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Analysis of Bioactive Compounds *Piper crocatum* as Inhibitors of Acetylcholinesterase *In Silico* and *In Vitro*

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Abstract

Alzheimer's disease, the leading cause of dementia in older adults, involves memory loss and cognitive decline, with β-amyloid plaques and neurofibrillary tangles (NFTs) as key features. Acetylcholinesterase (AChE), an enzyme that breaks down acetylcholine, plays a role in the formation of these plaques and tangles. AChE is a promising target for the development of small molecule inhibitors in Alzheimer's disease (AD) treatment. Indonesia's native red betel (Piper crocatum) contains bioactive compounds that inhibit AChE activity, as shown in previous research. This study aims to evaluate the AChE inhibitory potential of bioactive compounds from *P. crocatum* extracts (water, n-hexane and ethyl acetate) using an *in silico* approach (molecular docking) with 3 different docking software programs. Donepezil served as the reference compound, and the results were compared with *in vitro* AChE inhibition assays. The potential AChE inhibitors, based on molecular docking using PyRx, Autodock Vina and YASARA Structure, from each extract are SM05 (n-hexane extract), SM15 (water extract) and SM18 (ethyl acetate extract), with the most negative ΔGbind values, measuring −8.8, −9.2 and −11.2 kcal/mol (more negative than Donepezil's ΔGbind values). SM15 and SM18 Compounds show promise, based on its ΔGbind values, interactions with AChE, favorable pharmacokinetic properties, bioavailability, bioactivity and toxicity positioning both compounds as strong candidates for AD therapy. Both compounds were docked to the AChE substrate binding pockets (6O4W), forming hydrogen bonds with His447 and Phe297 at the "gorge" active site, and hydrophobic interactions with key amino acids in the peripheral anionic site (PAS) and substrate-binding sites (Tyr124, Phe297 and Phe338). This is consistent with *in vitro* assay results, which show that the ethyl acetate extract has strong inhibition, with an IC50 of 16.7908 ppm, while the water infusion extract yields a 26.621 % inhibition of AChE enzyme activity. In addition, the DIY extract exhibited the strongest AChE inhibitory activity with an IC50 of 40.799 ppm.

Keywords: Acetylcholinesterase inhibitors, Alzheimer's disease, Bioactive compounds, Columbin molecular docking, Piper crocatum, Pharmacokinetics

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by symptoms of dementia. Patients with AD progressively experience memory loss, impaired cognitive function, impaired communication skills and permanent personality changes [1-3]. People with AD are expected to double every 2 decades to 60 million by 2030 and around 115 million by 2050 [4]. AChE inhibitors (AChEIs) are the main drugs currently in use for treatment of Alzheimer's disease (AD), the most common form of dementia. The

inhibition of the enzyme acetylcholinesterase (AChE) increases the levels of the neurotransmitter acetylcholine and symptomatically improves the affected cognitive function. Inhibition of AChE (EC 3.1.1.7) activity can prevent and improve Alzheimer's condition [5]. AChE is an enzyme that hydrolyzes acetylcholine to choline and acetate in the synaptic cleft, ending the transmission of nerve signals from the acetylcholine neurotransmitter [2]. Acetylcholine is an important neurotransmitter that functions to deliver memory signals in the brain. The existence of various cholinergic transmission barriers and low levels of acetylcholine are the causes of decreased memory and cognitive function in people with AD [6].

A therapy that has been developed in the treatment of AD is AChE enzyme inhibition. AChE is the most potential therapeutic target for AD's symptomatic improvement. The inhibitory effect of AChE activity increases choline levels in the synaptic cleft which has a significant impact in increasing cholinergic transmission activity [2]. Several synthetic drugs have been shown to improve symptoms in AD patients by inhibiting the AChE enzyme mechanism. Synthetic drug compounds that have been approved by the Food and Drug Administration (FDA) for the treatment of AD include tacrine, rivastigmine, donepezil and galantamine [1]. None of the currently available medications can reverse, halt, or even slow the neuronal damage and destruction underlying the symptoms of Alzheimer's disease (AD) and its progression to fatality. Moreover, many of these medications are associated with potential hepatotoxicity, further limiting their long-term use and effectiveness. This critical unmet medical need, coupled with the substantial physical, emotional, societal and healthcare burdens associated with AD, has compelled the research community to advance their understanding and develop safer and more effective strategies to address this debilitating condition [7]. As a result, the exploration of compounds derived from natural sources has gained attention as a promising alternative for AD therapy due to their minimal side effects. Medicinal plants, in particular, have been shown to contain bioactive components that can support and enhance health, offering a potential pathway for addressing this debilitating condition [8]. One of Indonesia's native medicinal plants is red betel (*Piper crocatum*), which has garnered interest for its potential neuroprotective properties.

Recent studies indicate that red betel leaves possess significant acetylcholinesterase (AChE) inhibition activity, which is crucial for enhancing cognitive function by preventing the breakdown of acetylcholine, a neurotransmitter essential for memory and learning [9,10]. The active compounds identified in red betel include flavonoids, polyphenols and alkaloids, which not only exhibit antioxidant effects but also contribute to anti-inflammatory responses [10,11]. These properties are particularly relevant in the context of Alzheimer's disease, where oxidative stress and inflammation are known to exacerbate neuronal damage.

Advanced analytical techniques, such as Liquid Chromatography-Mass Spectrometry (LC-MS), have identified more than 200 active compounds in red betel (*Piper crocatum*) extracts, with certain fractions demonstrating significant acetylcholinesterase (AChE) inhibitory activity even at low concentrations. Detailed LC-MS analysis of secondary metabolites in the aqueous extract of *P. crocatum* has revealed a complex composition, including 9 distinct molecular weights in the flavonoid fraction, 7 in the tannin fraction, and 7 in the alkaloid fraction. These findings highlight the potential of *P. crocatum* as a promising source of bioactive compounds for therapeutic development [12]. The active compounds contained in betel leaf shown to have the ability to inhibit the beta-secretase (BACE1) enzyme which plays a role in catalyzing the formation of Aβ-amyloid peptide plaques [13]. Given the known role of acetylcholinesterase (AChE) in Alzheimer's disease pathogenesis, particularly its involvement in the aggregation of Aβ-amyloid plaques via the formation of stable AChE-Aβ complexes [1], inhibiting AChE has become a therapeutic target. *In vitro* studies of plant extracts, such as those from *Piper crocatum*, have shown promising potential in AChE inhibition. The water extract of red betel leaves (*P. crocatum*) is of particular interest due to its diverse secondary metabolites, including flavonoids, tannins and alkaloids, all of which are compounds with reported bioactivity [12].

Based on the results of the research, betel leaf has the potential as a drug for treating AD. Hence, this research attempted to predict the potential red betel leaf

as a drug candidate for AD through the AChE inhibition mechanism through *in sillico* analysis as an early stage in the development of betel leaf as an AD medication. This prediction combined virtual screening and molecular docking methods. The use of this method is expected to increase the ability to identify and optimize the potential active compounds contained in red betel leaves as an AD medication with an inhibitory mechanism against AChE. The aim of this research was to examine the inhibitory power of *P. crocatum* active compound against the AChE enzyme activity through a simulation of molecular docking.

Materials and methods

Identification of the active site of acetylcholinesterase (AChE)

Identification of the active site of the acetylcholinesterase enzyme is important before carrying out virtual screening and molecular docking. The stages of identifying the active site of AChE included the homology analysis of amino acid sequences and multiple sequence alignment. Homology of AChE amino acid sequences was analyzed using the BLASTp protein alignment program (Basic Local Alignment Search Tool of protein), https://blast.ncbi.nlm.nih.gov/Blast.cgi, by selecting the protein bank data (PDB) as the database. The amino acid sequences of AChE (PDB code of 6O4W) were downloaded from the protein data bank database, https://www.rcsb.org/search, then compared with the amino acid sequences obtained from PDB database through the BLASTp program to determine the homology of these sequences.

As many as 5 - 10 sequences of the homologous gene obtained from the results of BLASTp homology analysis were downloaded by accessing the accession number of these sequences for the multi-sequence alignment analysis stage. AChE amino acid sequence (PDB code of 604W) and its homologous gene sequences were added to the Clustal Omega multi-sequence juxtaposition program, https://www.ebi.ac.uk/Tools/msa/clustalo, by selecting the protein set type. The resulting multi-sequence alignment identified the amino acids that played a role in the AChE active site, which was the sequence motive or conserved sequence based on literature [14].

