recorded stabilized interactions of AChE-ligand complexes in the term of RMSD, RMSF, Rg, SASA, and hydrogen bond interaction. MMPBSA analysis revealed the binding energy of selected PSM ranging from −123.757 to −261.697 kJ/mol. Our study demonstrated the potential of 12 PSM (Sugiol, Margolone, 7-Hydroxy-3',4'- (Methylenedioxy) flavan, Beta-cyprone, Ethenone, Isomargolonone, Serpentine, Cryptolepine, Rotundone, Strictamin, Rotundenol and Nootkatone) as AChE inhibitors. Further in vitro and in vivo experimental evaluations with pure PSM could be beneficial for therapeutic uses.

1. Introduction
Dementia is a condition resulting in the impairment of the brain’s function by perturbing its structural and functional components (Geldmacher & Whitehouse, 1996). Dementia is represented by a group of disease among which Alzheimer’s disease (AD) is a top form which degenerates the nervous system by modulating different physiological and biochemical processes (Colovic et al., 2013). The pathological advancement of AD is directly or indirectly associated with age. The earlier assumption that pathological development of AD initiates only at an older age was ruled out. Recent studies evidenced that AD’s pathological features can initiate at the early age of 20 years (Gonneaud et al., 2017). The disease’s advancement and severity depend on various factors...
such as oxidative stress, food habits, metabolic disorders, etc (Craft, 2009; Tönnes & Trushina, 2017). It was estimated that one billion people might be affected by AD until 2030 (Qiu et al., 2009). Two structural features, such as β-amyloid deposition (induced by the activity of beta-secretase, gamma-secretase, and alpha-secretase, and a mutated form of amyloid precursor protein) and formation of tangles (due to the enhanced phosphorylation of the tau protein) are the significant indicators of the AD.

Additionally, the cholinergic system also plays a vital role in the mechanism inspired AD development that depends on the concentration of acetylcholine (ACh, neurotransmitter), as well as the activity of three enzymes such as Acetylcholine esterase (AChE), Butyrylcholine esterase (BChE) (catalyzation of reaction) and Choline-acetyltransferase (ChAT) (biosynthesis). Alzheimer’s disease is associated with some prominent features affected by the concentration of the ACh. In a recent study, Grimaldi et al. (2016) reported that the ACh favours the soluble peptide confirmation of β-amyloid plaques, thereby reducing its toxic effect.

Human AChE is a monomeric protein with 614 amino acids, composed of 12 beta-sheet and the 14 alpha-helix that formed a tertiary structure (Dvir et al., 2010). The catalysis of the ACh by the hAChE takes places by the involvement of several amino acid residues in, active site triad (Ser203, His447, Glu334), and Oxyanion hole (Gly121 Gly122, Ala194). Anionic subsites binding site, ASB (Trp266, Phe295, Phe297, Phe338, Gly448, Ile515), Acyl binding pocket, ABP (Trp236, Phe295, Phe297, Phe338), and Peripheral anionic site, PAS (Asp74, Tyr124, Ser125, Trp266, Tyr337, Tyr341). The enzymes catalyze the ACh into choline and acetate in the synaptic cleft, which was further recycled in neuronal cell bodies by the action of choline acetyltransferase (Waymire, 2020). AChE is one of the fastest enzymes of the human system, with a turnover ratio of $3 \times 10^7$ per min per molecule (Wilson & Harrison, 1961). Optimum activity of AChE is a prerequisite for regular cognitive brain function. Additionally, AChE also assists in developing nerve cells and reducing the size of dendritic extensions, axon outgrowth, and neuronal morphogenesis (Behra et al., 2002; Bigbee et al., 1999; Duval et al., 1992; Soreq & Seidman, 2001). Under physiological conditions, the hyperactivation of AChE is linked with their existence as a tetrameric form associated with the proline-rich attachment domain of either collagen-like Q subunit or proline-rich membrane-anchoring protein (Alvarez et al., 1997; Simon et al., 1998). The upholding activity of AChE participates in AD’s pathological advancement via reducing the concentration of ACh in the synaptic cleft and sustaining the aggregation of β-amyloid (Grimaldi et al., 2016; Koenigsberger et al., 1998).

Among the available strategies to manage AD, competitive inhibitory drug molecules against AChE are considered most promising. AChE competitive inhibitors slow down the enzyme activity, restoring ACh’s appropriate concentration at the synaptic cleft (Berg et al., 2002). In this regard, four drugs, such as tacrine, galantamine, rivastigmine, and donepezil, have been approved by the FDA, which reversibly inhibits AChE. However, long-term consumption of such drugs is reported to be adversely affecting the human’s different organs (cholinergic side effect, gastrointestinal disorder, and hepatotoxicity) (Colovic et al., 2013). Hence, the competitive or reversible AChE inhibitors of plant origin can be further explored for effective and safer AD treatment without adverse effects (Bi et al., 2009).

