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Dual Targeting of Protease and Helicase Domain of Dengue Virus Nonstructural 3 Protein from Multiple Bioactive Compounds as Novel Anti-Viral Candidate: Insight from Computational Study

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ABSTRACT

Dengue virus (DENV) is the leading cause of dengue fever, one of the neglected tropical diseases in tropical countries like Indonesia. Antivirus development is one of the priority programs in addition to vector control. One of the most critical proteins encoded by the DENV genome is non-structural protein 3 (NS3). This protein is commonly used to develop anti-virals because it has vital enzymatic activity in the DENV replication process. It also has sequences that are conserved among all DENV serotypes and consists of two domains: protease and helicase. This study aims to screen potential natural compounds targeting the DENV's NS3 using the molecular docking method on the two domains separately. The two domains were tested with 170 different compounds from various organisms in Indonesia. The study results show that compounds such as halishigamide A, arisugacin A, quinadoline B, and zingiberene have the potential to act as inhibitors in both domains at once. Quinadoline A and

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norquinadoline A had the most potential to be protease inhibitors and showed the best interaction results with protease. Halishigamide A and zingiberene had the most potential to be helicase inhibitors and delivered the best interaction results with the helicase residue. Based on these results, these compounds are thought to be potential inhibitors of DENV NS3, but further research and development are still needed to confirm these results.

Keywords: Anti-viral compounds; Bioinformatics; DENV NS3; NS3 protease; NS3 helicase

1. Introduction

Dengue fever is a severe health problem in the tropics caused by dengue virus infection (DENV) [1-4]. It requires a mosquito vector from the genus *Aedes*, causing the highest incidence of dengue fever in endemic mosquito areas [5-7]. It is found in more than 100 countries, and most of the worst cases are in developing countries, including Indonesia [8, 9]. With more than 300 million cases yearly, it is one of the worst vector- borne diseases in the world [4, 10, 11]. The WHO classifies dengue as a neglected tropical disease [3]. The main challenges to its eradication are the delicate handling of vectors that' s required and the absence of vaccines [12, 13].

The DENV genome is among the most studied viral genome from the flavivirus family. Besides the structural proteins which serve as the structural backbone of the viral particle, it also includes several non-structural proteins that are highly important, particularly for replication, maturation, and immune evasion processes. Among all seven non- structural proteins, one of the most important of them is non-structural protein 3 (NS3), due to its well- mapped enzymatic activity, as well as it being among one of the most conserved non-structural proteins in the flavivirus family [7, 14]. It consists of two main domains which serve different purposes during viral replication and maturation. The first domain is the protease domain, located in the N-terminal domain of the protein, which is responsible for cleaving the viral polyproteins into fully-functional proteins. It will bind to NS2B, which acts as a cofactor making essential conformational changes [15-18]. The second domain is the helicase domain, located in the C-terminal domain, responsible for unwinding the ssRNA and ATP utilization during replication [14, 19, 20].

Both domains have been commonly used as targets in developing anti- viral compounds in both DENV and other flaviviruses [3, 12, 21-24]. Several studies have shown that several compounds have potential as NS3 inhibitors. Some examples of potential NS3 protease inhibitors include ARDP0006, which indicates inhibition of the DENV-2 replication process in cultured cells. Another example is Aprotinin, which envelopes the enzyme and closes access to the protease's active site [25, 26]. Some research suggests suramin as a potent inhibitor for both domains of NS3, acting as a non- competitive inhibitor, and pyrrolone, which shows positive results in decreasing DENV replication in cultured cells [12, 27]. Most of the compounds discovered as anti-viral contenders are lab-based. However, researching anti-virals based on natural molecules is equally important because the majority of the lab-based compounds are derivatives of natural compounds. With this in mind, we believe there are natural chemicals which have the potential to act as NS3-specific inhibitors.

As a tropical country, Indonesia has a high rate of dengue infection, resulting in social and economic losses [9, 28]. On the other hand, it is blessed with huge natural resources, particularly biological ones, that can be explored to potentially solve the problem of the dengue epidemic [29, 30]. The groups of compounds that are widely tested and considered potential anti-viral compounds are flavonoids, phenolics, and

terpenoids, derived from various medicinal plants such as *Curcuma longa*, *Myristica fatua*, *Acorus calamus*, and *Cymbopogon citratus* [31, 32]. Indonesia's biological potential is not limited to these examples, there are still many natural compounds produced by other organisms that are still undiscovered and untested. This study aimed to test several compounds produced by a wider variety of organisms in Indonesia and Southeast Asia as potential anti-dengue agents.