Preparation of ligand and receptor structures

The ligands used in this research consisted of 2 types: Test and comparative ligands. The test ligands were natural compounds found in red betel leaf (*P. crocatum*), including water, ethyl acetate and n-hexane fractions obtained from LC-MS results based on previous research (**Table 1**). The 3D structure of natural compounds for each fraction was downloaded in the format *.sdf and *.mol on the Pubchem database site (https://pubchem.ncbi.nlm.nih.gov/) and Chemspider (http://www.chemspider.com/) by inputting the name of the molecule. The comparative ligand to compare the activity of natural compounds of red betel leaf against AChE inhibition was selected as active compounds obtained from DUD-E database (A Database of Useful Decoys: Enhanced) totaling 664 compounds in *.sdf format, which were known to play an active role against AChE. The geometry of the test ligand was optimized using YASARA structure software with the energy minimization method using NOVA force field. Energy minimization was conducted by using the em_runclean.mcr program on YASARA Structure. This resulted in the energy values between atoms reaching the minimum energy and forming a more stable and optimal molecular conformation. The 3D structure of test ligand from the geometry optimization result was saved in *.pdb file format. The file can be used for the analysis stage of virtual screening and molecular docking using Autodock Vina or YASARA Structure. Advanced preparation of the test ligand of 3D structure in the molecular docking stage using Autodock Vina was carried out by adjusting the atomic bond torsion of the molecule composition and converting to a *.pdbqt file.

The 3D structure of acetylcholinesterase (AChE) enzyme as a target receptor was obtained from the database of Protein Data Bank (PDB) code 6O4W. It was a homodimer docked by an inhibitor in the form of donepezil. Donepezil was separated from AChE 3-dimensional structure and stored in *.pdb format to be used in the virtual screening grid box validation stages and molecular docking through redocking. The preparation of the 3-dimensional AChE structure was conducted by removing water, adding hydrogen atoms to the correct amino acid, and removing the subunit of B polypeptide chain and other ligands without important roles in molecular docking. The preparation utilized

Autodock Tools 1.5.6 and YASARA Structure software. The files of AChE 3-dimensional structure prepared using Autodock Tools 1.5.6 software were saved in *.pdbqt format for virtual screening and molecular docking with Autodock Vina. The files prepared by YASARA Structure software were saved in *.s format for molecular docking with YASARA.

Table 1 Free energy binding values and Cd value bioactive compounds of *P.crocatum* were attached to AChE (6O4W).

Ligand code	Name of ligand	Source of ligand	Free energy binding value (Δ*G*) (Kcal/mol)			Cd (M)
			Virtual screening	Autodock vina	YASARA	
Refference Ligand	Donepezil	Native ligand of AChE (6O4W)	−4.85	−5.0	−4.9	0.002
SM01	Methyleugenol	n-Hexane Extract	−6.9	−7.1	−1.19	0.133
SM02	Protocatechuic acid	n-Hexane Extract	−6.4	−6.5	−1.25	0.121
SM03	Arginine Hydrochloride	n-Hexane Extract	−5.7	−5.8	−1.02	0.180
SM04	4-methoxyindole	n-Hexane Extract	−6.7	−6.7	−1.95	0.037
SM05	2-(3,4-Dimethoxyphenyl)-6-ethoxy-7-methoxy-1-naphthol	n-Hexane Extract	−8.8	−10.5	−3.44	0.003
SM06	1-(1,4-Dithian-2-ylmethyl)-3-(3-methoxypropyl)thiourea	n-Hexane Extract	−5.8	−6.2	−1.75	0.052
SM07	Leucylleucinamide-HCl	n-Hexane Extract	−7.3	−7.3	−4.26	0.001
SM08	N1-(5-methylisoxazol-3-yl)ethanediamide	n-Hexane Extract	−6.5	−6.4	−1.45	0.086
SM09	5-Isopropyl-3-pyrazolidinecarbohydrazide hydrochloride	n-Hexane Extract	−7	−6.7	−0.25	0.651
SM10	2,2,12,12-Tetramethyl-4,10-dioxo-3,11-dioxa-5,9-diazatridecan-7-yl methanesulfonate	n-Hexane Extract	−8	−7.6	No interaction	No value
SM11	2-(4-morpholinylmethyl)aniline sulfate hydrate	n-Hexane Extract	−3.6	−1.9	No interaction	No value
SM12	1H-Pirazol-1-carboximidamidhydrochloride	n-Hexane Extract	−5	−5	−1.10	0.156
SM13	1-Amino-3-(aminooxy)-2-propanyl N-(4,6-diamino-1,3,5-triazin-2-yl)glycinate dihydrochloride	n-Hexane Extract	−7.8	−6.8	No interaction	No value
SM14	3-(3,4-Dimethoxyphenyl)propionicacid	Water Extract	−7.2	−7.2	−1.20	0.133
SM15	Columbin	Water Extract	−9.2	−9.2	−3.58	0.002
SM16	Schisandrin B	Water Extract	−8.1	−7.5	−3.20	0.005
SM17	Ethyl-L-Serinate-HCL	Water Extract	−4.7	−4.7	0.67	0.323
SM18	Flemiphilippinin A	Ethyl Acetate Extract	−11.2	−11.1	−3.92	0.001

Ligand code	Name of ligand	Source of ligand	Free energy binding value (Δ*G*) (Kcal/mol)			Cd (M)
			Virtual screening	Autodock vina	YASARA	
SM19	Isocaviunin 7-O-gentiobioside	Ethyl Acetate Extract	−9.9	−8.8	No interaction	No value
SM20	(2E,5E)-2,5-Bis(2,4,5-trimethoxybenzylidene)cyclopentanone	Ethyl Acetate Extract	−9.7	−8	−5.55	
SM21	2-ethoxyethyl {[3-(4-tert-butylphenoxy)-4-oxo-4H-chromen-7-yl]oxy}acetate	Ethyl Acetate Extract	−9.3	−9	−2.43	

Validation of docking areas (grid boxes) for virtual screening and molecular docking

The docking areas (grid box) of test and comparative ligands were constructed by adjusting the natural ligand docking areas (inhibitor: Donepezil) on AChE, through selecting the center on ligand in Autodock Tools 1.5.6, and making a grid box around selected atoms: Donepezil in YASAR structure. Donepezil natural ligand was used for determining the best grid box by redocking the natural ligand to AChE. Grid box size validation through redocking with AutoDock Vina was repeated 100 times using the exhaustiveness parameter. Each run included local optimization (Broyden-Fletcher-Goldfarb-Shanno algorithm) with multiple evaluations of the scoring function and its derivatives. Results were automatically merged, refined, clustered and sorted to produce the final outcome [15]. Meanwhile, redocking using YASARA structure was repeated 999 times to maximizes the chances of finding the most favorable interaction between the ligand and receptor, ensuring a comprehensive search for the best binding conformation [16]. Several grid box sizes were analyzed for the accuracy of donepezil redocking through the evaluation of the molecule's position and conformation using the Root Mean Square Deviation (RMSD) value. The RMSD value was obtained by comparing the position and conformation of the redocked donepezil with the native donepezil bound to the thre3e-dimensional AChE structure (PDB 6O4W). The smaller the RMSD value (< 2.0 Å), the more precise the position and conformation of the ligand molecule would be than its original position and conformation.

The dimensions of the best molecular docking areas obtained from grid box validation using Autodock Vina were x, y and z of 60 at coordinates x = 88.991, y = 85.361 and z = −5.291. Meanwhile, the validation of docking areas utilized the YASARA structure in which the best molecular docking area size was 7.2 Å with angles x, y and z, each of 27.26 °. The obtained docking area size was then used for analyzing the interaction of the test and comparison ligands through virtual screening and molecular docking using Autodock Vina and YASARA structure methods (modified-Weni 2020) [17].

Virtual screening

Virtual screening was the initial step in identifying the potential inhibition of natural compounds of red betel leaves from water, ethyl acetate and n-hexane fractions against AChE (6O4W). Identification was carried out by comparing the natural compounds contained in betel leaves (**Table 1**) with 664 active compounds known to have AChE inhibitory activity based on DUD-E database as a comparative ligand. The geometry optimized for the test ligand (**Table 1**) and 664 comparative ligands that had been prepared were docked to AChE through virtual screening using PyRx software. The virtual screening was carried out on a grid box with the coordinates of the validation results based on Autodock Vina; i.e., coordinates of x = 88.991, y = 85.361 and z = −5.291 with 10 repetitions, and the

results were the binding affinity energy and RMSD values.

Molecular docking with Autodock Vina

Receptor and ligand preparations were the initial steps in molecular docking with Autodock Vina. Receptor preparation was the removal of water molecules, docked ligands and homodimer polypeptide subunits, as well as the addition of hydrogen atoms using the Discovery studio program and Autodock Tools 1.5.6, and they were saved in a *.pdbqt file format. Ligand preparation included the geometry optimization with YASARA Structure program, the addition of hydrogen atoms, and the bonding torque of the ligand constituent atoms using the Autodock Tools 1.5.6 program, then saved in *.pdbqt file format. The results of receptors and test ligand preparation were then used in the analysis of potential test ligands in AChE inhibition using Autodock Vina.

Molecular docking with Autodock vina utilized the validated grid box size and coordinates of x, y and z, each of 60 with the coordinates of x = 88.991, y = 85.361 and z = −5.291. The ligands docked to AChE (6O4W) were test ligands, namely natural compounds contained in the water, ethyl acetate and n-hexane fractions of red betel leaf (*P. crocatum*) (**Table 1**). The molecule docking was carried out with 20 exhaustiveness. Exhaustiveness was a function to control the accuracy in finding the minimum global. The higher the exhaustiveness used in the docking process, the more extensive the search results were; however, the docking process took longer duration. The results obtained were in the forms of free binding energy (ΔG) and RMSD values.

