Virtual screening of Indonesian flavonoid as neuraminidase inhibitor of influenza a subtype H5N1

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Abstract. Highly Pathogenic Avian Influenza (HPAI) H5N1 poses a significant threat to animal and human health worldwide. The number of H5N1 infection in Indonesia is the highest during 2005-2013, with a mortality rate up to 83%. A mutation that occurred in H5N1 strain made it resistant to commercial antiviral agents such as oseltamivir and zanamivir, so the more potent antiviral agent is needed. In this study, virtual screening of Indonesian flavonoid as neuraminidase inhibitor of H5N1 was conducted. Total 491 flavonoid compound obtained from HerbalDB were screened. Molecular docking was performed using MOE 2008.10. This research resulted in Guajavin B as the best ligand.

1. Introduction

Influenza is an acute respiratory illness caused by influenza viruses with symptoms off ever, sore throat, chills, cough, headache, muscle aches, and fatigue [1]. The disease is classified as an infectious disease caused by a virus and can infect humans as well as animals. Avian influenza is a significant threat to poultry and human health in the world. Genetic diversity possessed by the influenza virus causing the disease becomes a pandemic, mainly due to the incorporation of the gene(gene reassortment) of avian influenza virus with an influenza virus that commonly occurs in humans [2].

The subtype H5N1 virus included in the Highly Pathogenic Avian Influenza (HPAI) with morbidity and mortality that reaches 100%. The H5N1 virus was first identified in the early 1900s in Italy [3]. However, the virus is of international concern since the H5N1 infection in humans that occurs the first time at 1997 in Hongkong and cause a significant number of mortality [2]. In Indonesia, the number of H5N1 infections in humans since the beginning of 2005 until December 2013 reached 195 cases with 163 deaths [4]. H5N1 infection showed clinical symptoms such as high fever (up to 38°C), respiratory disorders, limfofenia, abnormalities on chest radiographs, and in some cases diarrhea and vomiting [5].

One precaution against H5N1 infection is through vaccination. However, vaccination has a low efficiency in protecting the body when the influenza virus infected [6] or as a result, of antigenic drift and antigenic shift happens to the virus. Therefore, various antiviral drugs have been developed for the treatment of influenza. There are two classes of antiviral drugs that have been developed at this time, the class of adamantane and neuraminidase inhibitor [7]. Adamantane class inhibits the action of the
M2 ion channel. Amantadine and rimantadine were developed in 1960 and had been accepted in many countries [7]. Nevertheless, this class limited to influenza A virus only. Influenza virus resistance to adamantane group is also quite high. Therefore, the use of adamantane class has been limited by the Centers for Disease Control and Prevention (CDC) [8]. Since 2010, oseltamivir (Tamiflu®) and zanamivir (Relenza®), which includes a neuraminidase inhibitor class of antiviral drugs, were recommended by CDC and the World Health Organization (WHO) for the treatment of influenza A and B infections [9].

Nevertheless, the threat of the Highly Pathogenic Avian Influenza H5N1 not only lies in a number of fatalities but also because of high resistance to the commercial drugs. The H274Y mutation has been shown to occur in H5N1 influenza virus, causing virus H5N1 resistant to oseltamivir [10]. Therefore, the emergence of influenza viruses resistant to commercial antiviral medication has led to the need for the development of more potent antiviral drugs [11].

Various kinds of compounds have been developed neuraminidase inhibitor for the treatment of influenza virus infection. Flavonoids are a large group of compounds of natural ingredients that can be found either in higher plants as well as the low level of the plant, including algae. Flavonoids are known to have pharmacological character among anticancer, antibacterial, anti-inflammatory, and antiviral [12]. In the context of a drug likeness and ability to be a drug, a flavonoid class of compounds that has a low molecular mass with good bioavailability is considered feasible. Meanwhile, Indonesia is the country with the second highest biodiversity in the world after Brazil. Of the 40,000 species of plants that exist in the world, 30,000 of who are in the Indonesian archipelago, and among the 30,000 species of plants in Indonesia, 9,600 of whom are known to have pharmacological activity [13-15]. Therefore, this study will carry out virtual screening natural materials of Indonesian flavonoid as neuraminidase inhibitors of influenza A virus subtype H5N1 via molecular docking.

