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In silico study to predicting Apyrase-ADP binding affinity from salivary gland of *Aedes aegypti* in inhibition of platelet aggregation by molecular docking

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ABSTRACT. Dengue hemorrhagic fever (DHF) caused by the dengue virus is an endemic disease in Indonesia. The dengue virus is transmitted to the human body by *Aedes aegypti* vector through the process of blood feeding. The success of dengue virus transmission through blood feeding is due to the presence of a protein that acts as an anti-coagulant, namely apyrase. Apyrase is an enzyme capable of degrading ADP in the blood feeding process. ADP has an important role as an inducer in platelet aggregation process. Therefore, our study aims to determine the strength of the interaction between Apyrase protein and ADP substrate in its activity as an inhibitor of platelet aggregation during blood feeding. *In silico* analysis on Apyrase and ADP ligands conducted by molecular docking method using AutoDock Vina software. Apyrase sequences of *Ae. aegypti* was taken from the UniProt database with accession number P50635. The three-dimensional structure of the ADP ligand was obtained from the PubChem database with accession number 6022. The study showed that interaction of apyrase enzyme with ADP ligand has good spontaneity based on the G value. The G value of these interaction are -9.6 kcal/mol (ADP sub unit 1) and -9.4 kcal/mol (ADP sub unit 2). The docking analysis showed the interaction of the apyrase enzyme with the ADP ligand bind exothermically. The active sites of the apyrase enzyme that interact with the ADP ligand are on the the amino acids HIS-49, ASP-98, ASN-130, HIS-131, HIS-257, HIS-259, ARG-370, GLU-451, ARG-453, and THR. -454.

Keywords: *Aedes aegypti*; Apyrase; blood feeding; *in silico*; molecular docking

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INTRODUCTION

Dengue hemorrhagic fever (DHF) is caused by dengue virus, the most arboviral disease globally with incidence rates reaching 390-400 million cases each year (Low *et al*., 2017; Cattarino *et al*., 2020). Dengue virus (DENV) is the viruses caused a tropical disease in the world which infects around 400 million people per year. This virus is a group of Arbovirus (Arthropod Borne Virus) which is transmitted to the human by female Aedes mosquitoes as vector (Barbosa-Lima *et al*., 2020). In 2020, there was an increase in dengue cases in several countries including Bangladesh, Brazil, Cook Island, Ecuador, India, Indonesia. Maldives, Mauritania, Mayotte (Fr), Nepal, Singapore, Sri Lanka, Sudan, Thailand, Timor-Leste, and Yemen (WHO, 2020). There are 10 provinces in Indonesia reported with the highest number of dengue cases, i.e., West Java, Bali, East Java, NTT, Lampung, DKI Jakarta, NTB, Central Java, Yogyakarta, and Riau (Kemenkes, 2020).

Distribution of DHF in Indonesia is influenced by *Aedes*. *Ae. aegypti* as a primary vector and *Ae. albopictus* as a secondary vector. Dengue virus transmitted to human by blood feeding. Blood feeding is an intake of nutrients, blood, and enlargement of blood vessels carried out by adult female mosquitoes (Wathon *et al*., 2016). Transmission of dengue virus through blood feeding can occur due to the presence of secretory proteins in the salivary glands of *Ae. aegypti* that act as vasomodulator and immunomodulatory factors (Titus *et al*., 2006). Some of these proteins are D7 family, serpine, apyrase, adenosine deaminase, purine nucleosidase, serine protease, -glucosidase, lectin, gambicin, lysozyme, defensins, Xa factor, and Aed a3 (Wasinpiyamongkol *et al*., 2012). The previous studies showed that there are two immunogenic proteins from the salivary glands of *Ae. aegypti* i.e., a protein

31 and 56 kDa (Oktarianti *et al*., 2014). Based on LC MS/MS analysis showed that the most abundant in 31 kDa protein is D7 protein, while 56 kDa is apyrase, both of them is involved in blood feeding (Oktarianti *et al*., 2015).