2. Materials and Methods

A literature review was conducted to determine which compounds should be tested for this study, aiming for compounds with known microbial or anti- viral properties. We then mined the list of eligible compounds with two primary databases, PubChem and ChemSpider. We only used 3D structural data in the SDF format compatible with AutoDock. We used 170 compounds from various literature mentioned, which have potential microbial and anti-viral effects [31-44].

Before conducting the test, all compounds underwent a preliminary examination using Lipinski's rule of five, aiming to select compounds that are genuinely eligible and that potentially exhibit anti-viral activity against the predetermined target proteins. This method was chosen because it could identify specific pharmacological properties along with specific chemical and physical properties that make a certain compound possible to use as a drug [45].

The NS3 data that we used in this experiment is divided into two parts, NS3 protease (PDB ID: 2FOM) [46] and NS3 helicase (PDB ID: 2BHR) [47]. Before the docking procedure, we removed all native ligands and water in the 3D structure of the target proteins. After that, both proteins and compounds are converted into AutoDock format. In this study, we used Autodock Vina integrated in PyRx as our main tool for docking (https://pyrx.sourceforge.io/). The docking process was carried out with ARDP0006 (CID: 3378440) as a control for NS3 protease and pyrrolone (CID: 14246795) as a control for NS3 helicase [12]. The protease (2FOM) had a docking coverage area (in Angstroms) of X: 50.5182, Y: 52.6502, Z: 42.5754, and a central area at X:0.4179, Y:-16.9884, Z:13.9911, while the helicase (2BHR) had a docking coverage area (in Angstroms) of X:51.3737, Y: 66.9738, Z: 59.6069, and a central area at X: -26.2840, Y:12.5976, Z:58.9679. Each docking process was repeated three times to minimize error. The docking results are the affinity value, the RMSD, the binding site location, and the protein-compound interaction, which will be known through the visualization process. Then, we visualized the top nine compounds based on the average affinity score from docking results.

The visualization process is carried out in two stages. The first stage is visualizing the protein-compound complex in 3D to see the compound's binding site using PyMOL (https://pymol.org/2/). The second stage is 2D visualization to determine the bonds between proteins and compounds schematically using LigPlot (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/) [48].

3. Results and Discussion

3.1 Results

The following docking results are the results of these two domains separately. The results in tables 1 and 2 are of the top nine compounds with the highest average affinity value (ΔG average) for each domain. All-natural compounds, whose results are shown in the tables, have a higher mean affinity value when compared with the control. Tables 1 and 2 also describe the 2D plotting results of the protein-compound complexes. In both tables, it can be seen that the compound with the highest magnitude affinity value is halishigamide A.

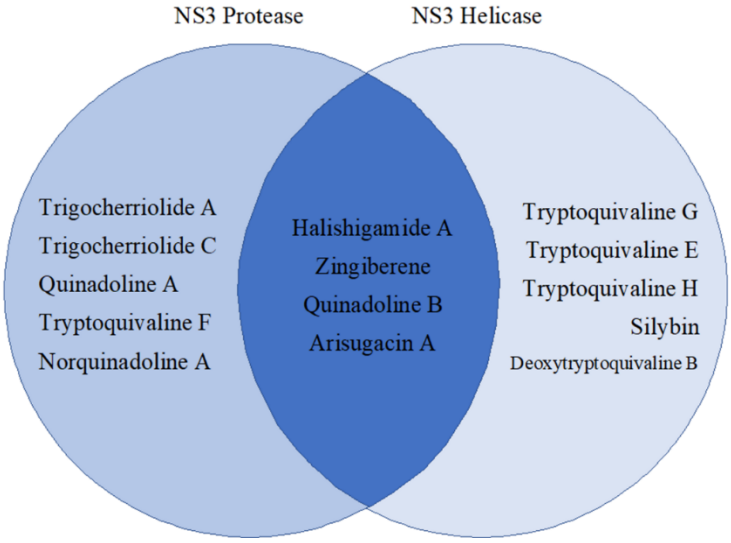

Fig. 1. A Venn diagram showing the similarity of potential compounds in the two NS3 domains.

The affinity difference between halishigamide A and the other compounds is 9.75% for protease and 49.1% for helicase. The docking results also indicated that several compounds had the potential to inhibit both NS3 domains. Fig. 1 shows that five of nine N-terminal compounds can also be applied to the other domains. It shows that four of the top nine compounds can target both NS3 domains simultaneously or separately.