Molecular docking with YASARA structure

Molecular docking with YASARA Structure utilized the receptor file prepared in a format of *.s; water molecules, docked ligands, and polypeptide B subunits were removed, and appropriate hydrogen atoms were added to amino acids. In the 3D AChE structure, a grid box was formed with the size of the validation results of 7.2 Å. Molecular docking was performed using the AMBER14 force field to calculate the energy value of the ligand-receptor interaction.

Analysis of docking results

The molecular docking using Autodock Vina and YASARA Structure were analyzed for ligand interaction on AChE in 2 dimensions with the LigPlot+ program, and in 3 dimensions with the PyMol program. Visualization of the ligand docking position on AChE was shown in 3 dimensions with the PyMol program.

Acetylcholinesterase inhibitory activity of red betel leaf extracts

The inhibition of acetylcholinesterase activity of red betel leaf extract was conducted using varying concentrations (10, 20, 30 and 50 ppm). In the AChE inhibitory activity assay, 3 treatments were utilized to evaluate enzyme activity and inhibition. Each treatment, including sample, positive control, negative control and blank was added to the microplate according to the volume The Blank treatment consisted of 50 µL of ACTh Mix, 50 µL of AChE enzyme and 50 µL of double-distilled water (ddH_2O), ensuring baseline measurements. For the Negative Control, 50 µL of ACTh Mix, 50 µL of ddH_2O and 50 µL of buffer were combined, excluding AChE or sample to confirm no enzymatic activity occurs without active components. Lastly, the Sample treatment included 50 µL of ACTh Mix, 50 µL of AChE enzyme and 50 µL of the tested sample, excluding buffer or ddH_2O, to assess the inhibitory potential of the sample. The reaction was incubated for 10 to 30 min at room temperature, protected from light. The measurement was performed using Ellman's method, with absorbance recorded at 408 nm. Enzyme activity and percentage inhibition are calculated and determined IC_{50} based on the linear regression of the following equation:

Inhibition % = [(negative control activity − sample activity)/negative control activity]×100 % (3).

Data analysis

Statistical methods were used to analyze the impact of red betel leaf extract on acetylcholinesterase inhibition. The analysis was carried out using Minitab 17 software. One-Way ANOVA was applied at a 95 % confidence level, with significant differences marked by a p-value < 0.05.

Results and discussion

Validation, structure and active site of AChE

The 3-dimensional structure of acetylcholinesterase used in this study is the structure with PDB code 6O4W, obtained from the Protein Data Bank. This structure was chosen due to the presence of the inhibitor, donepezil, which is naturally bound within the protein structure. The grid box is one of the important factors in producing accurate ligand-receptor interactions, both through virtual screening and molecular docking. Therefore, grid box validation is a crucial initial step in analyzing ligand-receptor interactions. The grid box validation is performed using 2 programs: AutoDock Vina for virtual screening analysis with PyRx and molecular docking with Vina, while the second program, YASARA Structure, is used for molecular docking analysis with YASARA Structure. Grid box validation is carried out by redocking the natural ligand from the 3D crystal structure of AChE (6O4W), which is the compound donepezil. Donepezil, which is a drug for Alzheimer's, is also used as a refference compound in determining the AChE inhibitor potential of the test ligands in this study.

The smaller the RMSD value, the more it indicates that the atoms between 2 compounds or macromolecules are in the same position. The more accurate the size and coordinates of the gridbox, the more accurate the position of the ligand docked to the AChE active site is. This is shown by the donepezil poses as the result of redocking which is similar to the native donepezil in AChE structure (6O4W) from the database shown in **Figure 1**. The greater similarity of the position of the redocked donepezil resulted in a lower RMSD value. The best grid box in validation was determined by several parameters including the accuracy of the ligand binding pose resulting from redocking on the native ligand attached to the receptor, and the RMSD values between the ligands resulting from redocking and native ligands on the receptor structure. RMSD is a measure of the average distance between atoms of the same compound, both of which are superimposed [18]. The greater similarity of the position of the redocked ligands resulted in a lower RMSD value. RMSD is commonly used in the accuracy assessment of the gridbox at the validation stage in the analysis of ligand-receptor interactions through molecular docking. The best gridbox is the one that produces a ligand fastening pose with an RMSD value of < 2.0 Å [19]. The gridbox validated by the Autodock vina produced a similar pose to the native donepezil attached to AChE structure (6O4W), with a small RMSD value by 0.4038 Å. RMSD value resulted from redocking by using the Autodock vina was smaller than using YASARA structure, which was valued at 2.0988 Å. The 2 gridboxes were then used in virtual screening and molecular docking process.

The active site of AChE is located inside the protein, 20 Å from the surface [20], and consists of the amino acids Ser203, His447 and Glu334. Along the substrate pathway to the active site, several other important amino acids are present, including the peripheral anionic site (PAS), the oxyanion hole, the anion subsite and the acyl binding pocket [1,20-22], which can be seen in **Figure 1**. Based on the redocking results of the donepezil compound, it can be seen that donepezil rebinds to the AChE active site with a conformation similar to the initial position before redocking, as indicated by the small RMSD value. The grid box is then used for docking the test ligands to AChE.

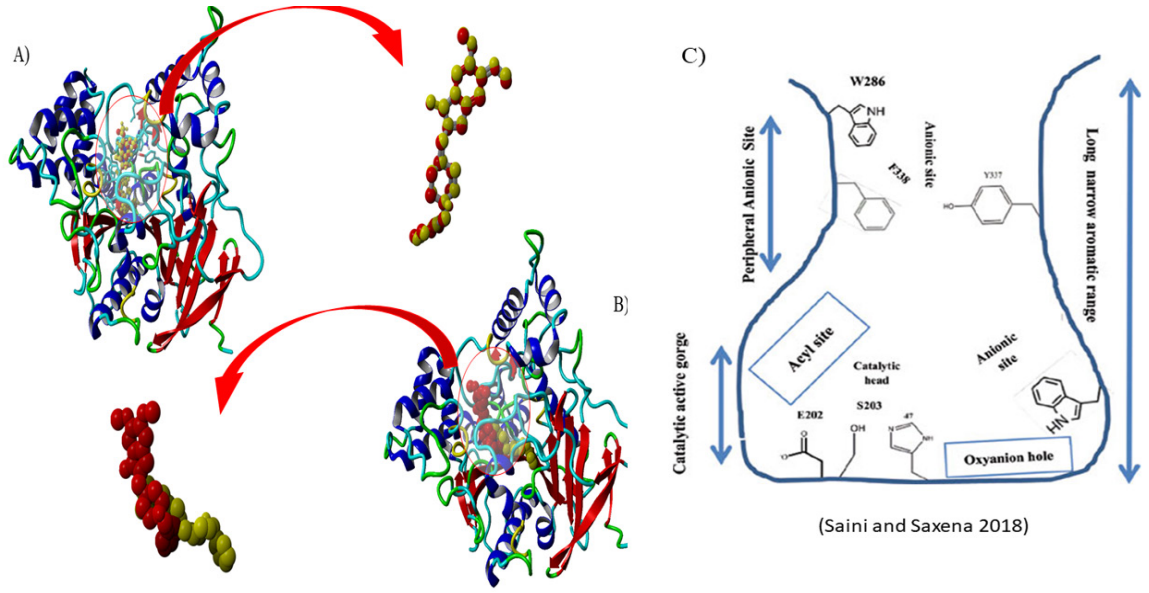

Figure 1 Redocking which is similar to the well-known ligand (*donepezil*) in AChE structure (6O4W) from the database; (A) Autodock vina method, (B) YASARA method and (C) Active site scheme "gorge" AChE.

Interactions of bioactive compounds of *P. crocatum* against AChE (6O4W) based on virtual screening

There were 21 active compounds from red betel leaves and 664 well-known ligands. Hence, the total of 685 compounds were attached to AChE (6O4W). The well-known ligand is a compound known to play an active role against AChE based on the DUD-E database. The results of the virtual screening of overall test and well-known ligands demonstrated quite diverse values of free energy of binding (ΔG) or binding affinity, ranging from weak binding affinity with an energy of > −5.0 kcal/mol to the strong one with an energy of < −10.0 kcal/mol. Referring to the results of virtual screening, 500 well-known ligands showed the strongest affinity for AChE (6O4W) with the lowest binding free energy value of < −10.0 kcal/mol, the 51 well-known ligands indicating quite strong affinity with binding free energy value of −9.1 to −10.0 kcal/mol, and the rest which had a weak binding affinity with free energy of > −9.0 kcal/mol.