Apyrase is an enzyme capable of degrading Adenosine triphosphate (ATP) or Adenosine 5'-diphosphate (ADP) in the blood feeding process. ADP has an important role as an inducer in the process of platelet aggregation through membrane receptors. Platelet aggregation by ADP causes blood clots. Therefore, the prevention of platelet aggregation by the apyrase enzyme is very important to trigger the inhibition of the blood clotting process so that it will make it easier for the vector to suck blood in the host's body (Fontaine *et al*., 2011). It is necessary to conduct in silico analysis to determine the strength of the interaction between Apyrase and ADP substrate in its activity as an inhibitor of platelet aggregation during blood feeding.

In silico method by molecular docking will show predictions of interactions and bond affinities as well as the conformation of protein-ligand compounds (Wardaniati & Herli, 2018). In this study used apyrase as a protein and the ligand is ADP. ADP is a ligand that is important inducer to platelets aggregation, as they will interact with P2Y1 and P2Y12 receptors (Wolska & Rozalski, 2019). *In silico* analysis to determine the bond strength between the apyrase enzyme and ADP in related inhibiting platelet aggregation during blood feeding process. These method as the basis before in vivo and in vitro analysis, the other hand there is advantage i.e., that the process takes a short time and costs less (Ruswanto *et al*., 2018).

MATERIALS AND METHODS

In silico study was carried out by performing molecular docking on apyrase and ADP ligand using Autodock Vina (Scripps Research Institute) docking software ver. 1.1.2.

Preparation of the apyrase 3D structure. The apyrase data downloaded from UniProt at https://www.uniprot.org/uniprot/P50635 in .pdb format. The next step is separated from the solvent and ligands or non-standard residues that are not needed (residue) in the molecular anchoring process using the PyMol application program (Fakih & Mentari, 2020). Then, it was optimized by using the AutoDock Tools application program. The optimization includes the addition of polar hydrogen atoms, Kolman charge and grid arrangement (X, Y, Z). The results of the process are in a pdbqt format for the process docking (Trott & Olson, 2010).

Preparation the ADP 3D structure. The ADP three-dimensional structure ligand was downloaded from PubChem at https://pubchem.ncbi.nlm.nih.gov/ in. sdf format, then edited using the Chem3D application program. Optimization with Chem3D was used to minimize ligand energy and saved in .pdb format. The ADP ligand was then optimized by adding hydrogen atoms, gasteiger charge, torsion tree with the help of the AutoDock Tools application and the results were saved in. pdbqt format (Sulastri *et al*., 2019).

Griding process. The griding process is carried out to indicate the area of the ligand to be docked. Grid box adjusted to the size of the ligand. Grid box data includes number of point dimension, spacing (angstrom), and center grid box, saved in a conf.txt format (Trott & Olson, 2010).

Docking process. The docking process was carried out using Autodock Vina program, run by the command prompt, then stored in log.txt and out.pdbqt formats (Trott & Olson, 2010). Validation needs to be done before docking analysis, by aligning the measurement of the root mean square deviation (RMSD) value (Arba *et al.*, 2020).

Analysis and visualization. The bond conformation analysis interaction of the active site of the apyrase (amino acid) and the ADP ligand can be visualized using the PyMol application program.

RESULTS AND DISCUSSION

The structure of Apyrase used is derived from *Aedes aegypti* with a length of 562 amino acids. This enzyme consists of two protein subunits (A1 subunit and A2 subunit) and cofactors that form a complex structure. The two subunits of the enzyme apyrase polypeptide chains have similar or

homodimers. In addition to the homodimeric structure, the apyrase enzyme also has a cofactor in the form of Zn in each subunit (Ribeiro & Valenzuela, 2003). Cofactors are inorganic compounds that have a certain level of oxidation number such as metal atoms. The cofactor is on the active site of the enzyme and functions to assist the catalytic biotransformation of the enzyme (Yang *et al*., 2018; Zhang *et al*., 2021). In addition, the crystal structure of the Apyrase enzyme also did not find any natural ligands (native ligands). Natural ligands from the crystal structure of a macromolecule are needed for the validation process of the docking method in order to be able to find the geometry of the experimental ligands and their physico-chemical interactions. It is necessary to search for the pharmacophores of the apyrase enzyme (Guedes & Maghales, 2015). The three-dimensional structure of the apyrase enzyme from the organism *Ae. agypti* downloaded from the UniProt database with the site address https://www.uniprot.org/uniprot/P50635 can be seen in Fig. 1.