Figs. 2 and 3 show the 2D and 3D visualization results more clearly. The 2D plotting results, visually and as described in Tables 1 and 2, show that some conserved residues were found in at least six docking complexes, as well as the control. They are Asp75 (B), Gly151 (B), Gly153 (B), His51 (B), Leu128 (B), and Tyr161 (B). In the helicase domain, the interacting residue tends to be unique, characterized by the low conserved residue. On the other hand, the residues that interacted with the controls were unique and did not come into contact with others. However, all of the top nine compounds interacting with the helicase domain had higher affinity than control, up to 200.

The results of further visualization in Fig. 4 show that eight out of the nine compounds tested against the protease domain are positioned in the same binding site pocket, including the control. This result confirms the 2D plotting results, which state that some residues are highly conserved because they are bound to the same pocket binding site. The visualization of the helicase complex in Fig.5 showed that the distribution of the potent compounds' binding sites tends to be scattered. It also showed that the control has different binding sites from others. However, we can see that the pyrrolone (control) has the same binding site as SO_4, a native ligand for the helicase domain, which interacted with Gly196, Lys199, Ala316, Pro195, Arg460, and Arg463. Even though the potential compounds did not occupy the same binding pocket, six of them interact with at least one residue from one of three helicase subdomains.

Considering the results, the top nine compounds in proteases have almost identical binding site pockets and a much higher affinity value when compared to control. These compounds may act as substitutes for control, the ARDP0006. The top nine compounds in helicase do have a much higher affinity value than pyrrolone; however, due to the fact that all of the top compounds interact with different binding site pockets other than control, their potential as an anti- viral substitution needs to be studied further.

