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Investigation of *Averrhoa bilimbi* fruit extract-derived compounds as potential acetylcholinesterase inhibitors in *Aedes aegypti*: Insights from molecular docking and molecular dynamics simulations

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Abstract
Aedes mosquitoes are primary vectors of human diseases such as dengue fever, chikungunya, and yellow fever. Their control heavily depends on chemical insecticides, but the rise of resistance in *Aedes aegypti* populations presents a significant challenge. This study explores the potential of bioactive compounds from *Averrhoa bilimbi* (starfruit) fruit as acetylcholinesterase (AChE) inhibitors for mosquito control. A total of 20 active compounds from *A. bilimbi* fruit extracts were identified using Gas Chromatography-Mass Spectrometry (GC-MS) and subjected to molecular docking analysis to evaluate their binding affinity toward AChE. Molecular dynamics (MD) simulations using YASARA software further assessed their stability and interaction dynamics. Among these compounds, ß-Amyrin (-9.4 kcal/mol) and Urs-12-ene (-9.3 kcal/mol) exhibited the strongest binding affinities, surpassing the reference inhibitor donepezil (-8.3 kcal/mol). However, MD simulations revealed that Urs-12-ene exhibited unstable binding and conformational fluctuations, making ß-Amyrin the most promising AChE inhibitor. This compound demonstrated strong and stable interactions with AChE, as supported by binding affinity, binding site analysis, and interaction stability. These findings suggest that ß-Amyrin from *A. bilimbi* fruit extracts holds promise as a natural AChE inhibitor for mosquito control. However, further in *vitro* and in *vivo* studies are necessary to validate its inhibitory potential and assess its efficacy and safety for practical application.

Keywords: *Aedes aegypti*, acetylcholinesterase inhibition, molecular docking, molecular dynamics simulation, *Averrhoa bilimbi*

Introduction
Mosquito-borne diseases continue to impose a significant global health burden, with *Aedes aegypti* serving as the primary vector for dengue fever, chikungunya, yellow fever, and other arboviral infections [1]. Resistance in *Aedes aegypti* to insecticides is a nationwide challenge for disease control programs in Indonesia [2]. The emergence of resistance poses a significant threat, as it can hinder efforts to combat mosquito-borne diseases [3, 4, 5]. This resistance is primarily attributed to detoxification mechanisms and reductions in target protein sensitivity [6]. Additionally, studies have shown that mutations in the voltage-gated sodium channel gene result in knockdown resistance (kdr), which contributes to pyrethroid (PY) resistance. This resistance has also been linked to fitness costs in various insect species, including *Aedes aegypti* [7, 8, 9]. Given the growing resistance problem, identifying new mosquitocidal agents and understanding their mechanisms of action are crucial for enhancing mosquito control strategies and mitigating insecticide resistance.
Acetylcholinesterase (AChE) is a critical enzyme in the nervous system, responsible for terminating nerve impulses by hydrolyzing the neurotransmitter acetylcholine [9]. Disruption of AChE activity leads to the accumulation of acetylcholine [10, 5], resulting in impaired neural function and, ultimately, insect mortality. As such, AChE represents an attractive target for insecticide development, particularly in the context of overcoming resistance mechanisms that limit the effectiveness of conventional agents.
Natural products offer a vast reservoir of structurally diverse compounds with potential

biological activity against various targets, including AChE. *Averrhoa bilimbi* (starfruit) is one such plant that has attracted scientific interest due to its rich phytochemical composition and traditional medicinal uses [11, 12, 13]. Recent advances in analytical techniques, such as Gas Chromatography-Mass Spectrometry (GC-MS), have facilitated the identification of bioactive compounds within *A. bilimbi* fruit extracts, providing a promising starting point for the discovery of new insecticidal agents [11, 12, 12].

In parallel, computational approaches most notably molecular docking and molecular dynamics (MD) simulations have emerged as powerful tools in drug discovery and the evaluation of ligand-protein interactions. These techniques enable a detailed investigation of the binding modes, affinities, and stabilities of potential inhibitors at the molecular level, thus offering valuable insights prior to in *vivo* experimentation. This study aims to explore the potential of bioactive compounds derived from *A. bilimbi* fruits as AChE inhibitors in *Aedes aegypti*. By integrating GC-MS-based compound identification with molecular docking and MD simulations, we evaluated the binding interactions between these natural compounds and the AChE enzyme, as illustrated in Fig.