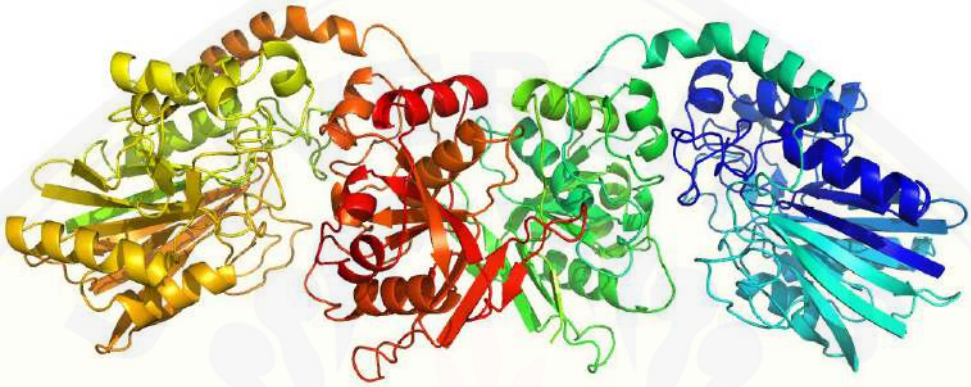


Fig. 1. Three-dimensional structure of the Apyrase from *Aedes aegypti* P50635 (https://swissmodel.expasy.org/repository/uniprot/P50635)

Pharmacophore is a structural feature of a compound that plays a role in biological effects (Qing *et al.*, 2014). Three-dimensionally, pharmacophores play a role in the specific binding of compounds to the target compound (Arba *et al*., 2020). Pharmacophore is a structural feature of a compound that plays a role in biological effects (Qing *et al.*, 2014). Three-dimensionally, pharmacophores play a role in the specific binding of compounds to the target compound (Arba *et al*., 2020). The pharmacophore location of the apyrase enzyme belonging to *Ae. aegypti was* obtained from homology modeling on the Swiss-Model expasy website to obtain *a* homologous 3D structure template (Brooksbank & Andrew, 2020).

The results of homology modeling showed that the Apyrase belonging to *Ae. aegypti* has similarities to the 5-ectonucleotidase in human with the accession code template 6xuq.1. The assessments used for selecting the model template are the Quality Model Energy Analysis (QMEAN) value, the global model quality estimation (GMQE) value, and the sequence identity. The QMEAN value in the template of the selected enzymes is -1.83 with the thumb symbol facing up. This shows that the reliability of the model used has a good quality. The QMEAN assessment function offers the quality of the model from several assessment elements to estimate the quality of the mode (Junaidin *et al*., 2019).

The 3-dimensional structural model is considered to have low quality if it has a QMEAN value of -4.0. The model marked with the thumb facing up indicates the better quality of the model, whereas the model marked with the thumb facing down indicates the poor model quality (Junaidin *et al*., 2019). The GMQE value is 0.65, where a value in between 0-1 indicates the good accuracy of the model between the apyrase enzyme and the 5-ectonucleotidase enzyme template. The GMQE assessment is an estimate of the accuracy/model fit between the target's 3-dimensional structure and the3-dimensional structure *template*. The higher the GMQE value is close to 1, the more accurate the results (Brooksbank & Andrew, 2020). Meanwhile, the value sequence identity shows 33.46%. The minimum value for sequence identity to be considered homologous is 30% (Pearson, 2014). Sequence identity between target and template above 30% indicates that there is a global structure accuracy

between the two sequences (Khor *et al*., 2015). in contrasts, the sequence identity which is lower than 30% indicates that the template is included in the zone category twilight, which requires preparation (Casadio *et al*., 2006).