Based on the results of virtual screening, 21 active compounds from the 3 fractions of red betel leaf also demonstrated various free energy binding values, ranging from −4.0 to −11.2 kcal/mol, as shown in **Table 2**. There were active compounds in each fraction indicating the strongest binding affinity among other compounds. The active compound of SM05 from n-hexane fraction showed the strongest affinity with the lowest binding free energy value of −8.8 kcal/mol. A number of natural compounds from the 3 fractions indicated similar binding affinities to the well-known ligands based on the binding affinity energy values; i.e., −8.8 to −11.2 kcal/mol. This showed that the natural compounds of n-hexane fraction (SM05; 2-(3,4-dimethoxyphenyl)-6-ethoxy-7-methoxy-1-naphthol), ethylacetate fraction (SM15; Columbin), and water fraction (SM18; Flemiphilippinin A) were predicted to have a good potential as drug candidates for AD through the mechanism of AChE inhibition based on virtual screening (PyRx). The lower the value of the free energy binding, the stronger the ligand binding affinity predicted on the target receptor [23-25]. SM05 is an active compound with the molecular name of 2-(3,4-dimethoxyphenyl)-6-ethoxy-7-methoxy-1-naphthol with the molecular formula of $C_{21}H_{22}O_5$ and a molecular weight of 355.15 m/z [26]. The active compound of SM15 showed the strongest affinity, and the binding free energy of SM15 compound was −9.2 kcal/mol. SM15 is a compound with the molecular name of Columbin, the molecular formula of $C_{20}H_{22}O_6$, and a molecular weight of 359.15 m/z [17]. The strongest affinity for the water fraction was evident in the active compound of code SM18 i.e., the Flemiphilippinin A compound with the molecular formula of $C_{30}H_{32}O_6$ and a molecular weight of 482.93 m/z [26]. SM18

compound had the lowest binding free energy of the 3 active compounds fractions by −11.2 kcal/mol. A negative value for free energy (ΔG) indicated that the ligand was bound to the target receptor. The more negative the free energy value (ΔG), the better the binding between the ligand and the target receptor [27]. In this *in silico* study, based on the binding free energy (ΔG) values obtained, the 3 active compounds (SM05, SM15 and SM18) from each P. *Crocatum* extract showed more negative binding free energy values compared to donepezil (**Table 1**). Based on this, the 3 active compounds have better potential as AChE inhibitors compared to donepezil, the reference compound, which is a commercial drug for Alzheimer's disease.

Table 2 Bioactive compounds of *P. crocatum* Interaction with AChE (6O4W).

Interaction	Interaction count			AChE essential amino acid		
	SM05	SM15	SM18	SM05	SM15	SM18
Hydrogen bond	1	2	2	(PAS) *Tyr124*	*(Active site)* His447 (PAS) *Asp74*	(*acyl binding pocket) Phe295*
Hydrophobik Interaction	9	12	14	(PAS) *Tyr72, Trp285, Tyr337, Tyr341; (Acyl binding pocket) Phe295, Phe297, Phe338;(oxyanion hole) Gly121*	(PAS) *Tyr72, Asp74, Tyr124, Ser125, Tyr341, Tyr337 (Anion subsite) Trp 86, Glu202, Gly448 (Oxyanion hole) Gly121*	(PAS) *Tyr72, Asp74, Tyr124, Trp286, Tyr341 Tyr337; (Acyl binding pocket) Tyr124, Phe297, Phe338;(Active site) His447*
Van der Waals	9	10	10	(PAS) *Tyr72, Asp74, Tyr124, Tyr337; (Acyl binding pocket) Phe295, Phe297; (oxyanion hole) Gly121*	(PAS) *Tyr72, Ser125, Tyr341 (Active site) Ser203, (Oxyanion hole) Gly121, Gly122*	(PAS) *Tyr74, Tyr337; (Acyl binding pocket) Phe297, Phe338;*
π-π Interaction	4	3	5	(PAS) Tyr341, Trp286; *(Acyl binding pocket) Phe 338.*	(PAS) *Tyr124, Tyr337; (Anion subsite) Trp86*	(PAS) *Tyr72, Tyr124, Trp286, Tyr341*

Interactions of the active compounds of red betel leaf against AChE (6O4W) based on molecular docking

The results of analysis found that the compounds from *P. crocatum* had a complex interaction with AChE essential amino acid (6O4W) (**Table 2**). The interaction with "gorge," the active site of amino acids, PAS, subsite anion, oxyanion hole and acyl binding pocket showed that the active compounds of the 3 red betel leaf fractions had a potential as inhibitors for AChE activity. The inhibition of AChE "gorge" active site made it possible to reduce the degradation rate of acetylcholine in patients with AD to prevent an increased risk of cholinergic inhibition. Natural ligand SM05, SM15 and SM18 of *P. crocatum* through virtual screening and molecular docking with Autodock vina and YASARA structure indicated a strong interaction with AChE PAS amino acid (6O4W) (**Table 2**, **Figures 2 - 4**). This highlighted that the 3 active compounds i.e., 2-(3,4-Dimethoxyphenyl)-6-ethoxy-7-methoxy-1-naphthol (SM05), Columbin (SM15) and Flemiphilippinin A (SM18) were potential drug candidates for AD therapy as an AChE inhibitor. The 3 active compounds i.e., 2-(3,4-Dimethoxyphenyl)-6-ethoxy-7-methoxy-1-naphthol (SM05), Columbin (SM15) and Flemiphilippinin A (SM18) were potential drug candidates for AD therapy as an AChE inhibitor. with a mechanism of inhibiting AChE catalytic activity in acetylcholine degradation and inhibiting the formation of Aβ-amyloid fibril plaque by blocking Aβ aggregation facilitated by AChE as stated by Alvarez *et al.* [28].

The SM05 compound was successfully docked to AChE substrate binding pocket as seen in the 3-dimensional visualization of **Figure 2(B)**. In the substrate binding pocket, SM05 forms hydrogen bonds with -OH group of Tyr124, the amino acid making up the peripheral anionic site (PAS). The hydrogen bond length was 2.88 Å, ideal for ligand-receptor interactions. SM05 compound interacted hydrophobically with a large number of amino acids; the amino acids of Tyr72, Asp74, Tyr124, Trp286, Tyr337 and Tyr341 in the peripheral anionic site (PAS), the amino acid Gly121 oxyanion holes and amino acids of Phe295, Phe297 and Phe338 in acyl binding pockets. The interaction between SM05 Van der Waals and AChE (6O4W) was with nine AChE amino acids; i.e., Asp74, Tyr72, Gly121, Tyr124, Ser293, Val294, Phe295, Phe297 and Tyr337. The interaction of π-π SM05 and AChE (6O4W) was between 4 amino acids from the anionic site, and PAS with SM05. The interactions of Van der waals, hydrogen bonds, π-π and hydrophobic interactions determined the ligand binding free energy values to the receptor.

SM15 compound was docked to AChE substrate binding pockets (6O4W) by forming a hydrogen bond between SM15 compound and Hys447 catalytic amino acid on the "gorge" active site, at the curve base of AChE "gorge" active site. Hydrophobic interactions were formed with a large number of amino acids making up the peripheral anionic site (PAS), oxyanion holes, anion subsites and Ser203 catalytic amino acid as seen in **Figure 3(C)** to form a strong hydrophobic environment. Van der Waals interactions were formed between SM15 and ten amino acids i.e., the amino acid anions, PAS and the oxyionine holes. The π-π interaction was formed with 3 AChE amino acids i.e., PAS and subsite anions. Based on the values of binding free energy (Δ*G*), dissociation constant and non-covalent intermolecular interactions with AChE amino acid, SM15 compound showed the strongest binding affinity compared to other compounds from the water fraction of red betel leaf based on virtual screening and molecular docking as seen in **Tables 1** and **2**.

SM18 compound showed the strongest binding affinity based on virtual screening and molecular docking with Autodock vina and YASARA with binding free energy (Δ*G*) values of −11.1 and −3.92 kcal/mol, respectively. Based on the 3-dimensional visualization in **Figure 9(B)**, SM18 was docked to the substrate binding pocket by interacting hydrophobically with a number of essential amino acids making up the peripheral anionic site (PAS), active sites and amino acid binding to substrates as seen in **Figure 4(C)**. SM18 had the most hydrophobic interactions with AChE amino acid (6O4W) i.e., 14 hydrophobic interactions compared to compounds from other fractions. SM18 also formed 2 hydrogen bonds based on the 3-dimensional visualization of interactions with Discovery studio programs; Ser293 and Phe295. Other interactions were Van der Waals interaction that occurred with ten AChE amino acids and π-π interaction with 5 amino acids.

The length of the hydrogen bond between the ligands and the receptors affected the binding free energy value (ΔG). The shorter the hydrogen bonds formed between the ligands and the receptors, the stronger the binding affinity for the ligands [23]. The aromatic groups in some amino acids were hydrophobic; therefore, they tended to interact hydrophobically with ligands that had high hydrophobicity [20].