2. Materials and methods

2.1. Materials
This research was using computational study (in silico), by including personal computer intel core i7 with 4 GB of RAM, MOE 2008.10, ACDLabsChemSketch 12, Chem 3D Pro, VegaZZ, Jalview 2.8.0b.1, Toxtree 2.6.0, UCSF Chimera 1.9 and several online software from internet web servers[16-22]. Chemical structures in this research such as neuraminidase were obtained from online database NCBI, EMBL-EBI, PDB, and HerbalDB (http://herbaldb.farmasi.ui.ac.id/v3/) [13].

2.2. Method

2.2.1. Preparation of Neuraminidase H5N1. This step included searching of neuraminidase sequence, multiple sequences alignment, searching, and validation of three-dimensional structure of neuraminidase influenza A subtype H5N1. Structure validation was done by evaluating QMEAN4 and QMEAN-Z as a result of SWISS Modeling[23]. Structure validation was verified by Ramachandran plot analysis, which could be accessed through http://mordred.bioc.cam.ac.uk [24].

2.2.2. Preparation of Ligand. Three-dimensional structures of Indonesian Flavonoid was obtained from the herbalDB (http://herbaldb.farmasi.ui.ac.id/v3/), developed by Faculty of Pharmacy, University of Indonesia. These structures were then converted into Molecular Design Limited (MDL) Molfile (.mol) using VegaZZ.

2.2.3. Molecular Docking Simulation. There were stages of the molecular docking, namely preparation of neuraminidase and ligands for docking, and molecular docking process of protein-ligand [25].
2.2.4. Molecular Docking Analysis. Gibbs free energy from docking with MOE could be seen on an output in the format of MOE database (.mdb). Complex with the lowest free energy was then prepared for relocking step by retaining increase with a factor of 100. Moreover, LigX and MOE features could be used to see the interaction between ligand inhibitor with amino acid residue on target enzyme. It was possible to identify which functional group from the ligand that would bind to the amino acid on the active site of the enzyme.

3. Results and discussion

3.1. Structure Validation of Three-Dimensional Neuraminidase. Neuraminidase is an enzyme that plays an important role in the life cycle of the influenza virus. The sequence of amino acids that constitutes the enzyme neuraminidase can be searched on the site National Center for Biotechnology Information (NCBI) through https://www.ncbi.nlm.nih.gov/ URL. One of the features of the NCBI Influenza Virus Resource is providing the data from the NIAID Influenza Genome Sequencing Project and GenBank, along with devices for the annotation and analysis of influenza sequences. NCBI Influenza Virus Sequence Database also provides protein sequences and nucleotide sequences encoding regions of the influenza virus. The search of neuraminidase influenza A virus subtype H5N1 sequences was done by determining search settings as follows: type A, human host, country / area Indonesia, NA protein, and subtype H5N1. A total of 90 sequences of proteins were obtained, 58 of whom completed (c), 1 noncompleted (NC), and 31 putative (p). Sequences that can be used is the completed ones.

The main goal of Multiple Sequences Allignment (MSA) is determining the conserved regions pattern of the sequences. The conserved region shows the identical area between the sequences. MSA was done to get consensus sequences representing the entire sequence of the H5N1 influenza virus neuraminidase. MSA was done by using Clustal Omega, which can be accessed online through http://www.ebi.ac.uk/Tools/msa/clustalo/ [26]. MSA results were then analyzed using software Jalview. Jalview is a program for editing, visualization, and analysis of the MSA results. By using Jalview, conservation of the region, the quality, as well as consensus sequences, could be obtained. Neuraminidase consensus sequences were obtained through this software in FASTA format. BLAST (Basic Local Allignment Search Tool) was performed on consensus sequences to search the biggest homology with sequences in the GenBank database. It was done using NCBI BLAST which can be accessed online through http://blast.ncbi.nlm.nih.gov/Blast.cgi [27]. Similarity searching through NCBI-BLAST can be done by uploading files consensus sequences in FASTA format. Protein-protein BLAST (BLASTP) was utilized to search for the protein sequence of the inserted protein sequences. Results of similarity searching on the server will show the percentage of identity, the maximum score, and protein access codes which have similarities with the input sequence. BLAST results show the high level of sequence homology with the consensus sequence of the protein in the GenBank database, which is 99-100%. From the results of BLAST, the chosen sequence is the one with access number ABI49398.1 (neuraminidase [Influenza A virus (A / Indonesia / CDC739 / 2006 (H5N1))]) because it has the highest score (925) and 100% identical to the consensus sequence.