Three-dimensional structure of the enzyme 5-ectonucleotidase used enzyme research crystallized by the method of *x-ray diffraction* at a wavelength of 1.97 Å of the human organism. The structure of the enzyme consists of subunit A1 and subunit A2 and there is a natural ligand in the form of O1T([[(2~{R},3~{S},4~{R},5~{R})-5-[6- chloranyl-4-[[(1~{S})-1-(4-fluorophenyl)ethyl] amino]pyrazolo[3,4-b]pyridine-1-yl]-3,4bis(oxidanil) oxolan-2- il] methoxi-oxidanil-phosphoryl] methylphosphonic acid), inorganic ligand or cofactor Zn and CA. The length of the amino acids that make up the structure is 542 amino acid residues. The removal of water molecules and natural ligands was used to obtain the secondary structure of the 5'-ectonucleotidase enzyme. In addition, the addition of polar hydrogen atoms and the calculation of the Kollman partial charge using the MGL software Tools were carried out. This stage aims to ensure that the bioactive peptide molecules can form a stable interaction with the enzyme macromolecules (Fakih & Mentari, 2020). The three-dimensional structure of the 5'-ectonucleotidase enzyme can be seen in Fig. 2.

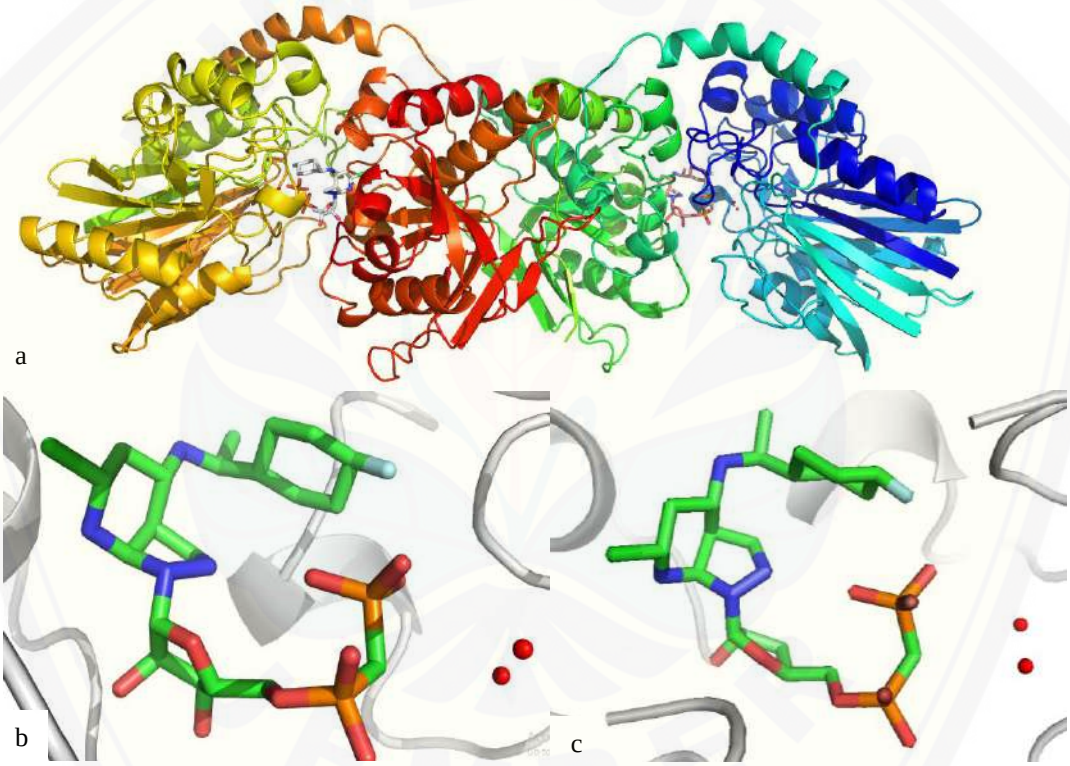


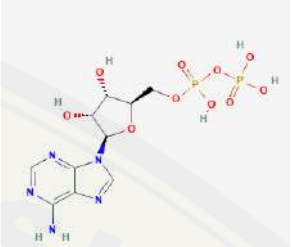
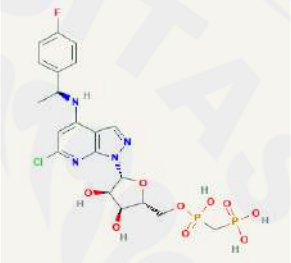
Fig. 2. Three-dimensional structure of the human 5'-ectonucleotidase macromolecule: a. Natural ligand sub-unit A1; b. Natural ligand sub-unit A2; c. Structure of red spheres: cofactor (https://swissmodel.expasy. org/templates/6xuq.1)

The 5-ectonucleotidase enzyme is the enzyme encoded by the NT5E gene. This enzyme has the activity of a phosphate group at nucleotide hydrolysis, namely the hydrolysis of AMP to adenosine (Minor *et al*., 2019). While the apyrase enzyme in *Ae. aegypti* also has the activity of hydrolyzing phosphate groups on nucleotides, namely the hydrolysis of ATP and ADP into AMP. The apyrase enzyme in *Ae. aegypti* is a group of enzymes belonging to the 5'nucleotidase group. There are three groups of enzymes apyrase in animals include 5'nucleotidase group, group-type Cimex apyrase, and a group of cell-surface apyrase (Masoud *et al*., 2020).