SM05 compound (2-(3,4-dimethoxyphenyl)-6-ethoxy-7-methoxy-1-naphthol) was predicted to have a strong potential as an AChE inhibitor in AD therapy based on several factors, including its value, binding free energy (ΔG), dissociation constant (Cd) and interaction with AChE essential amino acids. The value of binding free energy of SM05 was similar to that of the well-known ligands. The lower the Cd value, the slower the rate of ligand dissociation to the receptor due to high binding affinity [27]. SM15 compound had the potential as an AChE inhibitor in AD therapy. The potential of SM15 or columbin compound as a drug candidate in a therapy of a disease was known to be a potential candidate for diabetes therapy through computational inhibition of the alpha glucosidase enzyme [17]. Higher interaction caused the value of binding free energy (ΔG) of SM18 to be the most negative compared to other fractions [2] based on virtual screening and molecular docking.

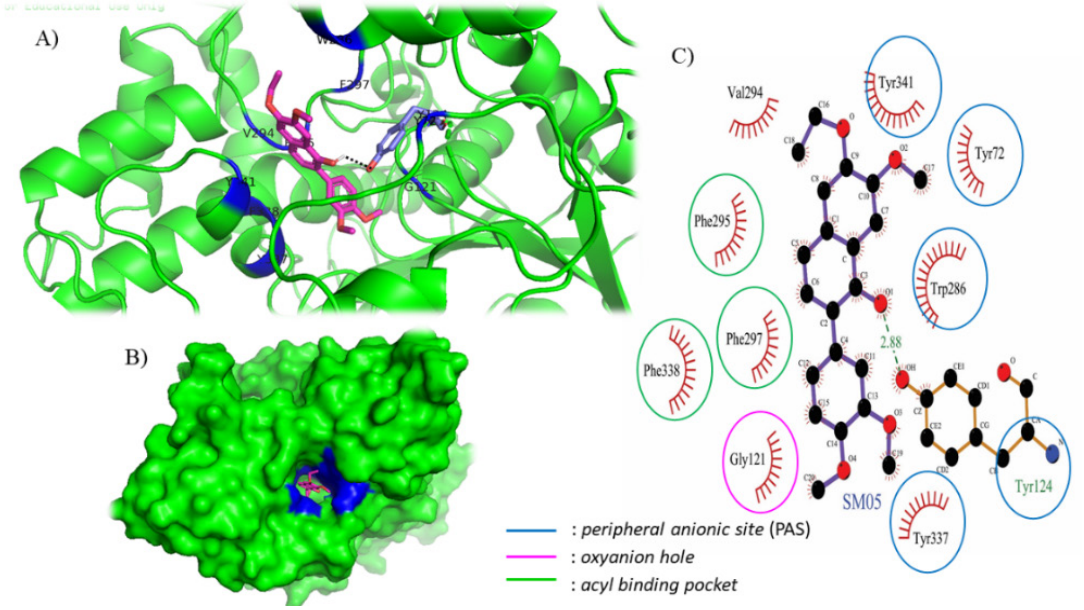

Figure 2 Complex of SM05 Compound and AChE (6O4W). (A) In the 3-dimensional visualization of SM05 interactions with essential amino acids (PyMol), yellow stick was SM05 compound, blue ribbon was hydrophobic interacting amino acids, black dotted line was hydrogen bonding, grey stick was AChE amino acid. (B) SM05 docking in the substrate binding pockets (PyMol). (C) In the 2-dimensional visualization of SM05 interaction with essential amino acids (LigPlot), the grey stick was SM05, brown stick was Tyr124 AChE amino acid, green dotted line was hydrogen bond with bond length, and red eyelashes were hydrophobic interactions.

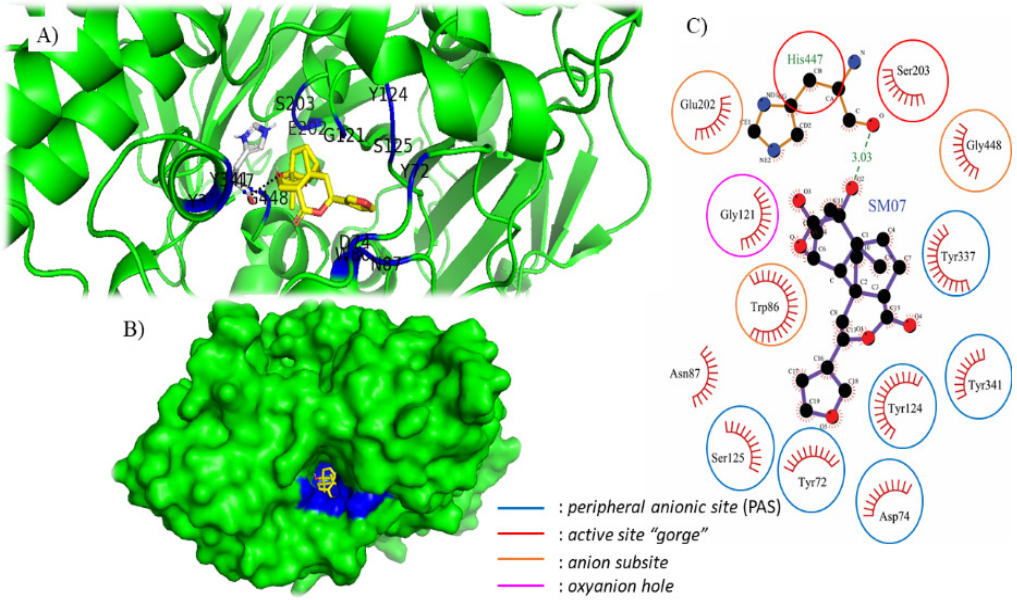

Figure 3 Complex of SM15 componds and AChE (6O4W). (A) In the 3-dimensional visualization of SM15 interactions with essential amino acids (PyMol), yellow stick was SM15 compound, blue ribbon was hydrophobic interacting amino acids, black dotted line was hydrogen bonding, grey stick was AChE amino acid. (B) SM15 docking in the substrate binding pockets (PyMol). (C) In the 2-dimensional visualization of SM15 interaction with essential amino acids (LigPlot), the grey stick was SM15, brown stick was His447 AChE amino acid, green dotted line was hydrogen bond with bond length, and red eyelashes were hydrophobic interactions.

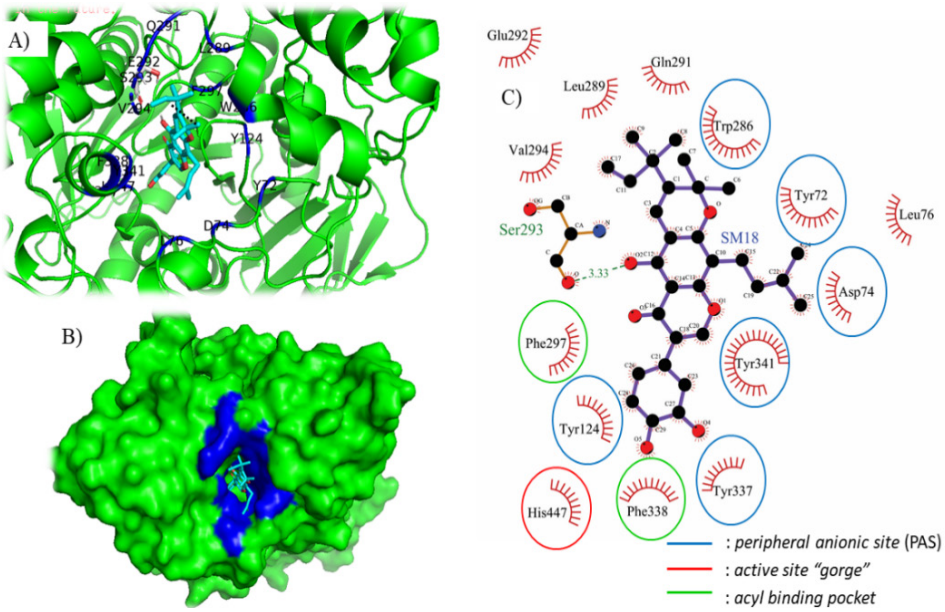

Figure 4 Complex of SM18 componds and AChE (6O4W). **(**A) In the 3-dimensional visualization of SM18 interactions with essential amino acids (PyMol), yellow stick was SM18 compound, blue ribbon was hydrophobic interacting amino acids, black dotted line was hydrogen bonding, grey stick was AChE amino acid. (B) SM18 docking in the substrate binding pockets (PyMol). (C) In the 2-dimensional visualization of SM15 interaction with essential amino acids (LigPlot), the grey stick was SM15, brown stick was Ser293 AChE amino acid, green dotted line was hydrogen bond with bond length, and red eyelashes were hydrophobic interactions.