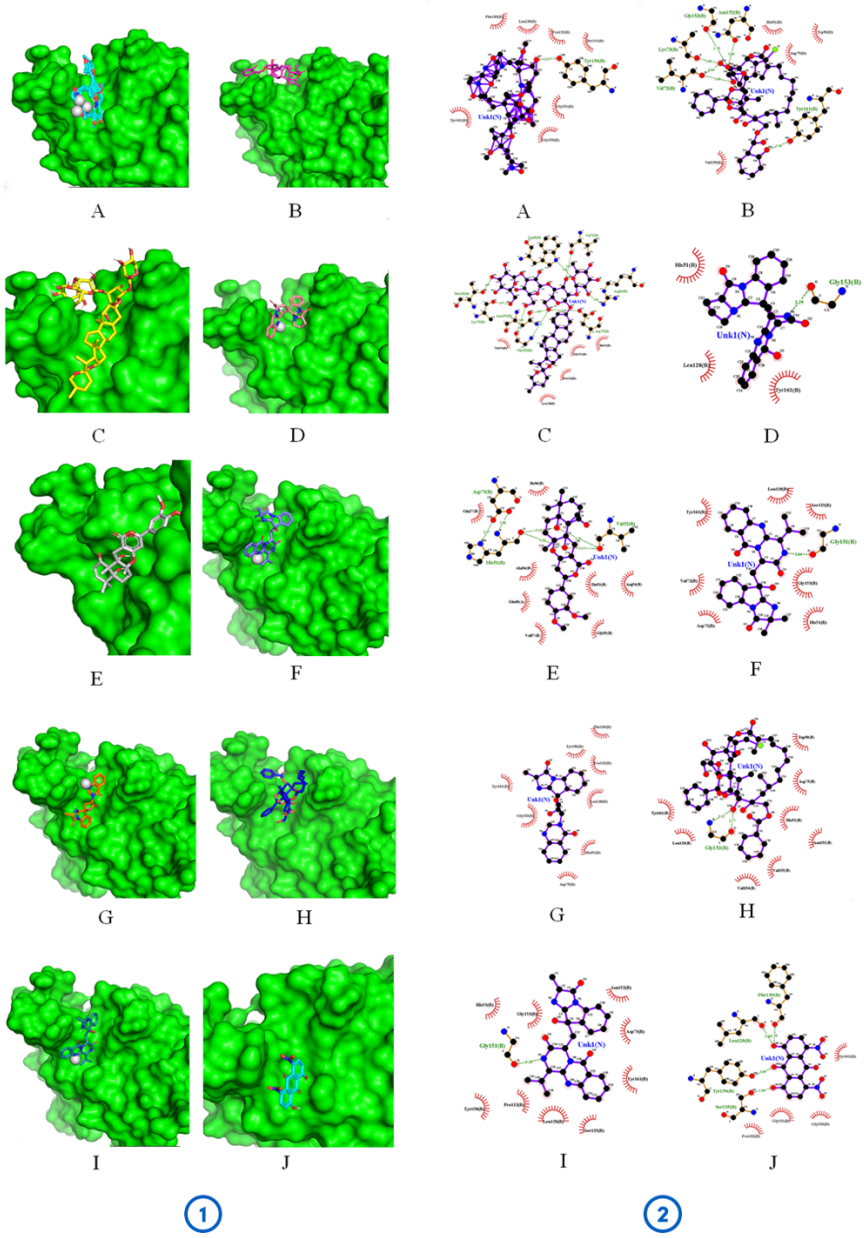

Fig. 2. (1) The results of the 3D visualization of the top nine compounds on NS3 protease, (2) The result of 2D plotting using NS3 protease. (A) Halishigamide A, (B) Trigocherriolide A, (C) Zingiberene, (D) Quinadoline B, (E) Arisugacin A, (F) Quinadoline A, (G) Tryptoquivaline F, (H) Trigocherriolide C, (I) Norquinadoline A, and (J) ARDP0006 as control.