The ADP ligand structure compounds is based on the results of previous studies (Oktarianti *et al*., 2015) which stated that ADP is a substrate of the Apyrase enzyme in the process blood feeding. Meanwhile, the natural ligand of the 5'-ectonucleotidase enzyme structure used in the validation process of the docking method is the O1T ligand. The ligand has a formula structure similar to that

of ADP. ADP consists of a $C_{10}H_{15}N_5O_{10}P_2$ structure, while O1T as a natural ligand has a structure $C_{20}H_{24}ClFN_4O_9P_2$. The use of natural ligands is intended as a reference for the ligands to be tested to make it easier to validate the docking method used (Santoso, 2017). Structures and profiles related to ADP and O1T ligands downloaded from PubChem on the website www.pubchem.com can be seen in Table 1.

Table 1. Profile of ADP and O1T ligands

Name	Accession number	Structure	Molecular formula
Adenosine 5'-diphosphate (ADP)	6022		$C_{10}H_{15}N_5O_{10}P_2$
[[(2~{R },3~{S},4~{R},5~{R})-5-[6-chloranyl-4-[[(1~{S})-1-(4-fluorophenyl)ethyl] amino]pyrazolo[3,4-b]pyridine-1-yl]-3,4bis(oxidanil)oxolan-2-yl]methoxi-oxidanil-phosphoryl]methylphosphonic acid (O1T)	130423739		$C_{20}H_{24}ClFN_4O_9P_2$

The validation of the docking method includes determining the position of the grid box and redocking. Determination of the grid box as a marker of the location of the three-dimensional coordinates of the interaction center of the ligand and protein follows the center of mass of the natural ligand (Santoso, 2017). The arrangement grid box used in this study is adjusted to the size of the O1T ligand molecule as a natural ligand for the 5'-ectonucleotidaseenzyme (Aswad *et al*., 2019). Setting grid box used in this study can be seen in Table 2.