Pharmacokinetic properties, drug-likeness and bioactivity of the active compounds of *P. crocatum*

Pharmacokinetic properties such as suitability of Lipinski's rules of 5, ADME (Absorption, Distribution, Metabolism and Excretion), drug-likeness and bioactivity are important parameters in assessing the potential of active compound as candidates of AD therapy drugs. Oral drug administration was preferred as it was easier to administer drugs to patients. Some processes occurring in active compounds after oral administration start from absorption, distribution, metabolism (detoxification by the liver), to systemic circulation towards the target [29,30]. The 3 compounds of *P.crocatum* (SM05, SM15 and SM18) had Log *p* values < 5 (**Table 3**); hence, these 3 compounds possibly had good lipophilicity and membrane permeability as drug candidates for AD. It means that they are generally better able to cross cell membranes, which are composed of lipid bilayers. This enhances the absorption and distribution of these compounds in the body, especially in tissues such as the brain. Therefore, compounds with a balanced Log P (not too high or too low) tend to be more effective as drugs. SM18 compound showed the lowest LogP value of −0.053, and its lipophilicity was predicted to be very strong to cross cell membranes as evidenced by the complex interaction with AChE important amino acid which was higher than other compounds (SM05 and SM15) as seen in **Table 2**. Hydrophobic amino acids surrounded the "gorge" active site area causing the compounds with high hydrophobicity like SM18 to have a more complex interaction with a strong binding affinity for the amino acid. However, in determining a good drug candidate, not only that it had strong interactions with the target receptor, it was necessary to assess other parameters. Based on the suitability of the properties with the Lipinski's RO5 parameter, the 3 active compounds of betel leaf fell into the drug-likeness category. Lipinski's rules of 5 (RO5) is a physicochemical parameter related to solubility and intestinal permeability of a compound as a basic characteristic in oral bioavailability. The parameters in RO5 include having 1) molecular mass less than 500 Dalton, 2) high lipophilicity (expressed as LogP < 5), 3) hydrogen bond donor of ≤ 5, 4) hydrogen bond acceptor of ≤ 10 and 5) molar reflection between 40 - 130. A compound does not meet the RO5 criteria, there may be a problem in oral bioactivity [31]. Based on the RO5 prediction results, the 3 active compounds

from the red betel leaf fractions showed conformity with the expected RO5. Log P is a parameter used to predict the permeation properties of a compound across the cell membrane and its lipophilicity. Log P is the membrane/water partition coefficient of a molecule. The value ≤ 5 indicates that the compound has good lipophilicity or hydrophobicity [32,33]. These compounds have good membrane permeability making them suitable for drug candidates. The drug compound used for CNS as a target should ideally have a LogP value of around 2 [32]. SM15 and SM18 had Log *p* values < 2, indicating that these compounds have the potential to access the CNS as Alzheimer's disease (AD) drugs.

The percentage of compound absorption in the body can be determined using the TPSA value. The absorption percentages of the 3 active compounds of red betel leaf (SM05, SM15 and SM18) were quite high by > 70 %. The highest absorption percentage of the 3 compounds was in SM05. SM05 compound had the lowest lipopolysity properties compared to other compounds, which presumably affected the high percentage of absorption in the intestine whereas SM18 had the lowest absorption percentage.

Based on water solubility, GI absorption and bioactivity parameters, SM15 compound was superior to other compounds. The advantages of SM15 over other compounds included high solubility in water and high gastrointestinal absorption affecting the bioavailability of these compounds. Based on the prediction of bioactivity, SM15 also showed high bioactivity with values of > 0.5, including the role of GPCR ligand, nuclear receptor ligand and enzyme inhibitor. These 3 roles were necessary in its use as a therapeutic drug for AD. Based on the information obtained, GPCR or muscarinic receptor played an important role in the transmission of cholinergic signals, and its activity decreased in patients with AD due to the decrease in neurotransmitter acetylcholine level and the receptor's expression. The role of SM15 as a GPCR ligand was very beneficial in AD therapy because it reduced the risk of cholinergic inhibition by stimulating multiple signal transmissions through GPCR activation.

TPSA (Topology Polar Surface Area) or Molecular polar surface area (PSA) was used to predict the properties of drug transportation. Polar surface area was defined as the surface number of polar atoms (oxygen, nitrogen and hydrogen) in a molecule. This parameter correlated very well with human intestinal absorption [33]. The ideal TPSA value for oral drugs is ≤ 140 Å^2 [33], which can be seen in Table 3; moreover, the 3 active compounds from the *P. crocatum* showed a TPSA value of ≤ 140 Å^2. This research revealed that the potential compound as a therapeutic drug for AD to be developed in further analysis was SM15 (columbin).

Another role of SM15 bioactivity was to act as a nuclear receptor ligand (NR). NR was a group of conserved transcription factors, and it regulated the transcription of certain genes when activated by lipophilic ligands including endogenous hormones such as estrogen, progesterone, androgens, glucocorticoids, vitamin A, vitamin D and other lipophilic compounds. Subsequently, the properties of SM15 compound had quite high lipophilic properties, so they were in accordance with the characteristics of NR ligand. The role prediction of SM15 as an NR ligand provided an opportunity for further analysis of the effect of SM15 on other metabolic aspects through its interaction with NR. In addition to the SM15 compound, SM18 also has the potential as a nuclear receptor ligand (NR), supported by its value of binding free energy (ΔG).

Another role of SM15 bioactivity prediction was as an enzyme inhibitor with a value of 0.57. The high bioactivity as an enzyme inhibitor strengthened the results of the analysis of SM15 potential as an inhibitor of AChE activity discussed based on various assessment parameters. Based on the predictions of pharmacokinetic properties, bioavailability, and bioactivity, among the 3 compounds of red betel leaf fractions (SM05, SM15 and SM18), SM15 was the most potential compound as a therapeutic drug for AD. Based on the oral toxicity prediction through ProTox3, the 3 compounds of red betel leaf fractions (SM05, SM15 and SM18) showed toxicity classes IV (for Columbin, SM15) and V (for SM05 and SM18) with respective LD50 values of 555 mg/kg (Columbin, SM15), 3,000 mg/kg (SM05) and 3,850 mg/kg (Flemiphilippinin A, SM18). This indicates that the 3 compounds of red betel leaf fractions are safe and have potential to be developed as oral drugs for Alzheimer's disease therapy. The oral toxicity properties of the 3 compounds from red betel leaf fractions are similar to the oral toxicity class of donepezil, which shows toxicity class IV with an LD50 value of 505 mg/kg (based on ProTox3 prediction). In

searching for drug candidates, the parameters for assessing the potential of a good compound as a drug were not only viewed from its strong binding affinity to the receptor, but also its suitability to the characteristics of the compound as a drug based on its pharmacokinetic properties, bioavailability, bioactivity and toxicity.

Table 3 Pharmacokinetic properties and bioactivity of the active compounds of *P. crocatum*.

Parameter	SM05	SM15	SM18
Molecular Weigth	353	358	312
Hydrogen bond donor	0	1	5
Hydrogen bond acceptors	5	6	6
LogP	4.789	0.746	−0.053
Molar reafractivity	86.08	82.48	77.15
Lipinski (drug likeness)	*Yes (0 violation)*	*Yes (0 violation)*	*Yes (0 violation)*
ADME			
TPSA	59.98 Å^2	85.97 Å^2	100.13 Å^2
%Absorption	88.32%	79.34 %	75.45 %
Water solubility	*Moderately soluble*	*Soluble*	*Poorly soluble*
GI absorption	*High*	*High*	*Low*
Log Kp (skin permeation)	−5.13 cm/s	−6.95 cm/s	−4.30 cm/s
Bioavailability score	0.85	0.55	0.55
Bioactivity score			
GPCR ligand	−0.05	0.64	0.00
Ion channel modulator	−0.09	0.11	−0.32
Kinase inhibitor	0.06	−0.26	−0.12
Nuclear receptor ligand	0.04	0.66	0.53
Protease inhibitor	−0.13	−0.05	−0.27
Enzyme inhibitor	−0.02	0.57	0.42
Oral toxicity prediction			
Predicted LD50	3000 mg/kg	555 mg/kg	3850 mg/kg
Predicted toxicity class	V	IV	V

Acetylcholinesterase inhibitory activity

The **Figure 5** presents the IC_{50} values (ppm) of red betel leaves water extract from 4 different regions: Bandung, Malang, DIY and Bogor. The IC_{50} values are as follows: Bandung (58.426 ppm), Malang (54.699 ppm), DIY (40.799 ppm) and Bogor (49.545 ppm). Lower IC_{50} values indicate higher inhibitory potency. The data reveals that the red betel leaves extract from DIY has the highest inhibitory activity (lowest IC_{50}), while samples from Bandung and Malang exhibit the lowest inhibitory activity, as indicated by their similar and higher IC_{50} values. The observed AChE inhibitory activity (IC_{50}) of red betel (*Piper crocatum*) extract remains lower than that of the commercial drug donepezil (0.035 μM). However, as a natural product, the red betel extract demonstrates an IC_{50} value with promising potential as an AChE inhibitor [34].

