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Original Article

In Silico Analysis of Inhibitor Potential of Punicalagin Compound in Pomegranate (*Punica granatum*) Against NS5 DENV-3 Protein

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ABSTRACT

Indonesia is one of the Dengue Virus (DENV) endemic areas which are dominated by DENV-2 and DENV-3. Until now, no specific drug therapy has been found to cure Dengue Virus Infection (DVI). Punicalagin is one of the active compounds that have the potential to be used as an antiviral. Unfortunately, not many studies have used punicalagin as a DENV antivirus. This study aims to determine the inhibitory potential of punicalagin compounds against NS5 DENV-3 protein through molecular docking. Molecular docking was performed using AutoDock Tools, ChemDraw, and Discovery Studio Visualizer. The target protein used is NS5 DENV-3 protein with PDB ID code: 4V0Q. The ribavirin compound was used as a positive control. The results obtained show that the punicalagin compound has the ability to attach to target receptors in the C-Terminal domain complex. This docking produces a bond free energy (ΔG) of -6.39 kcal/mol. This result is better than the ΔG of the control compound. Punicalagin's Inhibition Constant (Ki) value also showed better results than ribavirin. So it can be seen that the compound punicalagin effectively inhibits DENV replication and has the potential as a DENV drug candidate.

Keywords: Antiviral, DENV-3, *In Silico*, NS5 Protein, and Punicalagin.

Highlights: Add one short sentence of research's novelty and one short sentence of research's benefit.

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INTRODUCTION

Virus infection can occur to anyone and at any time, as it is still a global health problem.[1] One of the emerging virus is dengue virus (DENV) which can cause Dengue Virus Infection (DVI).[2] Dengue Virus (DENV) is a type of RNA virus that is transmitted through the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes. This virus has four types of serotypes with a rapid spread throughout the world in recent years.[3]

WHO states that DVI has increased cases by 30 times worldwide in the last five decades. The distribution of DENV is faster in areas with tropical and subtropical climates.[4] Indonesia is a tropical country which has a high humidity level so that mosquitoes can survive in almost all parts of Indonesia.[5] The majority of areas in Indonesia are DENV endemic areas with DVI cases which tend to increase every year. This has resulted a health problem in Indonesia that is challenging to resolve.[6]

The data from the Ministry of Health of the Republic of Indonesia[7] noted that there were 108,303 DVI cases in 2020 with the four DENV serotypes circulating throughout Indonesia.[8] The results of a serological survey on the distribution of DENV serotypes in Indonesia stated that DVI cases in Indonesia were alternately dominated by DENV-2 and DENV-3. This change in dominance of the two serotypes is thought to have occurred due to the persistence or inheritance ability of the DENV serotypes in the main vector before being transmitted to humans.[9]

Currently, the handling of DVI cases focused on the development of antiviral drugs. This is an urgent need considering there is no specific drug therapy that is effective in inhibiting the growth of DENV. The development of a drug requires several stages of testing which takes a long time.[10] One of the early stages of drug development is the *in silico* testing through molecular docking. This test was carried out to determine the interactions that occur between the test compounds and the target receptors.[11]

So far, there have been many *in silico* studies to determine the antiviral activity of a compound to inhibit DENV replication. Secondary metabolites commonly found in natural products, such as quercetin, catechins, mangiferin, and arthemicin have been shown to inhibit DENV protein replication. This inhibition is based on the value of the Gibbs free energy (ΔG) with the highest inhibition value occurring in the mangiferin compound. The ΔG value represents the strength of the ligand binding to the receptor. The lower the ΔG value, the stronger the bond between the ligand and the receptor.[12] Several Indonesian herbal plants have also been tested to determine their ability to anti-DENV activity. The results of an *in silico* study conducted by Rosmalena et al.[13] stated that the artesunic acid and homoegonol compounds found in *Myristica fatua* have the ability to bind to the NS5 DENV protein complex with bond energies of -7.2 kcal/mol and -7.1 kcal/mol.