Due to their structural and functional diversity, PSM is considered the repository of safe drugs that can prevent and cure several diseases and disorders of humans and animals. Earlier, the potential of crude/purified PSM has been successfully demonstrated to treat different life-threatening diseases such as cancer, malaria, Alzheimer’s, diabetes, etc (Bi et al., 2009; Calc et al., 2012; Esposito et al., 2006; Murray et al., 2013; Tafesse et al., 2017; Velander et al., 2017; Yi et al., 2015). Artemisinin is a sesquiterpene lactone obtained from Artemisia annua used as antimalarial drugs (Klayman, 1985). Nitisinone from the Callistemon citrinus depletes the tyrosine level, leading to tyrosinemia’s effective treatment (Das, 2017). Irwin and Smith III (Irwin & Smith, 1960) first reported the AChE inhibition potential of galantamine from Galanthus woronowii and Galanthus nivalis. Atropine (daturin) is an alkaloid obtained from the Atropa belladonna (Kamada et al., 1986), used under various conditions such as myopia during the surgery for improvement of slow heart rate, and depletion of saliva production, pesticides detoxification, and nerve agent by the reduction of the excess effect of ACh by the muscarinic antagonist effect (Yi et al., 2015). Cannabidiol is a phytocannabinoid obtained from Cannabis sativa, which reduces the hyperphosphorylation of the tau protein in the PC-12 cell lines and reduces β-amyloid inspired toxicity (Esposito et al., 2006). Capsaicin a pharmacologically important alkaloid obtained from Capsicum annuum, used to lower cholesterol levels (Srinivasan et al., 1980) and pain reliever (Anand & Bley, 2011). However, the possible use of PSM for AD treatment by employing them as enzyme inhibitors, disaggregation of the β-amyloid, and reducing phosphorylation of tau (τ) protein (Calcul et al., 2012; Velander et al., 2017) is sparingly studied.

The process of advancing in applying computational studies in drug discovery opened a new avenue towards screening many PSM and understanding their molecular interactions with the target site. Further, the same can be employed to predict the drug likeliness and bioavailability of PSM. In several recent research pieces, the computational tools were successfully employed to a large number of PSM against various diseases such as antiviral, antimicrobial, anticancer, and antidiabetic (Puttaswamy et al., 2020; Selvaraj, 2018; Tyagi et al., 2013), etc. The present study aims to integrate in vitro and computational studies to screen medicinal plant and their AChE inhibitory activity with the following objective (1) Analyzing the potential of AChE inhibitory activity of selected medicinal plants, (2) Creating PSM library of AChE inhibitory plants and analyzing their drug-like properties, and (3) Molecular docking and Molecular dynamics simulation of most promising PSM.

Creating PSM library of AChE inhibitory plants and analyzing their drug-like properties, and (3) Molecular docking and Molecular dynamics simulation of most promising PSM.
2. Material and methods

2.1. General experimental procedures

2.1.1. Plant material

Sixty-six samples belong to 58 plant species (Supplementary Table 1) were collected from different regions of India (Karnataka, Tamil Nadu, Kerala, and Delhi) and were identified based on their morphological characters following taxonomical keys (https://sites.google.com/site/efloraofindia/home). Moreover, the identity was also confirmed with the plant taxonomist (Dr. Sampat Kumar, University of Davangare, Karnataka), and samples were also sent to the Botanical Survey of India (BSI) Kolkata, India, in the form of herbarium for identification.

2.1.2. Chemicals

Acetylcholine esterase from *Electrophorus electricus* (electric eel) Type VI-S (SIGMA Cat No. C2888), Acetylthiocholine iodide (ATCI), 5,5-Dithiobis-(2-Nitro Benzoic Acid) (DTNB), sodium phosphate di and monobasic hydrates, and solvents were procured from Sisco Research Laboratories.

2.1.3. Equipment

Prestige stylo mixer grinder, 750 watts, Soxhlet apparatus, Rotary Evaporator Hei-VAP Core HL G3 XL, Buchi, Switzerland and Epoch Microplate Spectrophotometer, Biotek.

2.1.4. Plant material processing and metabolites extraction

The plant samples were chopped to 0.5-2.0 cm bits and dried in a hot air oven (Oven universal, India) at 45 °C for 3-5 days. The dried samples were coarsely powdered using a mechanical blender. The extraction of crude metabolites from powdered plant samples was performed in the Soxhlet apparatus using different solvents systems with increasing polarity (Hexane > Chloroform > Ethyl acetate > Acetone > Methanol > Water). Crude extracts were concentrated using rotavapor under reduced pressure. The dried and powdered crude extracts were weighed, and a stock of 10 mg/ml was prepared using methanol, and further dilutions were prepared using water. The stock solution and dilution of water extract were prepared in water.

2.1.5. In vitro acetylcholinesterase inhibition assay

The AChE inhibition assay was performed according to Elman’s method (Ellman et al., 1961) with minor modification. 170 μl AChE (0.85 U in 0.1 M sodium phosphate buffer pH 8) was incubated with 30 μl different concentrations (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 mg/ml) of plant extracts for 10 min at 37 ± 1 °C. Following this, 15 μl DTNB (14 mM in ethanol) was added to the reaction mixtures and incubated for 5 min. To this, 15 μl of ATCI (10 mM in water) was added and further incubated for 30 min at 37 ± 1 °C. The enzyme activity was recorded by measuring the OD at 412 nm in a microtiter plate reader. The reaction mixture where an equal volume of corresponding methanol replaced plant extract: water combination served as control. The enzyme inhibition was calculated with respect to the control, and results were interpreted in IC50 values.

The percentage inhibition of the plant extracts was calculated using the following formula:

\[
\text{Inhibition}(\%) = \left( \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100
\]

2.2. In silico studies

2.2.1. Virtual PSM library preparation

The plant extracts that inhibited the activity of AChE at a significant IC50 value (≤5 mg/ml) were selected to prepare the virtual PSM library. A list of 487 species-specific PSM was prepared by referring to previous literature (Silva et al., 2019). The metabolites’ structure was downloaded from the PubChem database as SDF format (Kim et al., 2016), converted to PDB using the Open Babel suite (O’Boyle et al., 2011). Molecules whose 3D structure was unavailable, the 2D structure was retrieved and converted to the 3D structure using Marvin suite [https://chemaxon.com/products/marvin].