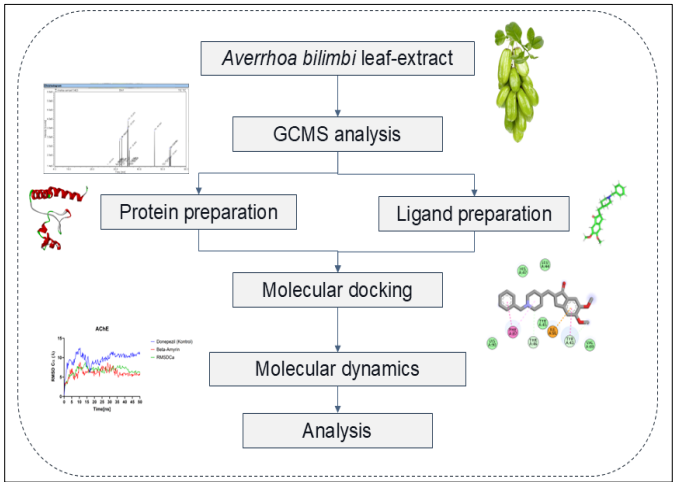

Fig 1: Study Workflow

2. Materials and Methods

2.1. Compound Data Mining

The 3D structure of Acetylcholinesterase (ACHE) protein with Uniprot identifier (uniprot ID) Q8MYC0 [13] was obtained from the Uniprot database (https://www.uniprot.org/) as a.pdb format file. The active compounds of *Averrhoa bilimbi* were obtained from the GC-MS results and downloaded through PubChem (https://pubchem.ncbi.nlm.nih.gov/) in the form of.sdf format files. Donepezil was used as a positive control compound [14].

2.2. Molecular Docking

2.2.1. Protein preparation

Protein molecule for AchE was prepared for analysis. The 3D protein structure was obtained from the UniProt (https://www.uniprot.org/) using UniProt ID Q8MYC0. The structure was prepared using the Discovery Studio Visualizer version 16 by removing water molecules and other ligands which still attached to the structure. Polar hydrogen atoms were added to the structure. The prepared structure was saved in *.pdbqt format using AutoDock Tools version 1.5.6.

2.2.2. Ligand Preparation

A total of 20 compounds were identified from the GC-MS result, all of which were evaluated as potential ligands. The 3D structure of the test ligand compound in *.sdf format was downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) and converted using PyRx 0.9.5 software [15] into *.pdb format. Furthermore, the ligand was optimized using OpenBabel tools integrated in PyRx 0.9.5 [15] software with the addition of hydrogen bonds and saved in *. pdbqt format.

2.2.3. Virtual screening

A total of 21 optimized and pre-prepared ligands, along with its control donepezil, were attached to AChE through virtual screening using PyRx software. Virtual screening was conducted on a grid box with the coordinates of the optimum validation results based on AutoDock Vina. The resulting binding free energy and root mean standard deviation (RMSD) values were then sorted based on the smallest to the most significant binding free energy values for the next step.

2.2.4. Docking Parameters

Validation was performed by specifying the grid box dimensions (x: 38, y: 20, z: 28; central point coordinates at −13,988, y: −43.906, z: 27109) [14]. The grid box was created by adjusting the dimensions of the control ligand-binding pocket (donepezil) in AChE by selecting the center on the ligand in AutoDock Vina. The complex of protein-ligand with binding affinity lesser than control (-8.3 kcal/mol) and lower than -7.0 kcal/mol [16, 17] then continued for ligand-residues interaction analysis using Discovery Studio 2019. Compound with a larger number of interactions and lower binding affinity within the active site of ACHE protein were directed into molecular dynamics analysis.

2.2.5. Molecular Dynamics

Molecular dynamics simulations were conducted to assess the stability of ligand-protein interactions over time [16, 18, 19]. The parameters analyzed included RMSD ligand conformation, RMSD ligand movement, RMSD backbone, root mean square fluctutation (RMSF), number of hydrogen bond, and binding free energy. Molecular dynamics were performed for 50 ns with AMBER14 forcefield [20] accompanied by the following parameters: pH 7.4; 0.9% NaCl concentration; 0.997 water density; 1 atm pressure; and 310oK temperature with cubic grid shape. All simulations were performed in YASARA software version 21 [21]. The binding energy calculations were also performed by fast boundary method with YASARA binding energy macros.