NS5 is the largest and most conserved protein complex (with more than 70% sequence identity among the four serotypes). The NS5 protein complex consists of two domains, namely the methyltransferase (MTase) domain at the N-terminal end and RNA-dependent RNA polymerase (RdRp) at the C-terminus. The high level of conservation in the NS5 protein structure makes it often used as a target for designing drugs with broad activity against several flaviviruses. The MTase domain (residues 1-265) plays a role in limiting viral RNA as well as N7 and 2'O ribose methylation activity. The RdRp domain plays a role in viral RNA replication. These two domains are connected by 5-6 residues (residues 266-271). The lack of activity of RdRp in host cells makes the NS5 complex a promising antiviral target for designing specific inhibitors with low toxicity.[14]

Punicalagin is a polyphenolic compound that is commonly found in pomegranate peels.[15] Punicalagin has been shown to have antiviral activity against the HSV-2 and SARS-CoV-2 viruses in silico. Until now, there has been no research regarding the effectiveness of punicalagin as a DENV-3 antiviral. Therefore, this study was conducted with the aim of knowing the potency of punicalagin inhibition against NS5 DENV-3 protein.

MATERIALS AND METHODS

Materials

The tool used for this research is a laptop device. The materials used for this study were three-dimensional files of punicalagin compounds and ribavirin compounds which were used as test and target ligands. The target receptor used in this study was the NS5 DENV-3 protein (PDB ID: 4V0Q).

Methods

This research was a descriptive observational study which aims to determine the ability of the punicalagin compound in pomegranates (*Punica granatum*) to bind the NS5 DENV-3 Protein using a pre-experimental one shot study design *in silico*.

Test of Physicochemical Properties

The physicochemical properties test refers to Lipinski's Five Laws or the Rule of Five. This test was conducted on the SwissADME website (http://www.swissadme.ch/).

Ligand Preparation and Optimization

Preparation begins by downloading the ligand and receptor structure data. Ligand structure data (punicalagin compounds and ribavirin compounds) are downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) in *sdf format. The downloaded data is converted to *pdb format using the ChemDraw Ultra application. The ligand optimization process was carried out in several stages including energy minimization, addition of H atoms, and addition of charge on the ligand structure. Ligand optimization was carried out using the AutoDock Tools and Chem3D Pro.

Receptor Preparation and Optimization

Receptor structure data (NS5 DENV-3 protein) was obtained through the Protein Data Bank database page (https://www.rcsb.org/) with PDB ID code: 4V0Q. Receptor preparation includes separation of native ligands and proteins, as well as other unnecessary molecules using the Discovery Studio Visualizer application. The final split result data is stored in *pdb format. Receptor optimization was carried out by adding H atoms and charges to the receptor structure using the Autodock Tools application. Optimization result data is saved in *pdbqt format.

Method Validation

Method validation was carried out by attaching native ligands to the protein structure using the AutoDock Tools application. The position of the grid box is placed at the midpoint of the ligand (X: 26.935; Y: 150.36; Z: 31.432) with dimensions X: 40; Y: 40; Z: 40. The method is said to be valid if the RMSD (Root Mean Square Deviation) value is below 2 Å.

Molecular Docking

The ligand binding process on the target protein was carried out using the AutoDock Tools application with the position of the grid box adjusted during method validation with dimension modification (X:126; Y:126; Z:126). The ligand binding process on the target protein will produce data and information that includes the bond interaction pattern formed,

the inhibition constant (Ki), and the bond free energy.

Visualization

The docking results are visualized using the Discovery Studio Visualizer application. The results are presented in the form of interaction patterns formed, inhibition constants (Ki), and bond free energy values (ΔG).

RESULTS AND DISCUSSION

Test of Physicochemical Properties

This test is based on the Rule of Five or Lipinski's Law of Five with 4 test parameters including the log P value, molecular weight, number of donor H atoms, and number of acceptor H atoms.[16] The results of the physicochemical properties of the test and control ligands are presented in the following table:

Table 1. Results of Test of Physicochemical Properties.

Test Parameters	Compound	
	Punicalagin	Ribavirin
Log P Value	-3,29	-2,94
Molecular Weight (g/mol)	1084,72	244,2
Number of H-Bond Donors	17	7
Number of H-Bond Acceptors	30	4

The results of the physicochemical properties test showed in Table 1 explained the punicalagin compound did not meet the 3 test parameters based on Lipinski's Fifth Law, while the ribavirin compound used as a control ligand fulfilled all the parameters of Lipinski's Fifth Law. Lipinski's Law of Five has 4 test parameters including Log P value <5, molecular weight <500 g/mol, number of H donors <5, and number of H acceptors <10. According to Lipinski's Rule of Five, a compound that has the potential to be used as a drug must meet the requirements for all parameters that have been determined.[17] This aura represents the level of capability of a compound to cross cell membranes.[16]