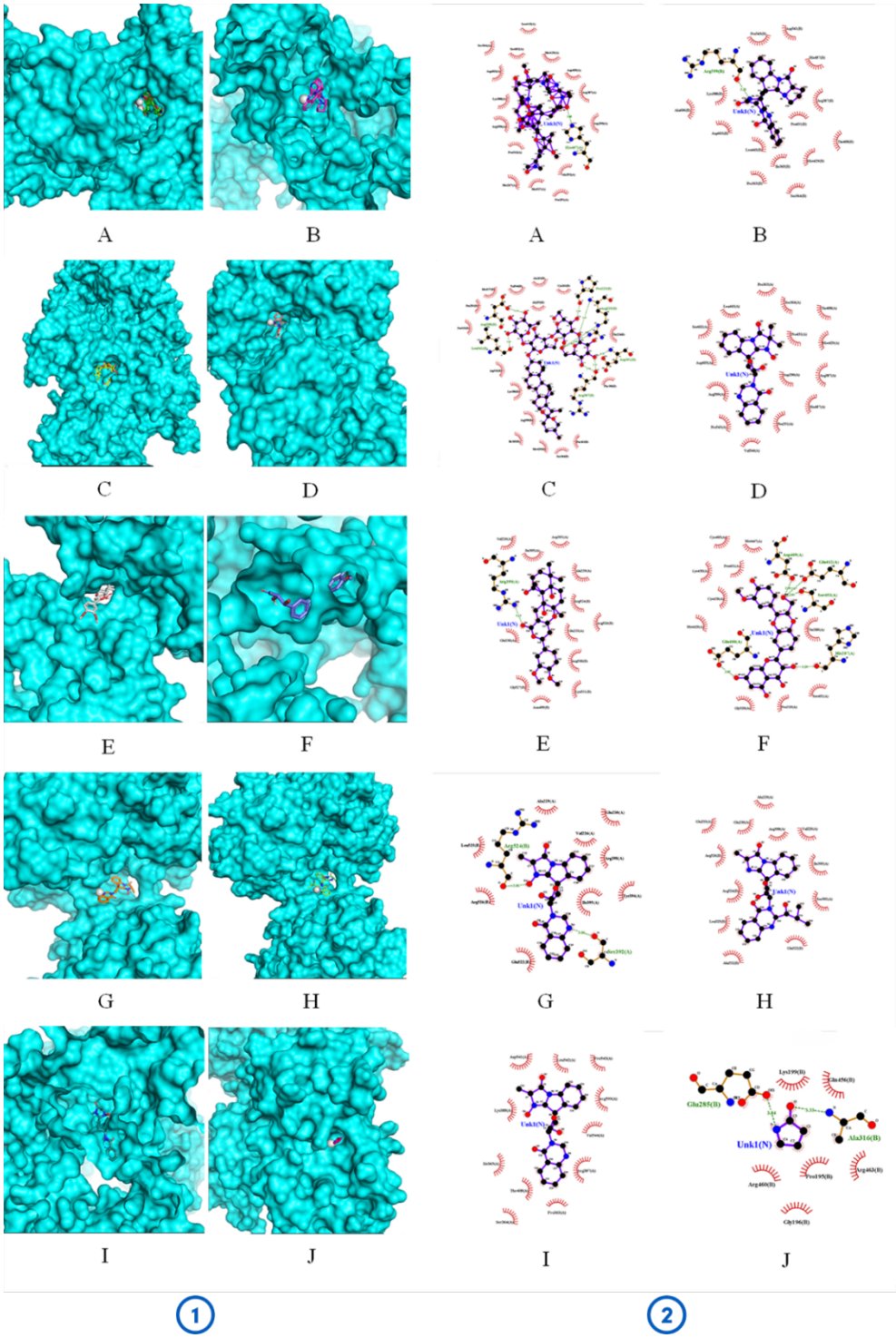

Fig. 3. (1) The results of the 3D visualization of the top nine compounds on NS3 helicase, (2) 2D plotting using LigPlot on NS3 protease. (A) Halishigamide A, (B) Quinadoline B, (C) Zingiberene, (D) Tryptoquivaline G, (E) Arisugacin A, (F) Silybin, (G) Tryptoquivaline E, (H) Deoxytryptoquivaline B, (I) Tryptoquivaline H, and (J) Pyrrolone as control.

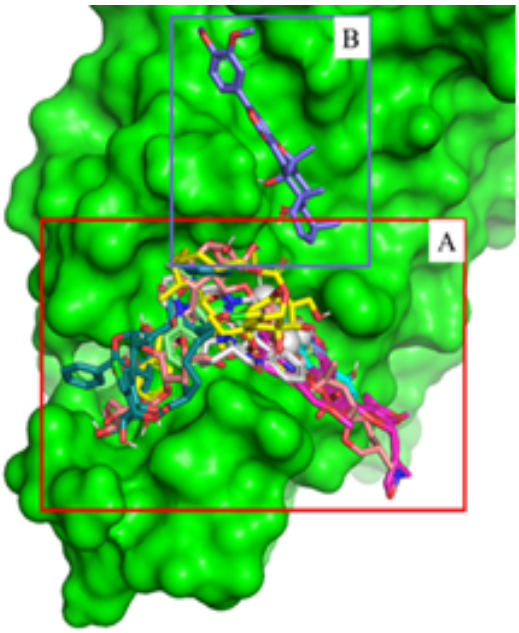

Fig. 4. The 3D visualization of the protease domains shows that 8 out of 9 tested compounds have the same pocket binding site (A), except for Arisugacin A (B).

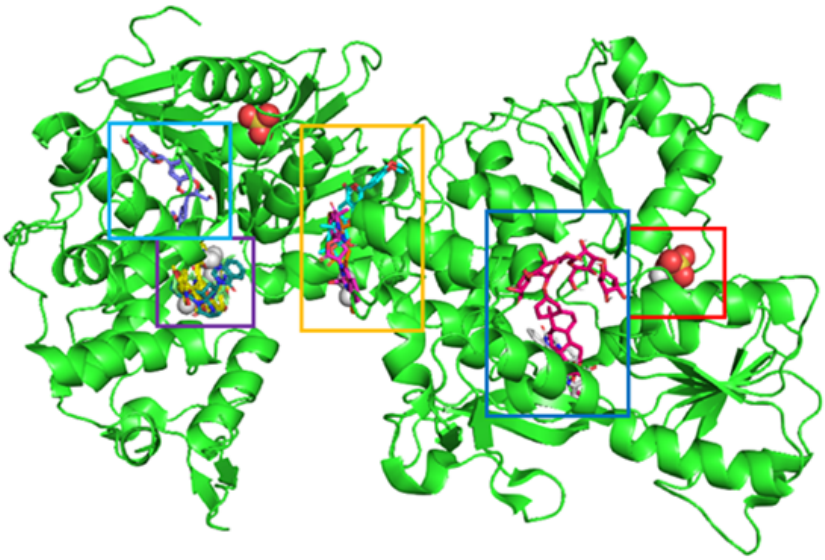

Fig. 5. The 3D helicase domain visualization shows the distribution of the binding site pocket of the tested compound molecule. The compounds tested were spread over five different binding site pockets where only one or two compounds occupied each pocket. It can be seen that the pyrrolone control occupies a different binding site from other compounds that also have the same pocket with the native ligand SO_4 (red box).

Table 1. Compounds docking result for DENV-2 NS3 protease.