3. Results and Discussions

3.1. Results

3.1.1. *Averrhoa bilimbi* fruit extract compounds

The GC-MS analysis identified 20 compounds in *A. bilimbi* fruit extract. Hexadecanoic acid was the most abundant compound, with relative area of 16.89%, followed by phytol at 6.44% and 2-Methyl-Z,Z-3,13-octadecadienol at 14.20% (Table 1). The 20 chemical compounds identified from the fruit extract were classified into terpenes, flavonoids, alkaloids, and saponins [11]. Three-dimensional (3D) structures of these ligands obtained from PubChem database are presented in Figure 2.

Table 1: Chemical compounds identified from *A. bilimbi* fruit extract

Retention time	Compounds	Relative area (%)	PubChem ID	Molecular Formula
26.420	d-Gala-l-ido-octonic amide	0.69	552061	C8H17NO8
29.96	E-2-Tetradecen-1-ol	1.42	5353006	C14H28O
30.09	3-Trifluoroacetoxypentadecane	0.35	534406	C17H31F3O2
30.600	Ethaneperoxoic acid	0.89	6585	C2H4O3
31.64	[1,1'-Bicyclopropyl]-2-octanoic acid	0.48	85247629	C14H24O2
31.72	Hexadecanoic acid	16.89	985	C16H32O2
32.07	Benzenepropanoic acid	0.91	107	C9H10O2
33.020	Octadecanoic acid	1.19	5281	C18H36O2
34.91	Methyl 9-cis,11-trans-octadecadienoate	0.98	11748436	C19H34O2
35.03	Cyclopropaneoctanoic acid	12.4	57346156	C19H36O2
35.25	Phytol	15.24	5280435	C20H40O
35.49	Heptadecanoic acid	1.66	10465	C17H34O2
35.86	2-Methyl-Z, Z-3,13-octadecadienol	14.2	5364412	C19H36O
36.23	Cyclopropanetetradecanoic acid	0.34	11323150	C17H32O2
46.48	Squalene	7.96	638072	C30H50
49.83	Ergosta-5,22-dien-3-ol,	0.42	5281327	C28H46O
50.57	9,10-Secocholesta-5,7,10(19)-triene-3beta,24,25-triol	0.56	5283748	C27H44O3
52.92	ß-Sitosterol	5.01	222284	C29H50O
53.06	Urs-12-ene	2.8	612603	C30H50
53.39	ß-Amyrin	4.88	73145	C30H50O
	Donepezil (control)		3152	C24H29NO3