Table 2. Setting grid box docking

Algorithm	X	Y	Z
Spacing	1000 Å	1000 Å	1000 Å
Dimension	20	20	20
A1 subunit Apyrase	-15 431	19 416	-32 709
A2 subunit Apyrase	-37 611	21 306	- 84,444

Method validation by docking the natural ligands of the enzyme crystal structure was carried out after setting the grid box (Boitttier *et al*., 2020). The parameter of the validation process is the Root Mean Square Deviation (RMSD). The docking considered valid by the protocol if a RMSD value is less than 2 (Arba *et al*., 2020). In this study, the RMSD value for the natural ligand of the A1 subunit is 0.981 and the natural ligand of the A2 subunit is 0.648 (Fig. 3), indicate the docking method is valid and can be used for docking on other ligands and to determine their binding activity.

Table 3. Binding affinity of apyrase and ADP ligand

Apyrase bond and ADP ligand	G value (kcal/mol)
A1 subunit apyarase and ADP	-9.6
A2 subunit apyrase and ADP	-9.4

The next parameters observed are free energy at the binding site (ΔG) and amino acid residues seen from visualization using software PyMol. The free energy value indicates the bond energy between the enzyme and the ligand (Primary & Aziz, 2019). The G value on the ADP ligand can be visualized in Table 3.

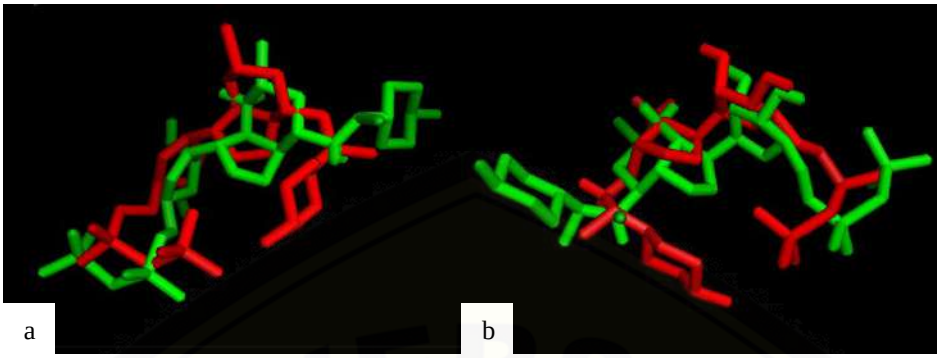


Fig. 3. RMSD conformity of the 5'-ectonucleotidase enzyme: a. The natural ligand of the A1 apyrase subunit; b. The natural ligand of the A2 apyrase subunit (B)

The results of docking between Apyrase and ADP showed that the binding affinity was high, it can be seen the G value between apyrase subunits A1 and subunit 2 with ADP ligand was relative similar. The negative G value indicates that ADP can interact with the apyrase enzyme exothermic, so it can be concluded that the inhibition of the platelet aggregation process by the apyrase enzyme that binds to the ADP ligand occurs spontaneously. Thermodynamically, the lower the free energy value of the ligand bound to the active site of the target protein, the more stable the molecule and the reaction will proceed spontaneously. Meanwhile, the greater the free energy value, the more unstable the complex structure formed (Primary & Aziz, 2019).

The docking visualization is to determine the amino acid residues that play a role in maintaining the stability of the interaction of the ligand compound with apyrase (Habiburrohman *et al*., 2021). The visualization result of the interaction of apyrase enzyme with ADP can be seen in Fig. 4.