The modeling was done by using SWISS-MODEL, which can be accessed online through the site http://swissmodel.expasy.org/. The comparative modeling or homology modeling is a reliable method for predicting the structure of the target and to obtain a three-dimensional structure of a homologous protein to use as a template [28]. Homology modeling was done in four stages, namely: identification of template structure, sequence alignment of targets and templates, modeling, and evaluation of the model results [23]. There are three modes that are commonly used in modeling, namely: automatic mode, alignment, and projects [23]. 3D modeling in this study was conducted in automatic mode, for identifying the residue amount between the target and template. The results can be trusted if the automatic mode showed that the similarity between common targets and templates is greater than 50% [23]. The template of 3ckz was obtained from the PDB. The results obtained through SWISS-MODEL modeling has a percentage of 97.14% identity to 3ckz template. Therefore, the
modeled 3D structure can be trusted because it has a high percentage of identity. Neuraminidase structure modeling results using SWISS-MODEL can be seen in figure 1. Based on the evaluation of residues in Ramachandran plot, there is only one aminoacid residue, glutamate (GLU), which is located in the outlier region, which is about 0.3% probability. It can be said that the structure of the generated 3D is good enough and can be used for the next stage of this research.

![Figure 1. Valid structure of neuraminidase H5N1](image)

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3.2. Visualization and Determination of Neuraminidase H5N1 Binding Site. The enzymes ABI49398.1 was visualized by using Gaussian Contact the Surface menu and maps of MOE2008.10. Visualization of ABI49398.1enzyme can be seen in figure 2a. Red tape showing α-helical secondary structure, yellow indicates β-sheet structure, green indicates loop structure, while the blue shows the structure of the turn, an area slightly transparent visualization of the surface of the enzyme. It can be seen that the active side ABI49398.1 is relatively hydrophobic, but still has some hydrogen bonding region. The active enzymes and proteins are generally in the form of a hydrophobic cavity and involve side chain amino acid residues. They are making up the active site, and can be seen through the finder site features contained in MOE 2008.10. Site finder feature will perform calculations toward the active side of the 3D coordinates of receptor atoms. Visualization of the binding of the enzyme to the zanamivir standard was also done through the docking process. Standard zanamivir was obtained from PubChem with CID60855 access number. The obtained binding sites are Agr98, Glu208, Glu257, Arg273, Tyr324, Arg348 and Tyr382 (figure 2b).

![Figure 2. (a) Visualization neuraminidase H5N1 using MOE and (b) binding site sequence ABI49398.1 with zanamivir standard](image)
3.3. **Molecular Docking Simulation and Analysis.** Flavonoid ligand optimization was done on the MOE database viewer (dv). The first stage in the optimization of ligands is to wash the flavonoids and standard ligand. The goal of Wash treatment is to improve the structure of the ligand and improve the position of the hydrogen atom in the ligand. Then, partial charge determination was done by using a partial charge command. A selected forcefield for the determination of the partial load is MMFF94 that can be used to validate the position of the hydrogen Tomi the ligand [29]. MMFF94 is a force field that parameterized for small organic molecules. The same forcefield is used for energy minimization of ligands. Minimization process was carried out by the RMS gradient of 0.001kcal/mole. The ligands set was then stored in .Mdb format and ready for the simulation of molecular docking.

In the molecular docking, the enzyme is made rigid while the ligand is in a state of the freedom of movement to seek for a stable conformation. The utilized placement method is Triangle Matcher with the 1000 number of rounds. Triangle Matcher shows the random movement of the ligand in the active site of the enzyme to produce an optimal bond orientation. After ligand interacts with the enzyme, the affinity between the receptor and ligand will be evaluated using a scoring function by estimating the value of the binding energy [30-31]. The utilized scoring functions in molecular docking are London dG, which is the default parameter for MOE software. London dG scoring function will show the value of the Gibbs free binding energy of the enzyme conformation and ligand[32].