The geographical variability in the AChE inhibitory activity observed among the red betel leaves from different regions can be attributed to several environmental factors that influence the phytochemical composition of plants. Factors such as soil composition, climate, altitude and local agricultural practices are known to significantly affect the levels of bioactive compounds in plants [35,36]. In our study, the red betel leaves from DIY exhibited the most potent inhibitory

activity, with the lowest IC_{50} value of 40.799 ppm. This could be due to the fact that Yogyakarta (DIY) is located at the lowest altitude (114 m above sea level) compared to other regions, such as Bandung (723 masl), Malang (350 masl) and Bogor (193 masl) [37-40]. Plants cultivated at lower altitudes often exhibit higher concentrations of bioactive phytochemicals. This suggests that agricultural practices in low-elevation areas may be more effective in producing crops with enhanced nutrient and phytochemical profiles [41].

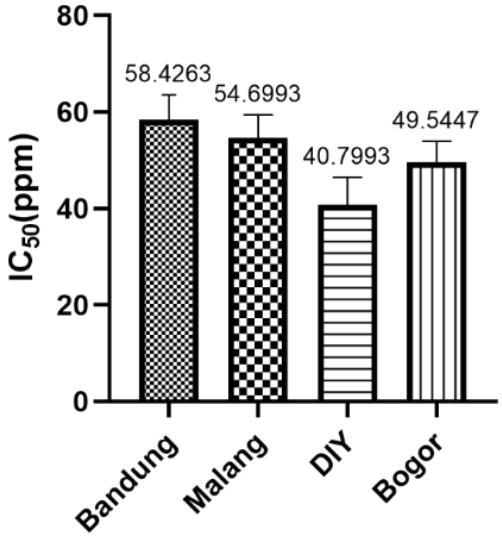

Figure 5 IC_{50} of red betel leaf water extracts in different region against AChE enzyme.

In addition to geographical conditions, the extraction methods and choice of solvent are also key factors influencing AChE inhibitory activity. Nurinsani *et al.* [10] provides further evidence of AChE inhibition by different red betel leaf extract fractions from Bogor region, where the ethanol extract displays the most substantial inhibition with an IC_{50} of 11.0965 µg/mL. This study also demonstrates a significant difference in inhibitory activity between various extracts, confirmed by a 1-Way ANOVA ($p < 0.05$). Finally, Huda *et al.* [42] observes that the water infusion extract of DIY red betel leaves yields a 26.621 % inhibition of AChE enzyme activity. Comparing across these studies, it appears that the extract type and preparation method substantially influence inhibitory potency, with ethanol extracts showing stronger activity than water extracts, indicating that red betel leaf's bioactive compounds may be more effectively extracted in polar solvents such as ethanol [43]. Ethanol's moderately polar nature allows it to extract a broader range of compounds, including flavonoids and phenolics, which are known for their strong AChE inhibitory effects [44]. In contrast, water, a highly polar solvent, may not be as efficient at extracting these non-polar or moderately polar compounds, leading to a higher IC_{50} value and weaker inhibitory activity. This phenomenon has been widely reported in phytochemical studies, where the choice of solvent significantly influences the yield and potency of extracted compounds [45]. The results of the computational study show that compounds SM15 [Columbin, water extract] and SM18 [Flemiphilippinin A, ethyl acetate extract] are the most potent compounds as Alzheimer's disease (AD) drugs based on binding free energy values, strong interaction with AChE, favorable pharmacokinetic properties, bioavailability and bioactivity. This is in line with the in vitro results, which show that the water extract and ethyl acetate extract have good AChE inhibition activity. The ethyl acetate extract has strong inhibition, with an IC_{50} of 16.7908 ppm, as reported by Nurinsani *et al.* [10], while the water infusion extract yields a 26.621 % inhibition of AChE enzyme activity [10,42]. Therefore, the use of different solvents in the extraction process significantly affects the biological activity of the plant extracts, including flavonoids and alkaloids that are key contributors to AChE inhibition [46].

Phenolic and Flavonoid are key indicators of antioxidant potential in various plant-based products, as they reflect the presence of bioactive compounds capable of scavenging free radicals. Phenolics and flavonoids are natural antioxidants that contribute significantly to health benefits, such as reducing oxidative stress and lowering risks of chronic diseases [47,48]. These findings highlight the importance of both regional environmental factors and solvent choice in determining the efficacy of plant-based extracts. Optimizing these factors in future research could enhance the therapeutic potential of red betel leaves, particularly for applications in enzyme inhibition and neuroprotective therapies [45,49].

Conclusions

The active compounds (SM05, SM15 and SM18) of *Piper crocatum* exhibit potential as inhibitors of AChE activity based on computational analysis. Among these, SM15 (columbin) and SM18 (Flemiphilippinin A) show the promise due to its strong interaction with AChE, favorable pharmacokinetic properties, bioavailability, bioactivity and oral toxicity making it a viable candidate for Alzheimer's disease therapy. Furthermore, the study highlights regional and methodological variations in AChE inhibitory activity of *Piper crocatum* extracts, with DIY samples exhibiting the highest potency (IC_{50}: 40.799 ppm) due to favorable environmental factors. These findings underscore the importance of optimizing environmental conditions and solvent selection to maximize the therapeutic potential of red betel leaves in neuroprotective applications.

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References

[1] SH Lu, JW Wu, HL Liu, JH Zhao, KT Liu, CK Chuang, HY Lin, WB Tsai and Y Ho. The discovery of potential acetylcholinesterase inhibitors: A combination of pharmacophore modeling, virtual screening, and molecular docking studies. *Journal of Biomedical Science* 2011; **18**, 8.

[2] PA Nogara, AR de Saraiva, DC Bueno, LJ Lissner, CLD Corte, MM Braga, DB Rosemberg and JBT Rocha. Virtual screening of acetylcholinesterase inhibitors using the Lipinski's rule of 5 and ZINC databank. *BioMed Research International* 2015; **2015(1)**, 870389.

[3] A Hálová, J Janoutová, L Ewerlingová, V Janout, O Bonczek, T Zeman, T Gerguri, VJ Balcar and O Šerý. CHAT gene polymorphism rs3810950 is associated with the risk of Alzheimer's disease in the Czech population. *Journal of Biomedical Science* 2018; **25**, 41.

[4] Alzheimer's Disease International. *World Alzheimer report 2023 reducing dementia risk: Never too early, never too late*. Alzheimer's Disease International, London, 2023.

[5] MS García-Ayllón, DH Small, J Avila and J Sáez-Valero. Revisiting the role of acetylcholinesterase in Alzheimer's disease: Cross-talk with P-tau and β-amyloid. *Frontiers in Molecular Neuroscience* 2011; **4**, 22.

[6] S Darvesh. Butyrylcholinesterase as a diagnostic and therapeutic target for Alzheimer's disease. *Current Alzheimer Research* 2016; **13(10)**, 1173-1177.

[7] R Khoury, N Grysman, J Gold, K Patel and GT Grossberg. The role of 5 HT6-receptor antagonists in Alzheimer's disease: An update. *Expert Opinion on Investigational Drugs* 2018; **27(6)**, 523-533.

[8] S Bordoloi, K Pathak, M Devi, R Saikia, J Das, VH Kashyap, D Das, MZ Ahmad and BA Abdel-Wahab. Some promising medicinal plants used in Alzheimer's disease: An ethnopharmacological perspective. *Discover Applied Sciences* 2024; **6(5)**, 215.

[9] RG Putri, M Safithri, H Husnawati and R Kurniasih. Penambatan molekuler senyawa aktif sirih merah (*Piper crocatum*) pada butirilkolinesterase sebagai kandidat antialzheimer (*in Indonesian*). *ALCHEMY Jurnal Penelitian Kimia* 2023; **19(1)**, 68-85.

[10] EYY Nurinsani, D Andrianto and M Safithri. Acetylcholinesterase inhibition activity and phytochemical screening of red betel leaf (*Piper*

crocatum Ruiz & Pav) as anti-dementia agents. *BIO Web of Conferences* 2024; **123**, 02009.

[11] World Health Organization. *Global accelerated action for the health of adolescents (AA-HA!)*. World Health Organization, Geneva, Switzerland, 2023.

[12] M Safithri and A Kurniawati. Formula of *Piper crocatum*, *Cinnamomum burmanii*, and *Zingiber officinale* extracts as a functional beverage for diabetics. *International Food Research Journal* 2016; **23(3)**, 1123-1130.

[13] S Wang, X Kong, Z Chen, G Wang, J Zhang and J Wang. Role of natural compounds and target enzymes in the treatment of Alzheimer's disease. *Molecules* 2022; **27(13)**, 4175.

[14] R Kurniasih. 2018, Struktur prediksi dan identifikasi substrat spesifik lakase isolat lokal neorspora crassa inaCC F226 (*in Indonesian*). Ph. D. Dissertation. Bogor Agricultural University, Bogor, Indonesia.

[15] R Agarwal and JC Smith. Speed vs accuracy: Effect on ligand pose accuracy of varying box size and exhaustiveness in AutoDock Vina. *Molecular Informatics* 2023; **42(2)**, 2200188.

[16] O Trott and AJ Olson. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry* 2010; **31(2)**, 455-461.

[17] M Weni, M Safithri and DSH Seno. Molecular docking of active compounds *Piper crocatum* on the a-glucosidase enzyme as antidiabetic. *Indonesian Journal of Pharmaceutical Science and Technology* 2020; **7(2)**, 64-72.