2.2.2. Physiochemical properties of metabolites

The metabolites were screened for Lipinski’s rule using the swiss-ADME tool (Daina et al., 2017). Molecules qualifying Lipinski’s rule were subject to Absorption, Distribution, Metabolism, Excretion, Toxicity (ADMET) properties using pkCSM (Pires et al., 2015). ADMET properties considered in the present study were, a) Caco-2 cell permeability (>0.90) to understand absorption characteristics of selected PSM, b) blood-brain barrier (BBB) permeability (LogBB > 0.3) to know the capabilities of selected PSM to reach target site, and c) Ames toxicity (Ames negative) to avoid the use of PSM with mutagenic potential (Castro et al., 2021; Pires et al., 2015).

2.2.3. Molecular docking and molecular dynamics simulation

2.2.3.1. Protein and ligand preparation.

The coordinates of hAChE (PDB ID: 6O5V, 2.15 Å) bound with the oxime reactivator RS-170B was downloaded from the PDB database (Gerlits et al., 2019), Oxime reactivator was removed from the complex using chimera (http://www.cgl.ucsf.edu/chimera). The explicit hydrogen atoms addition and 3D structure were optimized using UFF at 200 steepest descent algorithms in ligands. The geometry of protein and ligands was corrected using the clean geometry module of Discovery Studio (DS) 4.0 suite (San Diego, CA, USA). Polar hydrogen atoms and Kollaman charge were added into the protein structure using AutoDock MGL Tools 1.5.6 (Costa et al., 2020). The protein was then saved in pdbqt file format. Ligand pdbqt files were generated using the open babel tool (O’Boyle et al., 2011).

2.2.3.2. Molecular docking.

Site-specific docking screening of 79 compounds was done using Auto Dock Vina (Trott & Olson, 2010). The docking receptor grid was created by residues that are involved in the catalyzation of the substrates
of the AChE, such as active site triad (Ser\textsuperscript{203}, His\textsuperscript{447}, and Glu\textsuperscript{334}), as well as amino acid indirectly involved in the catalyzation of ACh molecules such as Oxyanion hole (Gly\textsuperscript{121}, Gly\textsuperscript{122}, Ala\textsuperscript{204}), Anionic subsites (Trp\textsuperscript{236}, Phe\textsuperscript{295}, Phe\textsuperscript{297}, Phe\textsuperscript{348}, Gly\textsuperscript{404}, Ile\textsuperscript{451}), Acyl binding pocket (Trp\textsuperscript{236}, Phe\textsuperscript{295}, Phe\textsuperscript{297}, Phe\textsuperscript{348}), Peripheral anionic site (Asp\textsuperscript{74}, Tyr\textsuperscript{124}, Ser\textsuperscript{125}, Trp\textsuperscript{337}, Tyr\textsuperscript{337}, Tyr\textsuperscript{341}). The dimensions of the grid box were 40 Å × 40 Å × 40 Å, and the center point coordinates were set as X = 97.8, Y = 47.402, Z = –23.194. The pose with the minimum binding energy (BE) and the corresponding interactions was selected and further visually inspected and analyzed in the 2-D interaction module of discovery studio [Discovery studio, BioVia]. Redocking was performed with crystallographic ligand (oxime reactivator RS-1708 (4-carbamoyl-1-3-(2-(E)-(hydroxyiminol)-methyl)-1H-imidazo-1-yl)-propyl)pyridin-1-ium) and the conformation obtained by re-docking to ensure that default searching and scoring parameters were enough to find poses with RMSD < 2 Å from crystallographic conformation (Supplementary Figure 1) (Gerlits et al., 2019).

2.2.3.3. Molecular dynamics simulation. The MD simulation studies were performed by using GROMACS 5.1.5 suite. The topology file of protein and ligand was generated using the GROMOS96 43a1 force field and PRODRG server, respectively (Schütte-Kopf & Van Aalten, 2004; van Gunsteren et al., 1996). The solvation of protein-ligand complexes was performed by using a cubic box (10 nm) with a simple point charge (SPC) of water, and the overall charge was neutralized by adding counter ions (Na – 9). Energy minimization was carried out to reduce the steric clashes with the help of the steepest descent algorithm for 50,000 iteration steps and cut off up to 1000 kJ/mol. After the energy minimization step, the system was equilibrated in two different phases for the 50,000 steps. The first phase of equilibration was done in NVT (constant number of particles, volume, and temperature), ensemble two fs for each step. The second phase of equilibration was performed in the NPT (constant number of particles, pressure, temperature) ensemble at 300 K. The covalent bond constraints in the equilibration step were determined by using the LINCS algorithm. The calculation of the Lennard-Jones and Coulomb interactions was performed by using a 1.4 nm radius cut-off. The Particle Mesh Ewald (PME) method was adopted for the long-range electrostatics calculation with the Fourier grid spacing of 1.6 Å. The box’s internal temperature was statute by using V-rescale, a modified version of the Berendsen temperature coupling method. The Parrinello-Rahman pressure was performed for the equilibration of NPT. The final step of MD simulation was performed for the 50 ns for each step of 2 fs. The trajectories were saved, and the results were analyzed by using xmgrace.

2.2.3.4. Molecular dynamics analyzes. The MD simulation results, such as RMSD, RMSF, the radius of gyration (Rg), solvent accessible surface area (SASA), and the hydrogen bond formation during the simulation, were analyzed by using Gromacs analysis modules. The g_h-bond utility of GROMACS was employed to compute the hydrogen bond numbers, distribution profiles of the complexes, and hydrogen bond occupancy (HBO) were determined using Python script. The MD simulation was performed using High-performance computing (HPC) facility, IIT Delhi, India.