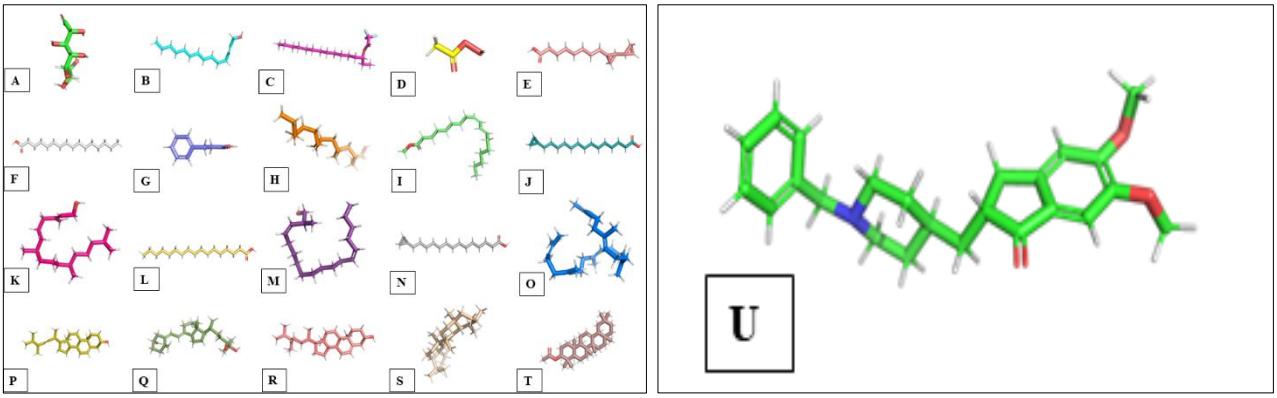

Fig 2: Three-dimensional (3D) structures of *A. bilimbi* fruit extract. (A) d-Gala-l-ido-octonic amide. (B) E-2-Tetradecen-1-ol. (C) 3-Trifluoroacetoxypentadecane. (D) Ethaneperoxoic acid. (E) [1,1'-Bicyclopropyl]-2-octanoic acid. (F) Hexadecanoic acid. (G) Benzenepropanoic acid. (H) Octadecanoic acid. (I) Methyl 9-cis,11-trans-octadecadienoate. (J) Cyclopropaneoctanoic acid. (K) Phytol. (L) Heptadecanoic acid. (M) 2-Methyl-Z,Z-3,13-octadecadienol. (N) Cyclopropanetetradecanoic acid. (O) Squalene. (P) Ergosta-5,22-dien-3-ol. (Q) 9,10-Secocholesta-5,7,10(19)-triene-3beta,24,25-triol. (R) ß-Sitosterol. (S) Urs-12-ene. (T) ß-Amyrin. (U) Donepezil (control).

3.1.2. Molecular docking results

Molecular docking was conducted on the 20 bioactive compounds against the AChE protein. It was found that only five compounds that have the lowest binding affinity (≤ -7.0 kcal/mol) [22] even when compared to control (-8.3 kcal.mol), which describes the most potent compound to interact with AChE. The lowest ligand binding affinity between five compounds was ß-Amyrin at -9.4 kcal/mol (Table 2). Fortunately, some compounds showed several hydrogen bonds accompanied by hydrophobic bonds and some van der Waals interactions. 9,10-Secocholesta-5,7,10(19)-triene-3beta,24,25-triol had the most interactions but mainly formed as Van der Waals interaction. Although d-Gala-l-ido-octonic amide had the most hydrogen bond, its binding residues were not at the binding site and differed from the control. Interestingly, ß-Amyrin and Urs-12-ene had lowest binding affinity (-9.4 kcal/mol and -9.3 kcal/mol, respectively) and formed some adequate hydrophobic bonds and Van der Waals interactions with all of the interacted residues found in the donepezil (Table 2&3).

Table 2: Docking results of *A. bilimbi* fruit extract

No	Senyawa	Binding Affinity
1	ß-Amyrin	-9.4 Kcal/mol
2	Urs-12-ene	-9.3 Kcal/mol
3	d-Gala-l-ido-octonic amide	-9,1 Kcal/mol
4	Ergosta-5,22-dien-3-ol,	-8.5 Kcal/mol
5	9,10-Secocholesta-5,7,10(19)-triene-3beta,24,25-triol	-8.3 Kcal/mol
6	Donepezil (Kontrol)	-8.3 Kcal/mol
7	ß-Sitosterol	-7.5 Kcal/mol
8	Squalene	-6.5 Kcal/mol
9	Benzenepropanoic acid	-5.8 Kcal/mol
10	[1,1'-Bicyclopropyl]-2-octanoic acid	-5.5 Kcal/mol
11	Cyclopropaneoctanoic acid	-5.2 Kcal/mol
12	Phytol	-5.2 Kcal/mol
13	3-Trifluoroacetoxypentadecane	-5.0 Kcal/mol
14	Methyl 9-cis,11-trans-octadecadienoate	-5.0 Kcal/mol
15	Hexadecanoic acid	-4.8 Kcal/mol
16	Cyclopropanetetradecanoic acid	-4.7 Kcal/mol
17	2-Methyl-Z,Z-3,13-octadecadienol	-4.6 Kcal/mol
18	Octadecanoic acid	-4.5 Kcal/mol
19	Heptadecanoic acid	-4.3 Kcal/mol
20	E-2-Tetradecen-1-ol	-4.2 Kcal/mol
21	Ethaneperoxoic acid	-3.7 Kcal/mol

The two most potential ligands with the lowest binding affinity values, Urs-12-ene and ß-Amyrin, exhibit favorable chemical interactions (Figures 4&5) and share the same amino acid residues as the control (donepezil) (Figure 3). Urs-12-ene forms nine van der Waals interactions with residues GLU 65, TYR 43, GLY 63, TYR 45, MET 61, VAL 60, THR 23, ASP 19, and GLY 18, along with five hydrophobic interactions with residues HIS 62, PHE 22, VAL 26, LEU 44, and LYS 15 (Figure 4& Table 3). Meanwhile, ß-Amyrin forms eight van der Waals interactions with residues VAL 60, GLY 18, ASP 14, ASP 19, LEU 44, THR 23, PHE 22, and GLU 65, as well as four hydrophobic interactions with residues LYS 15, VAL 26, HIS 62, and TYR 43 (Figure 5& Table 3).