Molecular docking simulations were carried out to generate data in the form of Gibbs free energy of binding ($\Delta G_{\text{binding}}$). The obtained $\Delta G_{\text{binding}}$ value is the result of intermolecular hydrogen interactions, electrostatic, and weak Van der Waals forces that occurred between the ligand and the enzyme in a pose. This value is the initial parameters in conducting virtual screening. This is because the Gibbs free energy shows stability and strength of enzyme-ligand interactions. The lower the value of the Gibbs free energy, the enzyme is more stable and thermodynamically favored. From 491 flavonoids ligand screened at the beginning, there is 149 ligand that has a lower value ($\Delta G_{\text{binding}}$) than the standard. Initial screening was done by retaining parameter of 30. Then, redocking was done against 149 best ligands to increase retain parameter to 100, in order to get the best conformation of each ligand. In order to expose better data representation, the best 20 results from the redocking can be seen in table 1.Hence, the complete results of the docking for whole 491 ligands and redocking of 149 best ligands could be seen in this following supplementary material: http://staff.ui.ac.id/system/files/users/aditya.parikesit/material/supplementary_material_10jcc.pdf. The best ligand for ABI49398.1 is M0000338 (Guajavin B) with $\Delta G_{\text{binding}}$ value of -67.2920 kcal/mol.

In general, the annotated interaction between guajavin B and neuraminidase is weak covalent ones (figure 3). Guajavin B is interacting with 10 amino acid residues on neuraminidase, namely: Glu99, Lys130, Asp131, Arg132, Trp159, Glu257, Arg273, Tyr324, Pro411, and Lys412 (figure 3). Guajavin B is included in the flavonol subclass of flavonoid. Flavanols hydroxyl substituent at position 3 and totally absent on double bond at C2-C3. This compound was formed from the reduction of flavanon [33].

Figure 3 shows that the ligand molecule is large enough to deter molecule entrance into the cavity of the enzyme. The formation of cation-aromatic interactions lies between the aromatic ring and Tyr324. Tyrosine will interact with aromatic ring due to delocalisation. Then, the hydrogen atoms in the side chains of Lys130, Arg132, Arg273, and Lys412 can form hydrogen interaction with oxygen atoms of hydroxyl groups on Guajavin B. Lysine is a positive polar (basic) amino acid due to the second primary amine group on the ε position of the aliphatic side chain.
Table 1. $\Delta G_{\text{binding}}$ of ligand

| No | Ligand ID       | $\Delta G_{\text{binding}}$ (kl/mol) |
|----|----------------|-------------------------------------|
| 1  | M00009338       | -67.2920                            |
| 2  | M00009213       | -64.7416                            |
| 3  | M00005901       | -63.8624                            |
| 4  | M00009105       | -63.5440                            |
| 5  | M00014080       | -63.4709                            |
| 6  | M00014082       | -61.9593                            |
| 7  | M00013884       | -59.5516                            |
| 8  | M00009106       | -59.5235                            |
| 9  | M00014066       | -58.6960                            |
| 10 | M00009235       | -58.5701                            |
| 11 | M00005487       | -58.4900                            |
| 12 | RMN00006834     | -58.3978                            |
| 13 | RMN00006832     | -57.1900                            |
| 14 | RMN00014803     | -56.4708                            |
| 15 | RMN00006843     | -56.4298                            |
| 16 | M00013746       | -56.2798                            |
| 17 | M00014329       | -55.9459                            |
| 18 | M00005900       | -55.0116                            |
| 19 | M00006246       | -54.9444                            |
| 20 | M00019135       | -54.7079                            |
| 21 | Oseltamivir     | -32.2472                            |
| 22 | Zanamivir       | -41.6318                            |

Figure 3: a) visualization of Guajavin B interaction with active site of neuraminidase, and the ligand entrance into the enzyme cavity based on (b) USCF Chimera 1.9 and (c) MOE 2008.10

While arginine is also positive polar (base) due to the guanidino group on the aliphatic side chain. Residues of Glu99, Asp131, and Glu257 have formed hydrogen bonding interactions with the H atom of the carbonyl group in Guajavin B to act as an acceptor of H atoms. Glutamate and aspartate are polar amino acids and have negative charges because of the second carboxyl group on
their side chains. In addition, the residue of Pro411 is also forming hydrogen interactions with the H atom of the hydroxyl group of the ligand by using the nitrogen atom of the main chain of Pro411.

4. Conclusion
The predicted structure of neuraminidase of H5N1 has been found. Thus, based upon virtual screening of Indonesian natural products, the ligand M0000338 (Guajavine B) has been determined as the best possible lead compound with $\Delta G_{\text{binding}}$ value of -67.2920 kcal/mol.

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