2.2.3.5. The binding free energy of the interaction between AChE-ligand. The Molecular Mechanics/Poisson-Boltzmann Surface Area (MM-PBSA) method has been adopted to calculate BE of AChE-PSM complexes and AChE-drug, which utilize ensembles acquired from the molecular dynamic simulation (Kumari et al., 2014). Following equations were used in the calculation:

\[ \Delta G = \langle \Delta G_{PE} \rangle - \langle \Delta G_{P} \rangle - \langle \Delta G_{L} \rangle \]

Here \( \Delta G \) and \( \langle \Delta G_{PE} \rangle \) denotes the binding and average free energy of the complex, respectively. At the same time, \( \langle \Delta G_{P} \rangle \) and \( \langle \Delta G_{L} \rangle \) depicts the free energy of receptor and ligand, respectively.

The above equation can also be approximately written as:

\[ \Delta G = \Delta E_{MM} + \Delta G_{psolv} + \Delta G_{npsov} - T\Delta S \]

Here, \( \Delta E_{MM} \) indicates the molecular mechanics interaction energy of the molecule calculated as the sum of the change in internal energy (\( \Delta E_{internal} \)), plus the change in electrostatics (\( \Delta E_{electrostatics} \)) and van der Waals (\( \Delta E_{vdw} \)) interactions upon ligand binding. \( \Delta G_{psolv} \) and \( \Delta G_{npsov} \) are the polar and nonpolar contributions to the solvation energy of a molecule, respectively. \( T \) denotes temperature and \( S \) is the molecule entropy. In MM-PBSA, the polar part of the solvation energy (\( \Delta G_{psolv} \)) is calculated by solving the Poisson-Boltzmann equation, while \( \Delta G_{npsov} \) is calculated by using the linear relation to the solvent accessible surface area.

The g_mmpbsa application of the GROMACS module was utilized to calculate distinct factors of the BE of AChE-ligand complexes. Binding energy is an average of the three energy terms apolar solvation energy, polar-solvation energy, and potential energy in the vacuum. In the present study, the snapshots at every 100 ps of the 40-50 ns simulation were collected to predict the BE using the MM-PBSA method.

3. Result and discussion

3.1. In vitro acetylcholinesterase inhibition assay

The AChE inhibitory potential of 390 extracts from 63 plant parts belongs to 58 plant species (Supplementary Table 1) was analyzed. Among 390 extracts, 38 extracts belong to 18 plant species were found inhibiting the activity of AChE (Figure 1). The range of inhibitory concentrations (IC\textsubscript{50} values) exhibited by the extracts were >0.0 to 1 mg/ml (5), >1 – 2 mg/ml (21), >2 – 3 mg/ml (4), >3 – 4 mg/ml (7), >4 – 5 mg/ml (1) and other extracts recorded IC\textsubscript{50} values higher than 5 mg/ml or IC\textsubscript{50} value never achieved or negative for AChE inhibition (Supplementary Table 2). The lowest IC\textsubscript{50} was recorded by acetone extract of Cyperus rotundus (rhizome) (0.5 mg/ml), followed by methanol extract of Terminalia arjuna (bark) (0.95 mg/ml) and, water extract of Acacia catechu (stem) (0.95 mg/ml).

In previous studies, extracts or metabolites from Terminalia arjuna (Suganthi et al., 2018), Terminalia chebula (Sancheti et al., 2010), Psoralea corylifolia (Somani et al.,
3.2. Virtual PSM library and drug-likeness properties of PSM

In silico drug-likeness analysis, consider many physicochemical and functional factors determining a given molecule’s probable drug-like behaviour. The physicochemical characters include mainly molecule size, molecular flexibility, lipophilicity, electronic distribution and hydrogen bonding characteristics. Whereas transport, absorption, metabolic stability, distribution, affinity to proteins, reactivity, toxicity, etc., the pharmacophoric characters determine the molecular interaction with the living system. Analysis of molecules using this information reduces the effort while screening a large pool of metabolites and reduced the probability of failures in in vivo experiments.

In our study, the virtual molecular library contains 487 PSM (after removing duplications) from 18 plants found positive for AChE inhibition in in vitro assay (Supplementary Table 2). When these PSM were analyzed for Lipinski’s rule, 283 molecules were found to have zero violations (Supplementary Table 3). Further, these molecules’ ADMET characterization yielded 78 PSM, which passed the required parameters, i.e. (a) Caco-2 cell permeability (>0.90), b) blood-brain barrier (BBB) permeability (LogBB > 0.3) and c) Ames toxicity (Ames negative). The results of Lipinski’s properties and ADMET characters of all studied molecules are presented in Supplementary Tables 4 and 5. Only those 78 PSM which satisfied the above conditions were taken to further studies. The results revealed the drug-likeness potential of these PSM and furthered their ability to cross the Gastrointestinal track and reach the target site (brain) without causing any mutagenic effect was predicted. Researchers have employed a similar approach of screening the molecules to eliminate those weak in their drug-like characters (Leão et al., 2020).

3.3. Molecular docking and molecular dynamics simulation of selected PSM

Molecular docking of selected 78 PSM along with substrates and 4 FDA-approved drugs (donepezil, galantamine, rivastigmine, and tacrine) were performed with the AChE. The PSM binding energy (BE) was distributed in the range of −3.40 to −10.90 kcal/mol (Table 1 and Supplementary Table 4). The binding energy of the substrates (ACh), donepezil, galantamine, rivastigmine, and tacrine were found −4.80 kcal/mol,
The top 12 compounds with BE less than –8.1 kcal/mol, standard drugs, and substrate were subjected for MD simulation for 50 ns. The results were analyzed using various GROMACS modules. The binding of ligands may introduce a wide range of conformational changes in the enzymes, such as loop or domain movement, conformational rigidity, etc., thus perturbing the enzyme’s conformational state. Although proteins require a moderate amount of conformational flexibility for their function, more significant domain movements may lead to the enzyme’s loss of function. Hence, to examine the ligands (substrate and inhibitors) induced structural changes in the protein and the ligand-bound complexes’ stability, we analyzed various parameters like RMSD, RMSF, Rg, SASA, Hydrogen bond number, hydrogen bond distribution, and hydrogen bond occupancy (HBO).