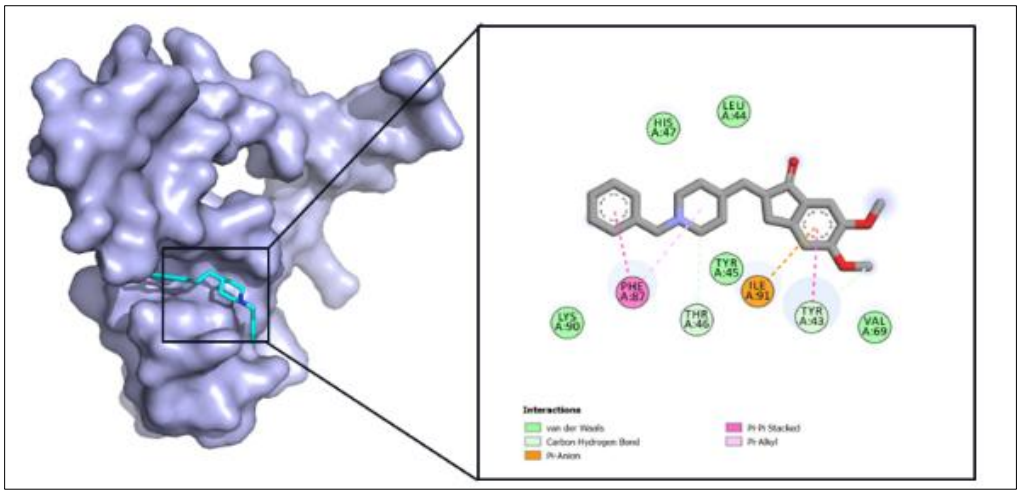

Fig 3: Molecular docking result of the AChE-donepezil complex. The left panel shows the overall binding of donepezil (blue) within the AChE binding pocket, while the right panel presents a 2D interaction map, including hydrogen bonds, van der Waals interactions, and hydrophobic bonds.

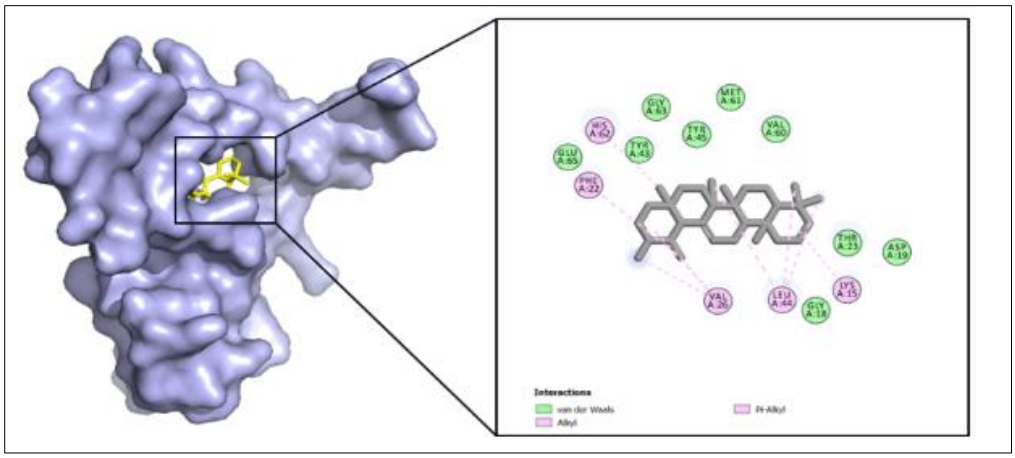

Fig 4: Molecular docking result of the AChE- Urs-12-ene complex. The left panel shows the overall binding of Urs-12-ene (yellow) within the AChE binding pocket, while the right panel presents a 2D interaction map, including van der Waals interactions and hydrophobic bonds.