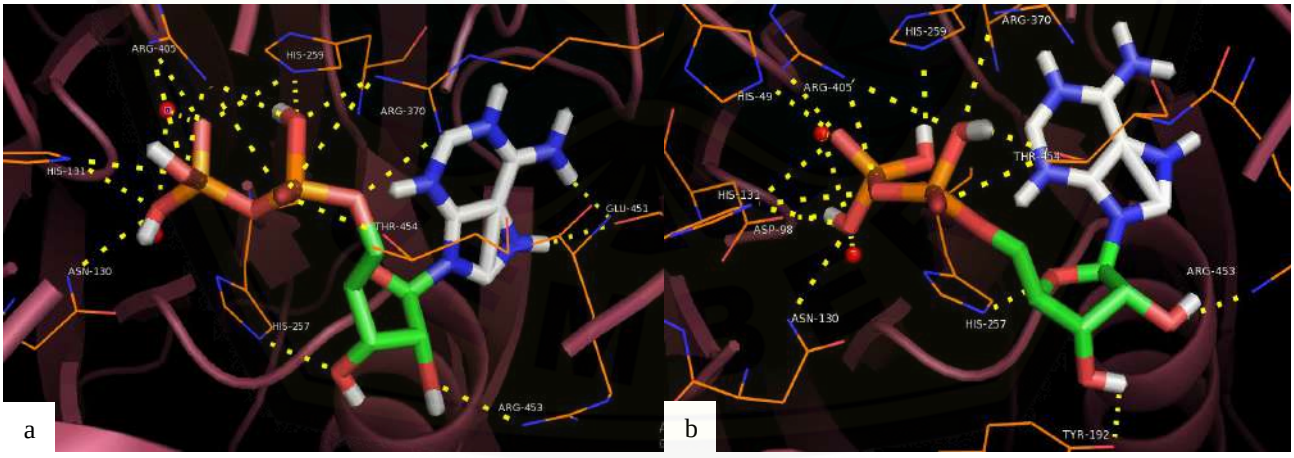


Fig. 4. The active site interaction: a. Apyrase sub unit A1 with ligand ADP; b. Apyrase sub unit A2 with the ADP ligand. The active sites of the apyrase enzyme that interact with ADP ligands are the amino acids HIS-49, ASP-98, ASN-130, HIS-131, HIS-257, HIS-259, ARG-370, ARG-405, GLU-451, ARG- 453, THR-454

The visualization using PyMol showed there is a polar hydrogen interaction between the ligand and the enzyme. The yellow dots in these figures indicate interactions involving hydrogen atoms with other atoms that have a high affinity. Hydrogen bonds can occur between atoms in different molecules or within the same molecule. An atom will donate its hydrogen covalent bond (donor) generally fluorine (F), nitrogen (N), or oxygen (O) atoms to atoms with unequal electrons (acceptors). The bonds formed (F – H, N – H, O – H) are polar, hydrogen atoms with high electron affinity tend to be

positively charged while electronegative atoms F, N, and O tend to be negatively charged. The electrostatic interaction is strengthened by the dipole-dipole interactions that occur between the proton donor atom and the proton acceptor atom. The ADP ligand with the amino acid apyrase enzyme subunit A1 and subunit A2 has 11 hydrogen bonds.

The visualization of figure 4 showed the presence of amino acid residues in the interaction of the ADP ligand with the Apyrase enzyme receptor A1 subunit and ADP with apyrase and A2 subunit. This indicates the active site of the apyrase enzyme that binds to the ADP ligand. The active site of the apyrase that interacts with the ADP ligand i.e., amino acids HIS-49, ASP-98, ASN-130, HIS-131, HIS-257, HIS-259, ARG-370, ARG-405, GLU-451, ARG- 453, THR-454.

The interaction of apyrase with ADP ligand from docking analysis showed stable and spontaneous bonding and the presence of an apyrase active site that interacts with ADP. This indicate that apyrase of the salivary glands from *Ae. aegypti* is able to hydrolyze ADP to inhibit platelet aggregation in humans so it facilitating blood feeding process of mosquitoes. It indirectly enhancing transmission virus dengue to human thus DHF cases increase.

CONCLUSION

In silico study by using docking method showed that the interaction of apyrase enzyme with ADP ligand has good stability. The G value of the apyrase enzyme interaction with ADP in the A1 subunit is -9.6 kcal/mol and the A2 subunit is -9.4 kcal/mol. This shows that ADP and apyrase enzyme can bind stably and spontaneously. The active site of the apyrase enzyme that interacts with the ADP ligand i.e., the amino acids ASN-130, ARG-405, ARG-370, ARG-453, GLU-451, HIS-131, HIS-257, HIS-259, THR-454, HIS- 49 and ASP-98.

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