No.	Compounds and Sources	ΔG Average (kcal/mol)	RMSD (Å)
1.	Halishigamide A/ CID: 44566626 *Halichondria* sp.	-13.8	1.218
2.	Trigocherriolide A/ CID: 71530649 *Trigonostemon cherrieri* (leaves)	-10.2	2.624
3.	Zingiberene/ CID: 102475150 *Curcuma xanthorrhiza*	-9.9	1.088
4.	Quinadoline B/ CID: 72547182 *Aspergillus* sp.	-9.9	1.814
5.	Arisugacin A/ CID: 10255275 *Aspergillus terreus* SCSGAF0162	-9.8	1.679
6.	Quinadoline A/ CID: 25146273 *Aspergillus* sp.	-9.8	1.347
7.	Tryptoquivaline F/ CID: 181786 *Apergillus fumigatus*	-9.8	2.531
8.	Trigocherriolide C/ CID: 71530651 *Trigonostemon cherrieri* (leaves)	-9.7	1.380
9.	Norquinadoline A/ CID: 386974765 *Cladosporium* sp.	-9.0	1.224
10.	ARDP0006/ CID: 3378440 Synthetic compound (Control)	-8.2	0.044

3.2 Discussion

The NS3 protein is often used as a target for anti-viral development and has several critical functions for the continuity of the infection process. It can function fully independently or form a complex with NS2B. Stand-alone NS3 could inhibit the RNA-sensing mechanism mediated by RIG-I by inactivating the RIG-I through interaction between the conserved RxEP motif and the 14-3-3ε protein, which will prevent the migration of RIG-I from the cytoplasm to the mitochondria. When NS3 forms a complex with NS2B, it has broader capabilities to inhibit and influence the IFN pathway. The NS2B3 complex can interact with the kinase domain of IKKε to inhibit the phosphorylation of NS3. It also significantly inhibits the mitochondrial fusion process by cleaving MFN1 and MFN2 and inhibiting the viral DNA's sensing process by cleaving the STING [51, 52]. Thus, the interaction between NS2B and NS3 is strongly suggested to be a promising target for antivirus development [46]. However, developing an antiviral compound that targets catalytic sites in the protease domain is difficult because it has a relatively flat contour, so it requires changing the NS2B or NS3 conformation for better interaction [15, 46, 51].

The main role of NS3 in the DENV replication process is to act as a protease for polyprotein processing, an RNA triphosphatase for capping nascent viral RNA, and a helicase for unwinding the double-stranded replicative form of RNA [52, 53]. One anti-viral compound with a strong potential to be used an anti-dengue drug is ARDP0006. The development of ARDP0006 so far has shown positive results where it acts as a competitive inhibitor on the catalytic site of the protease domain by interacting with the catalytic triad in the His51-Asp75-Ser135 [17, 54, 55]. One of the most conserved residues among DENV serotypes is Asn152. The results show that ARDP0006 as a control interacts with the active site, the Ser135. Apart from that, it also interacts with the P1 pocket residue, including Gly151 and Gly153, as an oxyanion hole, which plays a role in stabilizing enzymatic reactions primarily in the catalytic triad [56, 57]. The interaction between the catalytic triad and the oxyanion hole with ARDP0006 has a significant

chance of interfering with the protease domain's enzymatic performance. ARDP0006 interacts with Asn152, part of the S2 subsite, which interacts with the P2 pocket via hydrogen bonds. The interaction between ARDP0006 and the residues is suggested to significantly weaken the catalytic site [58].

Table 2. Compounds docking result for DENV-2 NS3 helicase.

No.	Compounds and Sources	ΔG Average (kcal/mol)	RMSD (Å)
1.	Halishigamide A/ CID: 44566626 *Halichondria* sp.	-16.7	2.073
2.	Quinadoline B/ CID: 72547182 *Aspergillus* sp.	-11.2	5.447
3.	Zingiberene/ CID: 102475150 *Curcuma xanthorrhiza*	-10.6	1.773
4.	Tryptoquivaline G/ CID: 76966159 *Aspergillus fumigatus*	-10.5	2.702
5.	Arisugacin A/ CID: 10255275 *Aspergillus terreus* SCSGAF0162	-10.4	5.934
6.	Silybin/ CID: 31553 *Silybum marianum*	-10.4	1.341
7.	Tryptoquivaline E/ CID: 76956260 *Aspergillus fumigatus*	-10.3	1.752
8.	Deoxytryptoquivaline B/ CID: 21125503 *Aspergillus fumigatus*	-10.2	2.323
9.	Tryptoquivaline H/ CID: 188425 *Aspergillus fumigatus*	-10.1	2.141
10.	Pyrrolone/ CID: 14246795 Antiviral drug (control)	-4.3	1.823