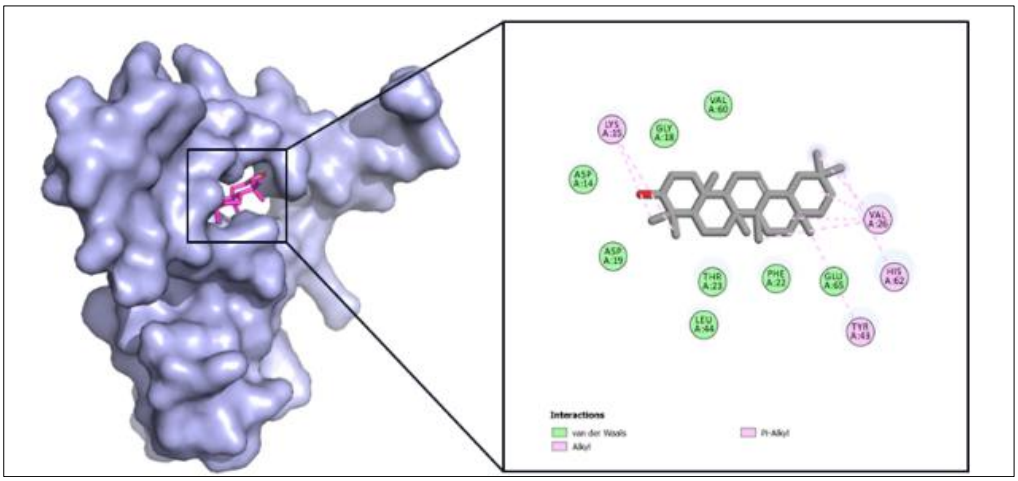

Fig 5: Molecular docking result of the AChE- ß-Amyrin complex. The left panel shows the overall binding of ß-Amyrin (pink) within the AChE binding pocket, while the right panel presents a 2D interaction map, including van der Waals interactions and hydrophobic bonds.

Table 3: Amino acid residues of AChE that interact with five potential ligands from *A. bilimbi* extract

No	Compounds	Hydrogen bond	Hydrophobic bond	Van der Waals
1	Donepezil (Kontrol)	THR 46, TYR 43	PHE 87, ILE 91, TYR 43	LYS 90, TYR 45, VAL 69, LEU 44, HIS 47
2	ß-Amyrin	N/A	LYS 15, VAL 26, HIS 62, TYR 43	VAL 60, GLY 18, ASP 14, ASP 19, LEU 44, THR 23, PHE 22, GLU 65
3	Urs-12-ene	N/A	HIS 62, PHE 22, VAL 26, LEU 44, LYS 15	GLU 65, TYR 43, GLY 63, TYR 45, MET 61, VAL 60, THR 23, ASP 19, GLY 18
4	d-Gala-l-ido-octonic amide	GLY 63, ASP 64, ASN 67, HIS 62, SER 49, TYR 80	N/A	ILE 66, TYR 68, MET 61, ASN 52, PRO 53
5	Ergosta-5,22-dien-3-ol,	TYR 43, TYR 45	LEU 13, VAL 17, PHE 22	ASP 14, MET 61, HIS 62, VAL 60, LEU 44, GLY 18, THR 23
6	9,10-Secocholesta-5,7,10(19)-triene-3beta,24,25-triol	GLU 65, GLY 63, TYR 41	PHE 22, TYR 43, VAL 26	MET 61, TYR 45, GLY 18, MET 42, PHE 29, ALA 30, VAL 60, ASP 64, HIS 62, THR 23, LEU 44

Notes: the bold typed amino acids described a similar binding site with the Donepezil (Control). "Hydrophobic bond" described the interactions involving Pi-Hydrophobic (Pi-Pi stacked, Pi-Pi T-Shaped, and amide Pi-Stacked), Alkyl Hydrophobic (Alkyl), and Mixed Pi/Alkyl Hydrophobic (Pi-Sigma and Pi-Alkyl). "Others" column described the unfavorable bonds. N/A means not available.

3.1.3. Molecular dynamics analysis

Molecular dynamics simulations were conducted to study the stability of the interaction between proteins and compounds from *A. bilimbi*. The RMSD ligand conformation shows that ß-Amyrin and Urs-12-ene are more stable compared to donepezil, indicating that these two ligands had potential as AChE inhibitor (Figure 6A). Meanwhile, The RMSD ligand movement indicates that Urs-12-ene exhibit higher fluctuations, suggesting greater movement within the binding site, while ß-Amyrin remains more stable with the least movement (Figure 6B). The RMSD backbone atoms (RMSDBb) also indicate that Urs-12-ene and ß-Amyrin form more stable complexes with AChE than donepezil, which shows their potential as AChE inhibitor (Figure 6C).