None of the ligands tested expelled out from the protein; throughout the simulation period. RMSD of all the test systems from its reference structure in due simulation time varies between ~0.25 to 0.4 nm, which reaches a plateau in 20 ns (Supplementary Figure 2). RMSF of the protein during the simulation period was found to be 0.2 to 0.3 nm. The closer inspection of the structure indicates that highly fluctuating regions correspond to the loop residues. In contrast, residues present in α-helix and β-sheet are mostly stable (Supplementary Figure 3). During the simulation period, the Rg variation in all cases was found 2.20 to 2.35 nm (Supplementary Figure 4). The low variation of Rg indicates that the ligand binding to AChE does not significantly change the enzyme’s integrity and compactness. SASA was found in the range of 220-190 (nm²), which indicated that ligands’ interaction with protein did not affect the protein folding (Supplementary Figure 5). Analysis of hydrogen bond numbers between AChE-ligand complexes recorded a range between 0 – 8 hydrogen bonds during the simulation.

Further, the maximum hydrogen bond distribution was in the distance of 0.25 to 3.4 nm (Supplementary Figure 6 and 7). The results obtained from the above analysis indicated the structural integrity of AChE and the stable interaction of ligands throughout the simulation period. The free energy calculation analysis helps assess the binding potential of
ligands as it provides a quantitative estimation of the BE. The van der Waals, electrostatics, polar solvation, and SASA energy contributing to the BE of the molecules are presented and total energy of the simulations were exhibited in Table 2.

Sugiol is an abietane diterpenoid commonly found in *Metasequoia glyptostroboides*, *Syzygium cumini*, *Azadirachta indica*, *Juniperus communis*, *Calocedrus formosana*, and *Lycium chinense* plant species (Bajpai & Kang, 2014; Chao et al., 2005). Previously sugiol has been reported for their anti-cancer (Hao et al., 2018), antileishmanial (Scariot et al., 2019), antimalarial activity (Bero et al., 2010), and inhibitors of α-glucosidase and tyrosinase activity (Bajpai & Kang, 2014). In the present study, sugiol record BE of −9.7 kcal/mol (docking studies) (Table 1) and formed pi interaction with amino acid residues of Peripheral anionic site (PAS) (Tyr^{124}, Trp^{286}, and Tyr^{341}) and Acyl binding site (ABS) (Phe^{297} and Phe^{338}) of AChE (Figure 2.1a and 2.1b). Binding energy determined through MMPBSA was found to be −184.734 ± 9.841 kJ/mol (Table 2). During the simulation, sugiol forms H bonds with Asp^{74} (91.32% HBO) and Arg^{296} (83.6% HBO) of PAS, indicating its potential to block the peripheral substrate binding site, thereby preventing the entry of substrate into the active site of AChE. Sugiol was found following all drug likeliness rules of Lipinski’s and ADMET properties with the bioavailability score (BAS) of 0.55 (Table 1).

Margolone, a diterpenoid obtained from the bark of *Azadirachta indica*, reported various biological activity such as antibacterial, antifungal, and antiviral properties (Ara et al., 1989). In the present studies, margolone record BE of −9.5 kcal/mol (docking studies) (Table 1) and found interacting with amino acid residues Phe^{295} (ABP) and Val^{294} in close vicinity by forming H bonds, and Pi interaction with Tyr^{124}, Trp^{286}, and Tyr^{341} residues of PAS of AChE (Figure 2.2a and 2.2b). Binding energy calculated through MMPBSA was −208.749 ± 13.005 kJ/mol (Table 2). During the simulation period, margolone was found to form an H-bond (75.1% HBO) with Arg^{296} residue present in the vicinity of ASB of AChE. In early literature studies, margolone was identified as one of the active compounds in extract responsible for their...
biological activities. However, no information is available on the effect of margolone in its pure form. Also, its biotransformation and bioavailability information is not available. Through swiss ADME, the BAS of Margolone was determined as 0.85 (Table 1). Hence, our study opens a new avenue for researchers to explore AChE inhibitory and other biological properties of margolone.

7-Hydroxy-3',4'-(Methylenedioxy) flavan is a natural flavonoid commonly found in *Terminalia bellirica* and *Zephyranthes ajax* (Nguyen et al., 2020; Valsaraj et al., 1997), which was reported for the antimicrobial and antifungal activity (Ali et al., 2017), and also reported for anti-HIV activity (Valsaraj et al., 1997). In the present studies, 7-Hydroxy-3',4'- (Methylenedioxy) flavan was recorded BE of $-9.4 \text{kcal/mol}$ (docking studies) (Table 1) and recorded pi interactions with Tyr$_{124}$, Trp$_{286}$, Tyr$_{337}$ and Tyr$_{341}$ residues belong to PAS of AChE (Figures 2.3a and 2.3b). Binding energy determined through MMPBSA for 7-Hydroxy-3',4'- (Methylenedioxy) flavan binds with AChE was $-186.510 \pm 11.443 \text{kJ/mol}$ (Table 2). During the simulation period, 7-Hydroxy-3',4'- (Methylenedioxy) flavan was found to

form H bond with Tyr$_{124}$ (26.5% HBO), Gln$_{291}$ (14.3% HBO), Phe$_{295}$ (21.9% HBO) and Tyr$_{337}$ (14.6% HBO) PAS residues of AChE. The results indicated the capability of 7-Hydroxy-3',4'- (Methylenedioxy) flavan to block the PAS, thereby preventing substrate entry to the active gorge. This molecule was found following all the drug likeliness parameters tested and recorded BAS of 0.55 (Table 1).