Visualization and plotting results show that eight out of the nine potential compounds occupy the same binding pocket as ARDP0006, with the highest affinity value being halishigamide A. Halishigamide A, a secondary metabolite produced by the *Halichondria* sponge genus, was the only animal-derivative compound tested this time [59, 60]. Based on the result, halishigamide A has an affinity value 68.3% higher than ARDP0006. It does not interact with the catalytic triad but with the oxyanion holes (Gly151 and Gly153), so it will affect the stability of the protease enzymes. It also interacts with Tyr150, highly predicted to be a key residue stabilizing the P1 side chain and the E2 strand in the C-terminal b-barrel of the NS3 protein [58]. Thus, it is strongly suspected that halishigamide A does not directly act as a competitive inhibitor but rather interferes with the catalytic reaction process by affecting the key stabilizer components, such as the oxyanion hole and Tyr150.

Alkaloids such as quinadoline A and norquinadoline A interact with all residues in the catalytic triad (His51, Asp75, and Ser135) and oxyanion holes (Gly151 and Gly153). It is thought that these two compounds can act both as competitive inhibitors of the catalytic triad and as non-competitive inhibitors by disrupting the interaction with oxyanion holes at a more effective rate. Besides, quinadoline A had a 19. 5% higher affinity than the control, while the affinity of its counterpart, norquinadoline A, was 9.75% higher than the control. Also, the control only interacts with a portion of the residue in the catalytic triad. Some research has stated that compounds from the quinadoline group could inhibit the replication, maturation, and release of new

viruses [61, 62]. This matches with results that indicate interaction between the quinadoline group compounds inhibits the catalysis process in the NS3 protease domain, thereby reducing the ability to replicate and the maturation of DENV particles.

Several other compounds are also known to interact with the catalytic triad and the oxyanion holes in P1, such as trigocherriolide A, zingiberene, quinadoline B, tryptoquivaline F, and trigocherriolide C. These compounds are secondary metabolites produced by several types of plants and fungi. Several previous studies stated that these compounds have promising potential as antimicrobe and anti-viral compounds for viruses such as chikungunya (CHIKV) and influenza A (H1N1) because they can significantly reduce the replication performance of the viruses. However, the working mechanism of these compounds to inhibit the virus replication process is still unknown [31, 37, 61, 63-69]. Although they did not fully interact with all residues at both sites, considering that the affinity values ranged from 18.3-36.6% higher than controls, these compounds should also be regarded as potential anti-viral agents for DENV. When we compared the results with previous studies, we suspect that the inhibitory effect could result from the interaction with the virus' protease, which affects its replication performance. It also indicates that these compounds have the potential to be developed into a broader spectrum of anti-virals.

The only compound not occupying the same pocket as ARDP0006 and other potential compounds is arisugacin A. Arisugacin A, which was isolated from the fungus *Aspergillus terreus* SCSGAF016, was initially recognized as an anti-acetylcholinesterase, but also showed potential as an anti-viral, especially for herpes simplex virus (HSV-1). Unfortunately, it is still unclear how this compound inhibits HSV-1 [70]. Although arisugacin A is not in the same pocket as other potential compounds, the plotting results show that it interacts with His51, which is part of the catalytic triad in the protease domain, suggesting that it is a potent compound able to act as an inhibitor in the catalysis process carried out by proteases. What distinguishes arisugacin A from other potential compounds is that it interacts with a residue at the NS2B binding site, the Gln27 [46]. The interaction between the protease and arisugacin A at this site is predicted to interfere with the formation of the NS2B3 complex, reducing its enzymatic activity.