ß-Amyrin formed more hydrogen bonds and closely similar to donepezil, indicating stronger and more stable interactions than Urs-12-ene (Figure 6D). Some hydrogen bonds in the protein-ligand complex also influence the stability and energy requirements of the protein-ligand interaction [23]. Hydrogen bond performs a robust and attractive force between protein and ligand through the formation of the bond through their functional groups, such as carboxyl, amino, or hydroxyl groups [24]. Remarkably, hydrogen bond comprises the primary interaction type of inhibitors of proteins in the PDB database [25], suggesting the significant role of this interaction in the discovery of protein antagonist. Moreover, ß-Amyrin and Urs-12-ene show lower RMSF values compared to donepezil, indicating greater protein stability. Urs-12-ene is the most stable, followed by ß-Amyrin (Figure 6E). Higher RMSF implies increased flexibility thus increasing the potential to interact with ligand molecules. The RMSF's of the two simulated ligands have values close to donepezil, although there are significant fluctuations in residues GLY 59, ASP 77, and ASP 83, but they do not have a major effect because they are not in the active site of the protein. For binding energy, Urs-12-ene and ß-Amyrin show higher and more stable binding energy than donepezil, indicating stronger interactions with AChE, with Urs-12-ene has the strongest binding (Figure 6F).

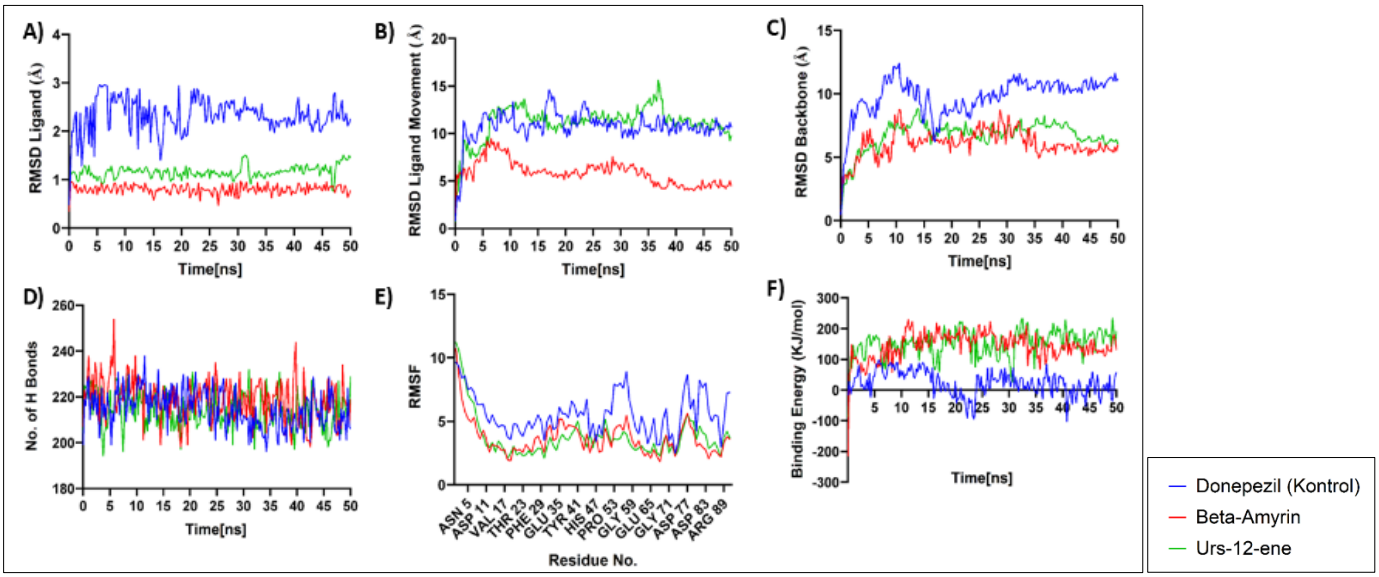

Fig 6: Molecular dynamics simulation on the interaction of AChE with the selected compounds: RMSD ligand conformations (A), RMSD ligand movements (B), RMSD backbone atoms (C), number of hydrogen bonds (D), root mean square fluctuation (RMSF) (E), and binding energy fluctuations (F).