ß-cyperone (Eudesma-4,6-dien-3-one), commonly found in *Cyperus rotundus* (Al-Snafi, 2016). α-cyperone, a structurally related molecule from *Cyperus rotundus* was reported as anti-inflammatory activity via decreasing COX-2 expression at mRNA and protein level (Jung et al., 2013). In the present study, ß-cyperone record BE of $-9.2 \text{kcal/mol}$ (docking studies) (Table 1) and pi interactions with Tyr$_{72}$, Trp$_{286}$, Phe$_{338}$ residues of PAS and Phe$_{338}$ residues of ABS of AChE (Figures 2.4a and 2.4b). BE determined through MMPBSA was
reported $-148.937 \pm 8.739$ kJ/mol (Table 2). During the simulation period, beta-cyperone was found to form H bonds with Trp$^{286}$ (5.4% HBO) of PAS and other amino acid residues like Tyr$^{72}$ (5.5% HBO), and Thr$^{75}$ (5.1% HBO) in the near vicinity of the AChE active site. Further, through swiss ADME, the BAS of beta-cyperone was determined as 0.55 (Table 1).

$1$-[5-Tert-butyl-5-hydroxy-3-(trifluoromethyl)-4H-pyrazol-1-yl]-2-(5-methyl-2-propan-2-ylphenoxy) ethanone obtained from the Ziziphus mauritiana (Siddiqui & Patil, 2015). It is pyrazoline-based compound containing fluorne, indicating its diverse biological activity such as anti-inflammatory, antimicrobial and antifungal (Naim et al., 2016). However, no attempt had been made to study the biological properties of this natural molecule in its pure form. In the present study, 1-[5-Tert-butyl-5-hydroxy-3-(trifluoromethyl)-4H-pyrazol-1-yl]-2-(5-methyl-2-propan-2-ylphenoxy) ethanone record BE of $-9.1$ kcal/mol (docking studies) (Table 1) and found interacting with amino acid residues (Asp$^{74}$, Ser$^{125}$, Tyr$^{337}$ and Tyr$^{341}$) of PAS through H bonds. The formed $\pi$ interaction with Tyr$^{124}$ and Trp$^{286}$ of PAS and Phe$^{397}$ and Phe$^{338}$ residues belongs to ABS of AChE (Figures 2.5a and 2.5b). Residues such as Ser$^{72}$, Trp$^{86}$, Asn$^{67}$ of anionic subsite shown halogen bond with fluorne atoms of 1-[5-Tert-butyl-5-hydroxy-3-(trifluoromethyl)-4H-pyrazol-1-yl]-2-(5-methyl-2-propan-2-ylphenoxy) ethanone (Figure 2.5a and 2.5b). Binding energy determined through MMPBSA was $-214.040 \pm 8.993$ kJ/mol (Table 2). During the simulation period, 1-[5-Tert-butyl-5-hydroxy-3-(trifluoromethyl)-4H-pyrazol-1-yl]-2-(5-methyl-2-propan-2-ylphenoxy) ethanone formed H bond with Asp$^{74}$ (13.1% HBO), Tyr$^{337}$ (46.7% HBO), Tyr$^{341}$ (5.2% HBO), and Tyr$^{124}$ (4.8% HBO) residue belongs to PAS of AChE. Further through swiss ADME, the BAS of 1-[5-Tert-butyl-5-hydroxy-3-(trifluoromethyl)-4H-pyrazol-1-yl]-2-(5-methyl-2-propan-2-ylphenoxy) ethanone was determined as 0.55 (Table 1).

Isomargolonone, a diterpenoid obtained from the Azadirachta indica, in several biological activities such as antifungal and antibacterial. Isomargalone was a suspected bioactive component present in the crude extract (Biswas et al., 2002). However, no biological activity has been reported with the purified compound. Through computational studies, isomargolonone was predicted as antibacterial via inhibiting New Delhi Metallo-$\beta$-lactamase (NDM-1), thereby potentiating beta-lactam antibiotics' activity (Thakur et al., 2013). In the present study, isomargolonone record BE of $-9.1$ kcal/mol (docking studies) (Table 1) and found interacting with amino acid residues Phe$^{295}$ and Arg$^{296}$ of ABS through H bonds, and pi interaction with Trp$^{286}$ and Tyr$^{341}$ residues belong to PAS of AChE (Figure 2.6a and 2.6b). Binding energy determined through MMPBSA was shown $-199.752 \pm 13.449$ kJ/mol (Table 2). During the simulation period, isomargolonone was found to form H-bond with Tyr$^{341}$ (44.9% HBO) and Asp$^{74}$ (43.9% HBO) of the PAS site, and 56.7% HBO was found with residue Ser$^{293}$ which in the vicinity of PAS. Further, through swiss ADME, the BAS of isomargolonone was determined as 0.85 (Table 2).