The Log P value is a parameter that shows the level of solubility of a compound in water or fat.[18] Compounds with a high level of hydrophobicity also have a high level of toxicity due to the inability of these compounds to penetrate the lipid bilayer and will spread widely in the body which results in a reduced level of selectivity of compounds for target receptors.[19] Log P values can still be tolerated at a ratio of -0.4 to 5.[20]

The molecular weight of a compound affects the permeability of a compound in penetrating the cell membrane. A compound having a molecular weight > 500 g/mol is unable to diffuse across the cell membrane. The number of hydrogen bond donors and acceptors is a test parameter in Lipinski's Fifth Law which aims to determine the number of hydrogen bonds needed for a compound during the absorption process.[18] The number of hydrogen bonds in a compound will be directly proportional to the amount of energy required during the absorption process.[21]

The process of designing a drug must be carried out carefully in order to avoid toxic effects and to optimize the effectiveness of the drug so that it can interact properly in the body. The physicochemical property test aims to minimize the toxic effects that arise from a drug on the basis of Lipinski's Five Laws. In addition, this law can also be used in predicting whether a compound can be given orally or not. Based on the 4 test parameters, the punicalagin compound did not meet the parameters of Lipinski's Fifth Law, while the ribavirin compound fulfilled all of the parameters of Lipinski's Fifth Law. A compound that does not meet the test parameters of Lipinski's Rule of Five cannot be administered orally. However, these compounds can still be given by injection.[20]

Method Validation

The method validation process is carried out before starting the belay process using the test compound. The docking method is acceptable and is said to be valid if the RMSD value obtained is less than 2.00 Å from the result of native ligand binding with the receptor.[22] RMSD (Root Mean Square Deviation) is a value that represents the relative deviation level when a ligand is tethered to the active site of the receptor.[19] The results of the method validation show that the method used is valid with an RMSD value of 1,659 Å. RMSD value < 2 Å indicates a stable bond between the ligand and the receptor. The smaller the RMSD value indicates the position of the atomic bonds in the ligand the better and closer to the original conformation.[22]

Molecular Docking

Molecular docking was performed with the AutoDock Tools 1.5.6 application. The test ligands used were punicalagin compounds, and ribavirin compounds as control ligands and glycerol as native ligands were used for comparison. The docking process was carried out 10 times to obtain the best conformation from the interaction between the ligand and the receptor. The docking results of the three ligands show different positions of the ligands and bonds formed. The binding positions of the three ligands are presented in the following figure:

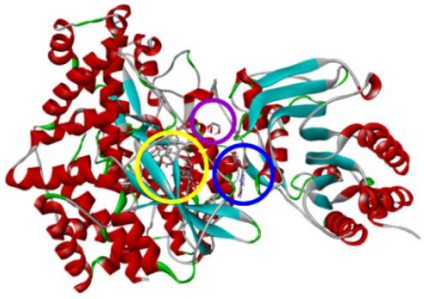

Figure 1. Binding Position of Test Ligand, Control Ligand, and Native Ligand on NS5 DENV-3 Protein.*

*Description: yellow: test ligand position, blue: control ligand position, purple: native ligand position

Figure 1 is the result of the binding of three ligands to the NS5 DENV-3 protein structure with different binding sites. The NS5 DENV-3 protein is a protein complex that plays a role in the DENV-3 replication process. This protein complex includes the largest protein complexes with the most durable protein components. The NS5 DENV-3 protein complex is composed of 900 amino acid residues which are divided into two active sites, namely the N-Terminal domain complex at residue range 1-262, and the C-Terminal domain complex at residue range 273-900.[23]

The NS5 DENV-3 protein complex is commonly known as a conserve protein. This is due to the important role of this protein complex in DENV-3 replication. For this reason, the NS5 protein complex is often used as a target receptor in *in silico* studies for the development of drug candidates.[24] Visualization of conserved proteins can be used to predict the bonds formed from the results of molecular docking (Figure 2).

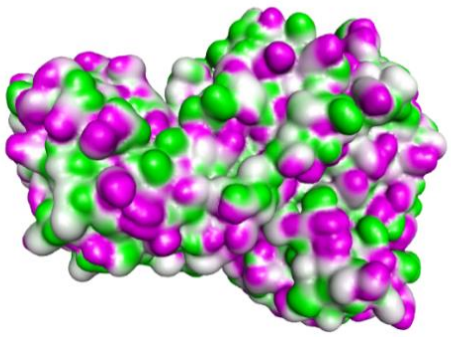

Figure 2. Representation of Conserved Protein on NS5 DENV-3 Protein.