3.2. Discussions

This study identified 20 compounds derived from *A. bilimbi*, with Hexadecanoic acid being the most abundant compound. These ligands were then subjected to molecular docking to predict inhibitory potential against AChE protein. It was found that only five ligands exhibited favorable binding

affinity compared to control (-8.3 kcal/mol): ß-Amyrin (-9.4 kcal/mol), Urs-12-ene (-9.3 kcal/mol), d-Gala-l-ido-octonic amide (-9.1 kcal/mol), Ergosta-5,22-dien-3-ol (-8.5 kcal/mol), and 9,10-Secocholesta-5,7,10(19)-triene-3beta,24,25-triol (-8.3 kcal/mol). The two most promising ligand based on binding affinity and chemical interactions, ß-Amyrin and Urs-12-ene, were further analyzed using molecular dynamics to assess their stability. Among six MDS parameters performed, ß-Amyrin outperformed in four (RMSD ligand conformation, RMSD ligand movement, RMSD backbone, and hydrogen bonds), suggesting it as the most potential AChE inhibitor.

Acetylcholinesterase (AChE) is an enzyme responsible for hydrolyzing the cholinergic neurotransmitter acetylcholine (ACh) in synaptic transmission. When a drug molecule inhibits AChE activity, acetylcholine levels in the synaptic cleft increase, which can ultimately lead to fatal consequences [25]. ß-Amyrin and Urs-12-ene were found to interact with tyrosine (Tyr) residues, which are predominantly located in the catalytic binding sites of AChE. Tyrosine plays a crucial role in regulating plasma neurotransmitter levels and contributes to neurotransmitter initiation [26, 27].

Various insecticides, including organophosphates, carbamates, and the insect repellent N,N-Diethyl-meta-toluamide (DEET), are known to target acetylcholinesterase (AChE), leading to the accumulation of acetylcholine at synapses and ultimately causing mosquito mortality [28, 29]. Studies have demonstrated that limonoids, such as azadirachtin from neem, can inhibit AChE in *vitro* in *Nilaparvata lugens* [30]. Additionally, pyrimidine trione furan (PTF) derivatives have shown inhibitory activity against AChE1 in *Culex pipiens* and *Anopheles gambiae*, even in populations resistant to organophosphates and carbamates [31]. Moreover, 1-nitro-2-phenylethane, extracted from *Aniba canelilla* plant oil, has demonstrated AChE inhibitory effects in electric eel [32].

Earlier investigations have reported that the extract from *A. bilimbi* was effective in killing *Aedes aegypti* larvae, where using a higher concentration of ethanol as a solvent to extract compounds from the fruit and leaves of Wuluh starfruit (*Averrhoa bilimbi* L.) led to increased larval mortality [11]. The mortality of the test larvae can be attributed to the chemical compounds present in the juice of Wuluh starfruit (*A. bilimbi* L.). The fruit and leaves of *A. bilimbi* contain alkaloids, saponins, and flavonoids [11]. According to Mawuntyas' research, alkaloids have insecticidal properties [33]. In their natural form, alkaloids from fresh leaves and fruit impart a bitter taste and, when present as salts, can break down and damage cell walls. Saponins, a class of triterpenoid compounds, also exhibit insecticidal activity. When ingested by insects, saponins interfere with digestive enzyme activity and nutrient absorption, acting as a stomach poison. Similarly, flavonoids, a type of phenolic compound, possess antimicrobial, antiviral, antifungal, and insecticidal properties. A previous study also support the findings of this research, demonstrating that triterpenoids, such as ß-Amyrin, exhibited strong interactions with NSP1 of DENV-2 in virtual screenings, suggesting their potential antiviral properties [34].

One limitation of this study is its reliance on an in silico approach, which constrains the interpretation of the results. The lack of in *vitro* or in *vivo* validation raises concerns regarding the accuracy and reliability of the predictions. Experimental validation is essential to confirm the computational findings by comparing them with real biological data. Additionally, the identification of *A. bilimbi*-derived compounds was based on available references without direct experimental isolation, which may introduce bias and potentially overlook other bioactive compounds. Future studies should incorporate comprehensive compound isolation and biological assays to validate the predicted AChE inhibitory activity.

4. Conclusion

Among 20 active compounds from *A. bilimbi*, ß-Amyrin and Urs-12-ene have the best binding affinities and interaction chemistry than other compounds. However, Urs-12-ene showed unstable conformation and binding during molecular dynamics analysis. Therefore, ß-Amyrin were the most potent compound as AChE inhibitor. This compound have worthy interaction with the AChE according to the binding affinity, binding site, and interaction stability. Nevertheless, further in *vitro* and in *vivo* studies are warranted to confirm its inhibitory activity.

5. Acknowledgment

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