Serpentine is an indole alkaloid obtained from the Catharanthus roseus, Rauwolfa serpentina, and Rauwolfa tetraphylla (Rohela et al., 2016). Serpentine proved to be anticancerous via intercalation reaction with topoisomerase II-DNA complex and inhibiting PI3Ks (Dassonneville et al., 1999; Sharma et al., 2017). In the present study, serpentine record BE of $-9$ kcal/mol (docking studies) (Table 1) and found forming pi interactions with Tyr$^{72}$, Leu$^{16}$, Tyr$^{124}$, Trp$^{286}$, Leu$^{289}$ and Tyr$^{341}$ amino acid residues belong to PAS or adjacent to PAS of AChE (Figure 2.7a and 2.7b). Binding energy determined through MMPBSA was $-261.697 \pm 14.678$ kJ/mol, which was highest amongst all the PSM studied (Table 2). During the simulation period, serpentine was found to form an H-bond with Gln$^{291}$ (11.6% HBO), Ser$^{293}$ (14.2% HBO), and Tyr$^{72}$ (4.3 HBO) residues adjacent to PAS of AChE. Our studies correlate with Pereira et al. (Pereira et al., 2010), where they reported in vitro AChE inhibitory activity of serpentine in its pure form with IC$_{50}$ of 0.77 $\mu$M. Further, the authors reported the serpentine's inability to change diaphragm contractions even at higher concentrations tested (100 $\mu$M), indicating their low affinity for neuromuscular nicotinic receptors. In our study, serpentine was found to pass all drug likeliness parameters and recorded higher BAS 0.85 (Table 2). In contrast, the study previous study (Chitra & Kumar, 2009) suggested the incapability of serpentine to penetrate tissues and deep neuromuscular synapse, indicating their low bioavailability in ex vivo preparations.

Cryptolepine is an organic hetero-tetracyclic alkaloid compound commonly found in Sida cordifolia, Cryptolepis Buchananii, and Cryptolepis sanguinolenta (Cimanga et al., 1997; Pande et al., 2006). It has a role as an antimarial and anti-neoplastic (Pande et al., 2006), anti-inflammatory (Olajide et al., 2010), and cysteine protease inhibitor (Cimanga et al., 1997). In the present study, cryptolepine record BE of $-8.7$ kcal/mol (docking studies) (Table 1) and pi interactions with Trp$^{286}$ residues belong to PAS of AChE (Figure 2.8a and 2.8b). Binding energy determined through MMPBSA was found to be $-192.101 \pm 23.444$ kcal/mol (Table 2). During the simulation period, cryptolepine was found to form an H-bond with Gln$^{291}$ (0.1% HBO) residues of AChE, which is the least among all the ligands studied. However, another type of interaction, such as ionic, Van der wall, hydrophobic, etc., may stabilize the complex throughout the simulation period.

In vivo bioavailability of cryptolepin is a significant hurdle in using it as a drug molecule. In our studies, swiss ADME analysis revealed the bioavailability score of cryptolepine as 0.55 with high Gl absorption (Table 2). According to Stell et al. (Stell et al., 2012), the active form of cryptolepine (against Plasmodium falciparum) oxidized into inactive cryptolepine-11-one by rabbit liver aldehyde oxidase under in vitro conditions. Further, Forkuo et al. (Forkuo et al., 2017) observed a similar cryptolepine metabolism pattern in humans and rats. In addition to this, direct glcuronidation of cryptolepine was recorded in humans. Upon oral administration, some of the metabolized products of cryptolepine were identified in plasma and urine. The parental compound detected in urine is negligible, and the plasma half-life was reported to be 4.5 h.

Rotundone is a bicyclic sesquiterpene commonly found in many plant families, especially different grapes and pepper species, as an aromatic compound (Zhang et al., 2016). Though rotundone is widely studied for its aroma, its other
Possible biological activities are neglected. In the present study, Rotundone record BE of $-8.7$ kcal/mol (docking studies) (Table 2) and found interacting with amino acid residue Phe$^{295}$ and Arg$^{396}$ of ABS of AChE forming H bonds and Tyr$^{72}$, Trp$^{286}$, Phe$^{338}$, and Tyr$^{141}$ residues belong to PAS of AChE (Figure 2.9a and 2.9b). $-205.461 \pm 10.255$ kJ/mol of BE was recorded as MMPBSA for rotundone (Table 2). During the simulation period, rotundone was found to form H bond with Asp$^{74}$ (6.7% HBO), Ser$^{293}$ (3.3% HBO) and Trp$^{286}$ (3.2% HBO) Ser$^{239}$ (1.7% HBO), Phe$^{295}$ (0.6% HBO), Tyr$^{337}$ (0.6% HBO), Thr$^{341}$ (2.2% HBO) of PAS, and Tyr$^{72}$ (1.5% HBO), Gly$^{122}$ (0.1% HBO) of oxyanion hole. Further, through swiss ADME, the rotundone BAS was determined as 0.55 (Table 1).

Strictamin is an alkaloid obtained from the Catharanthus roseus, Vinca minor, and Alstonia scholaris (Kaushik et al., 2011). Tan et al. (Tan et al., 2019) reported antioxidant, neoplastic, and anti-snake venom properties of the Alstonia macrophylla bark's crude extracts. Further alkaloid composition analysis revealed the presence of strictamin as a bioactive compound. The antimicrobial potential of strictamin was reported by Skariyachan et al. (Skariyachan et al., 2019) through molecular docking studies against enzymes involved in the amino acid biosynthesis pathway of Acinetobacter baumannii. In the present study, strictamin record BE of $-8.3$ kcal/mol (docking studies) (Table 1) and found interacting with amino acid residue Phe$^{295}$ of ABS formed H-bonds, and $\pi$ interaction with the Tyr$^{72}$, Trp$^{286}$, Arg$^{296}$, and Leu$^{289}$ residues belongs to PAS or adjacent to PAS of AChE (Figure 2.10a and 2.10b). Binding energy determined through MMPBSA was $-192.012 \pm 13.133$ kJ/mol (Table 2). During the simulation period, strictamin was found to form H bond Trp$^{286}$ (0.1% HBO), Leu$^{289}$ (0.1% HBO), Pro$^{290}$ (0.1% HBO), Gln$^{291}$ (0.7% HBO), Glu$^{292}$ (3.6% HBO), Ser$^{293}$ (1.4% HBO), Arg$^{296}$ (1.1% HBO), Gly$^{342}$ (0.1% HBO) and Lys$^{348}$ (1% HBO) amino acid residues belongs to PAS or vicinity of PAS of AChE. Further, through swiss ADME, the BAS of strictamin was determined as 0.55 (Table 1).

