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Binding Mode, Absorption, And Distribution Profile, Mutagenicity and Carcinogenicity of Asam Gelugur (*Garcinia atroviridis* Griff.) Fruit Phytoconstituents as Influenza H7N9 Neuraminidase Inhibitor

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Abstract
The purpose of this study was to predict the bond mode, absorption and distribution profile as well as the mutagenicity and carcinogenicity of the asam gelugur fruit compounds which have the best inhibitory activity in neuraminidase influenza subtype H7N9 active pocket by in silico. Pharmacophore modeling and molecular docking using Ligandscout software. Absorption and distribution profiles using PreADMET software. Mutagenicity and carcinogenicity using Toxtree software. The results showed that 10 of the 13 compounds of asam gelugur fruit formed hydrogen bonds with important amino acid residues in the active sac neuraminidase H7N9. Organic acid group compounds have good absorption and distribution profiles. All acid gelugur fruit compounds are predicted not to be mutagenic and carcinogenic. Hydroxycitric acid is a compound that is predicted to have the best inhibition with the lowest Ki value of 1.09 µM. Hydroxycitric acid is a compound that is predicted to have the best inhibition with the lowest Ki value of 1.09 µM.
Keywords: Neuraminidase Inhibitor, Influenza, H7N9, Hydroxycitric Acid

1. Introduction

Influenza "flu" is an acute respiratory infection caused by influenza viruses that can threaten life such as pneumonia [1]. Based on laboratory data from the National Influenza Centers (NIC) for the period 26 November - 09 December 2018, there were 10,520 positive cases of influenza virus infection. Most influenza virus infections are caused by influenza type A, which is 9,970 (94.8%) and influenza type B infections are 550 (5.2%). Cases of influenza deaths in Indonesia alone range from 65-100% during the period 2005 to 2017, with the highest cases occurring in DKI Jakarta, West Java and Banten provinces [2].

The H7N9 influenza A sub-type included in avian influenza was reported to have first infected humans in China in 2013 with 786 cases of infection and 307 deaths (CFR 39%) [3]. Influenza H7N9 viruses are considered to have pandemic potential because of their ability to bind sialic acid receptors that are similar in poultry (α2.3) and humans (α2,6) [4,5,6]. Currently, there is no vaccine available to prevent the infection of influenza A H7N9 virus and the effectiveness of the neuraminidase inhibitor group as the main choice of treatment for avian influenza is not known clinically for patients infected with this virus [7]. In addition, evidence of the resistance of the neuraminidase inhibitor by H7N9 has been shown based on *in vivo* testing [8]. Other studies also showed a decrease in viral susceptibility to oseltamivir isolated from patients with influenza A H7N9 infection [9].

Neuraminidase (NA) is a glycoprotein that is the main target for the prevention and treatment of influenza infections [10].

Influenza neuraminidase is divided into 9 subtypes which are differentiated based on their antigen characteristics [11]. The catalytic mechanism begins with the binding of neuraminidase influenza with an endogenous ligand namely sialic acid found on the surface of the host cell. There are 3 arginine residues in the active sac which function to stabilize the carboxylic group and directly form the ring of pyranoside conformation. Amino acid residues that act to form the catalytic active side with ligands are Arg371, Arg292, Arg118, Arg224, Asp151, Glu276 and Tyr406 [12,13,14].

Oseltamivir (Tamiflu®) is a derivative of ethyl esters from sialic acid which was developed to increase its bioavailability and become the first neuraminidase inhibitor (INA) approved for oral use. Oseltamivir acts as a prodrug which is the active form of the oseltamivir carboxylic metabolite by the esterase enzyme in the liver [15,16]. The change in protein in the INA target can cause resistance which will limit the prevention and treatment of influenza. The evolution of influenza viruses can cause the immune system to escape the virus strains with new antigen characteristics so that the immune response is inadequate and leads to drug resistance [17,18].