Visualization of conserved protein NS5 DENV-3 shows the potential for 2 types of bonds. The green and purple colors in figure 3 represent the hydrogen bonds and hydrophobic bonds that can be formed in the NS5 DENV-3 protein complex. Hydrogen bonds and hydrophobic bonds are types of

bonds resulting from the interaction of the ligand with the receptor which play a role in maintaining the stability of the conformation of the ligand and receptor bonds.[25]

The interaction that occurs due to tethering of the test ligand on the target receptor produces hydrogen bonds and electrostatic bonds with amino acid residues in the range 340-737. This is different from the results of the binding of the control ligand which forms hydrogen bonds with amino acid residues in the range 67-582, and hydrogen bonds with amino acid residues in the range 300-355. The bond formed from the docking of the test and native ligands occurs in the C-Terminal domain complex of the NS5 DENV-3 protein, while the control ligand binds to amino acid residues in the N-Terminal and C-Terminal complexes.

The N-Terminal and C-Terminal complexes are protein complexes that play a role in the multiprotein replication process found in the NS5 DENV-3 protein. The N-Terminal complex has a methyltransferase enzyme that functions in the RNA translation phase into polyproteins in the host cell, while the C-Terminal complex contains an RNA polymerase enzyme that helps speed up the process of RNA replication.[23]

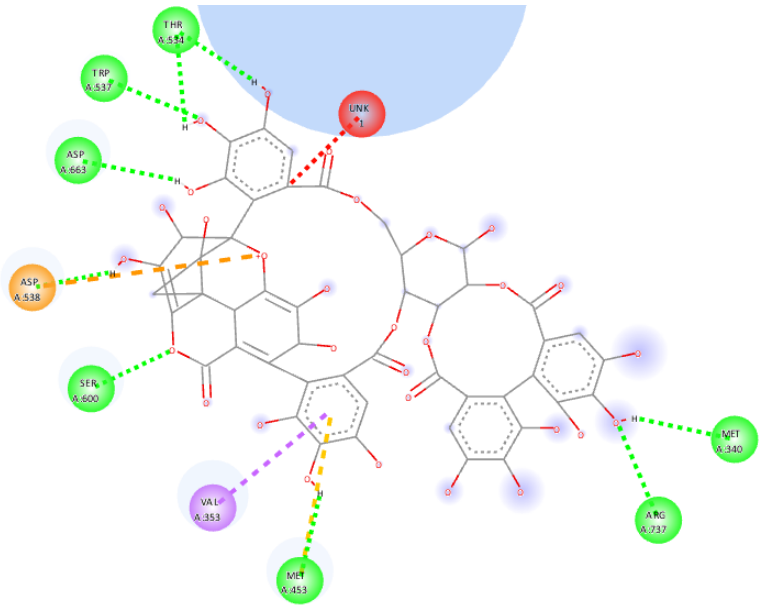

Figure 3. Bonds Formed by Molecular Docking of Test Ligand and Receptor.

Attachment of the test ligand to the target receptor results in three different types of bonds, namely electrostatic bonds (orange), hydrophobic bonds (purple), and hydrogen bonds (green) (Figure 3). These three bonds support the stable conformation of the ligand binding to the receptor. Electrostatic bonds are bonds that occur due to the distribution of electrons resulting in positive and negative charges on a molecule.[26] This type of bond helps to increase the conformational stability of the ligand bond with the receptor.[27] It also forms hydrogen bonds. Hydrogen bonds are said to be strong if they have a bond length above 1.85 Å.[28] This bond supports the stability of the protein structure.[29] Most of the hydrogen bonds formed from the interaction of the tested ligand and the receptor have a bond length of above 1.85 Å so that they have strong hydrogen bonds.

The hydrophobic bond formed from the interaction of the test ligand with the receptor also helps in reducing interactions with water molecules through alignment of the positions of non-polar compounds, thereby helping to maintain protein stability.[25] This interaction is formed with

the residue of the amino acid valine at point 353. Valine is a non-polar amino acid that is hydrophobic.[30] The binding position of the test ligand on the active site of the protein in the C-Terminal complex will interfere with the work of the RNA polymerase enzyme so that the process of viral replication cannot occur.