The second domain that makes up DENV NS3 is helicase. The vital role of the helicase in the dengue infection mechanism is to denature the double-stranded RNA strands to be used as a template for the replication process by the NS5 [80, 81]. There are at least two critical components in the helicase, the first component is the enzymatic active site, and the second is a helicase subdomain. The active site of the enzymatic helicase is found in the residues of Lys199, Arg284, Glu285, and Arg460-Arg463 [73]. The second component is a subdomain of C-terminal helicase with three subdomains: I, II, and III. The first one consists of Pro223, Arg225, Asp290, Gln243, Thr244, Cys261, Thr264, and Thr267; the second one consists of Pro363, Ile365, Lys366, Arg387, Thr408, Asp409, Leu429, and Arg538; and the third one consist of Asp54, Arg599, and Asp603. These subdomains function primarily as the location for binding of single-stranded RNA [71, 72, 74]. They are further divided into several motifs, namely subdomains I-VI. Motif I is a P-loop, motif II is DExH, and motif VI is DEAH. These motifs generally influence ATPase activity [72]. Arg-225 (motif Ia), Thr244, Thr264, Ile365, Lys366 (motif IV), Asp290, Arg-387, Lys388, Asp409, Thr408, Arg-538, Asp541, and Arg599 interact directly with components of ssRNA, whether on the phosphate backbone, ribose, or nitrogenous base [71, 72].

The 3D visualization results show that each compound's binding site location is not concentrated explicitly in one pocket. It also shows that pyrrolone (control) is entirely separate from other potential compounds. The control occupies the same binding site as the native ligand on the helicase's active site. This suggests it acts specifically as an inhibitor of ATPase by cleaving the ATP used for the enzymatic processes carried out by helicase, and significantly reduces helicase activity and overall viral replication ability [12, 25]. Meanwhile, most potential compounds interact with the helicase subdomain component; none of them interact directly with the helicase active site as pyrrolone does.

The results demonstrated by 2D plotting indicate that halishigamide A, quinadoline B, zingiberene, tryptoquivaline G, silybin, and tryptoquivaline H interact with at least two subdomains. Halishigamide A, the compound with the highest affinity value, shows interactions on all helicase subdomain residues. The combination of high-affinity values and interactions with each of the three subdomains simultaneously strongly indicate that halishigamide A can act as a competitive inhibitor of ssRNA. This means that the ssRNA cannot bind and occupy the helicase domain, so it will suppress the helicase's functionality and performance [71, 72]. Also, this interaction may suppress the ATPase activity because the subdomains I and II are also shared with the ATPase [72, 75]. Other compounds with similar properties but lower affinity values w ere zingiberene and tryptoquivaline G. Zingiberene has more stable interactions with subdomains I and II because it has hydrogen bonds with Pro233, Arg225, Arg387, and Asp290 with a distance range of 2.73-3.33Å. It is suspected that the zingiberene's chance to bind to the same site with ssRNA is relatively high even though its affinity value is 37% lower than halishigamide A.

On the other hand, tryptoquivaline G also interacts with all the subdomains but with an affinity value that is 37% lower than halishigamide A. Therefore, the interaction between tryptoquivaline G and all the subdomains is presumably not as strong as halishigamide A and zingiberene. However, these compounds' potential as competitive inhibitors of ssRNA in helicase needs further study. Other compounds like quinadoline B and tryptoquivaline H interact with subdomain II and III, respectively. Meanwhile, syblin only interacts with subdomain II. These three compounds each have affinity values 235-260% higher than pyrrolone. Quinadoline B and silybin have at least one hydrogen bond with a residue on a subdomain, whereas all of the bonds in tryptoquivaline H are hydrophobic contacts. It is assumed that with a small affinity range of around 11%, the interaction between quinadoline B and silybin is stronger than the interaction of tryptoquivaline H, due to the additional hydrogen bonds. However, because the affinity differences are not significant and the interaction model differs from only one hydrogen bond, it is assumed that the chances of these compounds acting as ssRNA inhibitors, especially in subdomains II and III, are more or less equal.

Some compounds did not bind to residues on any subdomains when plotted using LigPlot, such as arisugacin A, tryptoquivaline E, and deoxytryptoquivaline B. These compounds have affinity values 237-242% higher than the control. The plot showed no interaction between the subdomain components or the helicase's active site. Thus, although it is known that these compounds have a much higher affinity value than the control, it is not yet clear how these compounds act as inhibitors for NS3 helicase.

4. Conclusion

This study suggests halishigamide A, arisugacin A, quinadoline B, and zingiberene to be potent inhibitors in both NS3 domains.

On the other hand, quinadoline A and norquinadoline A were specifically predicted as potent protease inhibitors because they showed the best interaction with protease. Halishigamide A and zingiberene are suspected to be potent helicase inhibitors. Based on these results, those compounds are considered potential inhibitors of DENV NS3 in general, but further comprehensive research and development are still needed to verify these results.

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