[18] AS Abouzied, S Alqarni, KM Younes, SM Alanazi, DM Alrsheed, RK Alhathal, B Huwaimel and AM Elkashlan. Structural and free energy landscape analysis for the discovery of antiviral compounds targeting the cap-binding domain of influenza polymerase PB2. *Scientific Reports* 2024; **14(1)**, 25441.

[19] BD Rosiarto, AR Puspaningtyas and D Holidah. Studi aktivitas antioksidan senyawa 1-(p-klorobenzoiloksimetil)-5-fluorourasil dengan metode molecular docking dan metode DPPH (antioxidant activity of 1-(p-chlorobenzoyloxymethyl)-5-fluorouracyl using molecular docking and DPPH method) (*in Indonesian*). *E-Jurnal Pustaka Kesehatan* 2014; **2(1)**, 95-99.

[20] H Dvir, I Silman, M Harel, TL Rosenberry and JL Sussman. Acetylcholinesterase: From 3D structure to function. *Chemico-Biological Interactions* 2010; **187(1-3)**, 10-22.

[21] J Cheung, MJ Rudolph, F Burshteyn, MS Cassidy, EN Gary, J Love, MC Franklin and JJ Height. Structures of human acetylcholinesterase in complex with pharmacologically important ligands. *Journal of Medicinal Chemistry* 2012; **55(22)**, 10282-10286.

[22] C Seniya, GJ Khan and K Uchadia. Identification of potential herbal inhibitor of acetylcholinesterase associated Alzheimer's disorders using molecular docking and molecular dynamics simulation. *Biochemistry Research International* 2014; **2014(1)**, 705451.

[23] XY Meng, HX Zhang, M Mezei and M Cui. Molecular docking: A powerful approach for structure-based drug discovery. *Current Computer Aided-Drug Design* 2011; **7(2)**, 146-157.

[24] M Atanasova, N Yordanov, I Dimitrov, S Berkov and I Doytchinova. Molecular docking study on galantamine derivatives as cholinesterase inhibitors. *Molecular Informatics* 2015; **34(6-7)**, 394-403.

[25] I Doytchinova, M Atanasova, I Valkova, G Stavrakov, I Philipova, Z Zhivkova, D Zheleva-Dimitrova, S Konstantinov and I Dimitrov. Novel hits for acetylcholinesterase inhibition derived by docking-based screening on ZINC database. *Journal of Enzyme Inhibition and Medicinal Chemistry* 2018; **33(1)**, 768-776.

[26] F Ramadani. *Aktivitas antioksidan, total tanin ekstrak dan fraksi daun sirih merah (Piper crocatum) dan identifikasi dengan LC-MS* (*in Indonesian*). Bogor Agricultural University, Bogor, Indonesia, 2018.

[27] CK Joshi and HK Joshi. Molecular docking analysis of acetylcholinesterase with phytocompounds from medicinal plants. *Multilogic in Science* 2015; **4**, 164-168.

[28] A Alvarez, R Alarcon, C Opazo, EO Campos, FJ Munoz, FH Calderon, F Dajas, MK Gentry, BP

Doctor, FGD Mello and NC Inestrosa. Stable complexes involving acetylcholinesterase and amyloid-β peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimer's fibrils. *The Journal of Neuroscience* 1998; **18(9)**, 3213-3223.

[29] P Paixão, LF Gouveia and JAG Morais. Prediction of the human oral bioavailability by using *in vitro* and *in silico* drug related parameters in a physiologically based absorption model. *International Journal of Pharmaceutics* 2012; **429(1-2)**, 84-98.

[30] GD Stanciu, A Luca, RN Rusu, V Bild, SI Beschea Chiriac, C Solcan, W Bild and DC Ababei. Alzheimer's disease pharmacotherapy in relation to cholinergic system involvement. *Biomolecules* 2019; **10(1)**, 40.

[31] CA Lipinski. Lead- and drug-like compounds: The rule-of-5 revolution. *Drug Discovery Today: Technologies* 2004; **1(4)**, 337-341.

[32] SK Bhal. LogP-making sense of the value. *Tropical Journal of Natural Product Research* 2007; **4(12)**, 1208-1214.

[33] SM Alwan. Computational calculations of molecular properties and molecular docking of new and reference cephalosporins on penicillin binding proteins and various β-lactamases. *Journal of Pharmacy and Pharmacology* 2016; **4(5)**, 212-225.

[34] F Li, ZM Wang, JJ Wu, J Wang, SS Xie, JS Lan, W Xu, LY Kong and XB Wang. Synthesis and pharmacological evaluation of donepezil-based agents as new cholinesterase/monoamine oxidase inhibitors for the potential application against Alzheimer's disease. *Journal of Enzyme Inhibition and Medicinal Chemistry* 2016; **31**, 41-53.

[35] D Andrianto, S Hermita and S Haryanti. The classification of betel leaves (*Piper betl*e) from 15 ethnics in eastern Indonesia based on phytochemicals fingerprint analysis. *Biodiversitas Journal of Biological Diversity* 2019; **21(1)**, 252-257.

[36] EW Purnama, M Safithri and D Andrianto. Clusterization of red betel leaves (*Piper Crocatum*) from various regions in Indonesia based on secondary metabolite fingerprint analysis and cytotoxicity values. *Indonesian Journal of Applied Research* 2023; **4(2)**, 170-182.

[37] F Kurniawan. Review of geographic factors support mapping in Yogyakarta related to food security conditions on the physiological quality of talented athletes. *Advances in Health Sciences Research* 2022; **43**, 289-294.

[38] R Ismail and A Ismail. Spatial analysis on influence of elevation to coronavirus disease-19 infection fatality ratio in Bandung Regency and Bandung City. *IOP Conference Series: Earth and Environmental Science* 2024; **1404(1)**, 012050.

[39] R Jannah, H Helmi and S Sufardi. Soil physical and chemical properties in Patcholi planting area in Teungoh Geunteut Village, Kecamatan lhoong, Aceh Besar. *Jurnal Ilmiah Mahasiswa Pertanian* 2022; **7(3)**, 431-439.

[40] W Abd Karim, A Farajallah and B Suryobroto. Exploration and prevalence of gastrointestinal worm in buffalo from West Java, Central Java, East Java and Lombok, Indonesia. *Aceh Journal of Animal Science* 2016; **1(1)**, 1-15.

[41] S Shukla, N Kumar, P Bhardwaj, P Pandita, MK Patel, MS Thakur, R Kumar, M Rawat and S Saxena. Effect of cold arid high-altitude environment on bioactive phytochemical compounds of organically grown Brassicaceae vegetables for nutri-health security in mountainous regions. *Scientific Reports* 2024; **14(1)**, 15976.

[42] AS Huda, AEZ Hasan and M Safithri. Acetylcholinesterase enzyme inhibitor and antioxidant activities from a mixture extracts of black tea, red betel, Cinnamon and Curcuma. *Current Biochemistry* 2023; **9(2)**, 63-72.

[43] B Kharel, L Rusalepp, B Bhattarai, A Kaasik, P Kupper, R Lutter, P Mänd, G Rohula-Okunev, K Rosenvald and A Tullus. Effects of air humidity and soil moisture on secondary metabolites in the leaves and roots of Betula pendula of different competitive status. *Oecologia* 2023; **202(2)**, 193-210.

[44] L Gali and F Bedjou. Antioxidant and anticholinesterase effects of the ethanol extract, ethanol extract fractions and total alkaloids from the cultivated *Ruta chalepensis*. *South African Journal of Botany* 2019; **120**, 163-169.

[45] A Plaskova and J Mlcek. New insights of the application of water or ethanol-water plant extract rich in active compounds in food. *Frontiers in Nutrition* 2023; **10**, 1118761.

[46] H Khan, S Amin, MA Kamal and S Patel. Flavonoids as acetylcholinesterase inhibitors: Current therapeutic standing and future prospects. *Biomedicine & Pharmacotherapy* 2018; **101**, 860-870.

[47] RR Elfirta, PR Ferdian, I Saskiawan, TH Handayani, KFG Mandalika, R Riffiani, K Kasirah and UMS Purwanto. Antioxidant properties of kombucha beverage infused with *Ganoderma lucidum* and green tea from *Camellia sinensis* (L.) Kuntze with several fermentation times. *Karbala International Journal of Modern Science* 2024; **10(1)**, 12.

[48] N Marliani, IM Artika, M Rafi, M Syukur, RY Galingging and W Nurcholis. Phenolic and flavonoid content with agro morphological characters of 12 accessions of Justica gendarussa grew in Indonesia. *Biodiversitas Journal of Biological Diversity* 2022; **23(10)**, 5101-5107.

[49] JE Lee, JTM Jayakody, JI Kim, JW Jeong, KM Choi, TS Kim, C Seo, I Azimi, J Hyun and B Ryu. The influence of solvent choice on the extraction of bioactive compounds from Asteraceae: A comparative review. *Foods* 2024; **13(19)**, 3151.