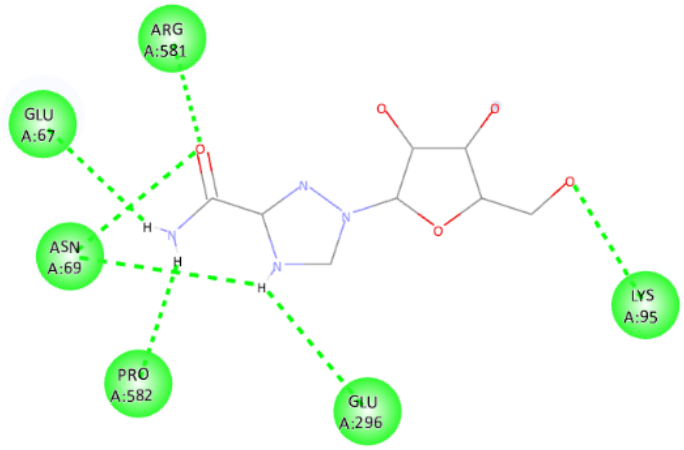

Figure 4. Bonds Formed by Molecular Docking of Control Ligand and Receptor.

Molecular docking of the control ligand with the target receptor produces only one type of bond, namely a hydrogen bond (Figure 4). The hydrogen bonds formed have a bond length above 1.85 Å. The strength of this bond contributes to maintaining the stability of the conformation of the ligand with the protein.[29]

The binding position of the control ligand in the C-Terminal and N-Terminal complex will interfere with the work of the RNA polymerase and methyltransferase enzymes so that the process of viral replication cannot occur.

Analysis of Molecular Docking Results

Molecular docking results were analyzed by comparing several data parameters, including inhibition constant (Ki), bond free energy (ΔG), and bonds formed from the docking process of test ligands, native ligands, and control ligands. This analysis was conducted to assess the level of effectiveness and potential of the tested ligands as drug candidates.

Table 2. Results of Molecular Docking.

Ligand	ΔG (kcal/mol)	Ki (μM)	Bond Type		
			Hydrogen	Hydrophobic	Electrostatic
Test	-6.39	20.67	MET340 MET453 THR534 TRP537 SER600 ASP538 ASP663 ARG737	VAL353	ASP538
Control	-6.09	34.52	GLU67 ASN69 LYS95	-	-

Native	-3.11	5.26	GLU296 ARG581 PRO582 LYS300 LYS355	-	-

The data presented in Table 2 is the best result from the 10x binding process of each ligand to the receptor. The inhibition constant (Ki) values of the three ligands have different values. The Ki value represents the level of strength of a compound in inhibiting the rate of action of the target receptor. The smaller the Ki value, the greater the inhibitory strength31. The test ligand Ki value was between the control and native ligand values. This shows that the inhibitory power of the tested ligands was lower when compared to the native ligands, but higher than the control ligands.

The bond free energy value (ΔG) is a value that indicates the degree of stability of the ligand conformation with the receptor. The ΔG value is inversely related to the level of affinity of the ligand for the receptor. The smaller the ΔG value, the greater the affinity of the ligand for the receptor.[22] Based on the results of the ΔG values of the three ligands, the ΔG value of the tested ligands was the best at -6.39 kcal/mol. This indicates that the bond of the test ligand with the target receptor is more stable than the control and native ligands. The binding free energy of the tested ligand is negative indicating that the tested ligand can interact with the receptor so that it can be used as a DENV-3 inhibitor. The magnitude of the ΔG value is influenced by several factors, such as differences in the number and types of bonds formed from the interaction of the ligand with the receptor[32], as well as the flexibility of the ligand structure during the binding process.[33]

STRENGTH AND LIMITATION

The strength of this research was that the punicalagin compound has a better binding energy than the control compound for the NS5 DENV-3 protein. This research was limited to computational tests only, so further *in vitro* tests are needed.

CONCLUSIONS

Based on the results obtained in this study, it can be concluded that the punicalagin compound is able to bind to the NS5 DENV-3 protein which is characterized by the presence of electrostatic bonds, hydrophobic bonds, and hydrogen bonds with amino acid residues in the C-Terminal domain complex which contains the RNA polymerase enzyme. The value of the inhibition constant of the punicalagin compound showed better affinity than the control compound, but lower than the ligand compound. Bond free energy (ΔG) values of punicalagin compounds showed the best results compared to native and control ligands. Therefore, the punicalagin compound is effective and has the potential to be used as a DENV-3 drug candidate.

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CONFLICT OF INTEREST

All authors in this research confirmed that there is no conflict of interest.

AUTHOR CONTRIBUTION

RK, SR, THS, MD, and AHR performed in charge of collecting data. RK writing article. YR is a principle investigator who provides study ideas and validates data.

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