Rotundenol is a sesquiterpene found in Cyprus rotundus and other related plant species (Irawanto et al., 2020). In the present study, rotundenol record BE of $-8.1$ kcal/mol (docking studies) (Table 1) and found interacting with amino acid residue Tyr$^{141}$ through H bonds, and $\pi$ interaction with the Tyr$^{72}$, Leu$^{76}$, and Trp$^{286}$ residues belong to PAS and in the vicinity (Figure 2.11a and 2.11b). Binding energy determined through MMPBSA was $-143.973 \pm 9.765$ kJ/mol (Table 2). During the simulation period, rotundenol was found to form H bond Arg$^{296}$ (39.3% HBO), Gln$^{291}$ (34.4% HBO), and Ser$^{293}$ (78.8% HBO) amino acid residues at the vicinity of PAS of AChE. Further, through swiss ADME, BAS of rotundenol was determined as 0.55 (Table 1).

Nootkatone is a sesquiterpene obtained from the Cyprus rotundus (Seo et al., 2011). Seo et al. (Seo et al., 2011) observed the anticoagulation activity of nootkatone in rat platelet aggregation ex vivo and in vitro experiments. In the present study, Nootkatone record BE of $-8.1$ kcal/mol (docking studies) (Table 1) and found interacting with amino acid residue Tyr$^{124}$ with H bonds and $\pi$ interaction with the Tyr$^{72}$, Trp$^{286}$, and Tyr$^{141}$ residues belong to PAS of AChE (Figure 2.12a and 2.12b). Binding energy determined through MMPBSA was $-123.831 \pm 11.440$ kJ/mol (Table 2). During the simulation period, nootkatone was found to form an H-bond with Phe$^{295}$ (63.3% HBO) amino acid residue of ABS and Tyr$^{124}$ (0.1% HBO) amino acid residues of PAS of AChE. Further, through swiss ADME, the BAS of nootkatone was determined as 0.55 (Table 1).

Among the four FDA-approved drug molecules, the highest BE of $-10.9$ was recorded by Donepezil, which was found interacting through H-bond with Phe$^{295}$ (ABS) and Tyr$^{137}$ (PAS) $\pi$-$\pi$ interactions with His$^{447}$ of the catalytic triad. Further donepezil recorded the highest MMPBSA (-269.465 \pm 12.579) among all the drug and PSM tested. It also recorded Arg$^{296}$ (36.3% HBO), Gln$^{291}$ (71.2% HBO), Phe$^{295}$ (7.2% HBO) (ABS), Trp$^{286}$ (PAS) (8.2%). Except for donepezil, none of the tested drug and PSM was found interacting with a catalytic triad (Ser203, Glu334, and His447). Most of these molecules were found interacting with the amino acid residues of PAS and ABS, indicating their ability to prevent/reduce ACh's chances of entry into the gorge of the AChE.

Overall, the PSM was found interacting with amino acid residues of active site triad, Oxyanion hole, Anionic subsites binding site, Acyl binding pocket, and Peripheral anionic site through hydrogen bonding or $\pi$ interactions during molecular docking or simulation. Some PSM also interacted with amino acid residues adjacent to above-mentioned sites, possibly hindering the substrate accessibility by the active site. Sugiol, which recorded the highest BE in molecular docking, showed pi interaction with amino acid residues of PAS (Tyr$^{124}$, Trp$^{286}$, and Tyr$^{141}$) and ABS (Phe$^{295}$ and Phe$^{339}$) of AChE. H bonds were formed with Asp$^{74}$ and Arg$^{296}$ of PAS during the simulation, indicating its potential to block the peripheral substrate binding site. Serpentine, which recorded the highest MMPBSA in MD simulation, was found forming $\pi$ interactions with Tyr$^{72}$, Leu$^{76}$, Tyr$^{124}$, Trp$^{286}$, Leu$^{289}$, and Tyr$^{141}$ amino acid residues belongs to PAS or adjacent to PAS of AChE. Whereas, during the simulation period, serpentine was found to form an H-bond with Gln$^{291}$, Ser$^{293}$, and Tyr$^{72}$ residues adjacent to PAS of AChE.

5. Conclusion

From the results obtained, it could be concluded that high throughput screening of plant extracts and in-silico studies provides a better opportunity to screen structurally diverse PSM to find AChE inhibitors. The present study reported three plant species and 11 PSM as potent AChE inhibitors. The selected PSM passed all the drug-likeness (Lipinski's rule and ADMET) parameters, enhancing confidence in researchers to subject them for further in vitro and in vivo studies. As these top-ranked 12 molecules are reported to present in various edible plants, their use is considered safe compared to other synthetic drug molecules, and they can be subjected to a drug development path with minimal effort. The possible use of these edible plants (with AChE inhibitory molecules) as a part of the diet of affected patients may be further explored. The major drawback of the present study is...
that it does not provide information about the nature of inhibition by the PSM as only competitive inhibitors are preferred in reducing the AChE activity. Hence, further studies are required with purified PSM to understand the nature of enzyme inhibition.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Author contribution

Conceptualization Hariprasad P.; Data curation Gourav Choudhir; Formal analysis Gourav Choudhir and Hariprasad P.; Investigation Gourav Choudhir and Hariprasad P.; Project administration Hariprasad P.; Resources Hariprasad P. and Satyawati Sharma; Software Open access; Supervision Hariprasad P. and Satyawati Sharma; The manuscript was written through contributions of all authors. All authors have approved the final version of the manuscript.

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