One source of natural ingredients that has the potential to overcome influenza is asam gelugur (*Garcinia atroviridis* Griff.). Asam gelugur is widely used as a flavoring dish by Malay people. The fruit is often used as a giver of sour taste and when boiled with sugar can be made jam [19,20]. Testing of influenza viruses showed that methanol extract from the fruit of Asam Gelugur gave IC_{50} of 9.43 µg / mL and 5.15 µg / mL respectively for testing neuraminidase of the bacteria C. perfringens and neuraminidase H5N1 [21]. Other results also showed that the ethyl acetate fraction of Asam Gelugur fruit gave the highest IC_{50} to neuraminidase H5N1 of 50.92 µg / mL [22].

Pharmacophore modeling can facilitate identification of target compounds through the structural characteristics of ligands that are actively bound to target proteins or receptors. Pharmacophore itself is a molecular framework which is an important part of a compound that is responsible for providing biological activity. One device that supports pharmacophore modeling between the protein-ligand complex is Ligandscout. This device can provide information about 3D structures including areas of lipophilic, donors, and hydrogen bond receptors [23].

Computational chemical methods, known as molecular docking, aim to obtain optimized conformations from intermolecular interactions of protein ligands and to obtain a relative orientation based on a terminalized free energy system (Tripathi and Misra, 2017). The interactions that occur between protein ligands involve several types of non-covalent interactions such as hydrogen bonds, ionic bonds, hydrophobic, and van der Waals. Some steps of molecular docking include the preparation of 3D protein structure, ligand preparation, estimation of bonding energy of the protein-ligand complex, and analysis and interpretation of results [24].

2. Materials and methods

Materials

Material or data used is 3D neuraminidase structure of influenza A H7N9 complex with oseltamivir downloaded from Protein Data Bank with PDB ID: 5L15. The 2D structure of 13 compounds (ligands) from asam gelugur fruit.

Tools

The equipment used in this study consists of hardware in the form of a PC with specifications: Intel® Core ™ i5-450M processor (2.30 GHz), 4 GB DDR3 SODIMM PC-8500 memory, OS: Ubuntu 14.04 LTS Linux. Software (software): LigandScout 4.01, PreADMET, Toxtree, R 3.2.2.

Molecular Docking Simulation

The 3D crystal structure of the macromolecules used in this study is the H7N9 influenza neuraminidase receptor complex with oseltamivir downloaded from the Protein Data with PDB ID: 5L15 obtained through X-ray diffraction with a resolution value of 2,4Å. The format of the crystal structure file

that is downloaded is .pdb. with a complex ligand on the Ligand Binding Domain (LBD), Oseltamivir which acts as an antagonist or influenza neuraminidase receptor inhibitor. The next step is the separation of ligands that have been complexed from the neuraminidase structure and then stored as files in the .pdb format. and .mol2. Molecular docking and interaction of modes of bonding of asam gelugur fruit compounds were performed using the Ligandscout application. The docking results are then validated by re-docking. Scoring functions obtained from the program are calculated as free binding energy (Ei), then each correlation is calculated using RMSD.

Pharmacophore Modeling

The results of the docking of the asam gelugur fruit compounds on neuraminidase were used in determining the pharmacophores. Validation of 3D structure-based pharmacophore modeling methods and interaction feature models was carried out using LigandScout software. Validation was carried out by screening 200 active sets and 6000 decoys sets of neuraminidase obtained from Directory of Useful Decoys enhanced (DUDe). The result of this process is a pharmacophore-fit score with the calculation of the quality of the pharmacophore through the ROC curve.

Prediction of Absorption and Distribution Profiles, Mutagenicity and Carcinogenicity

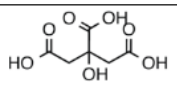
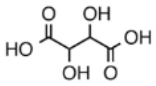
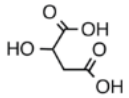
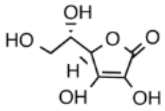
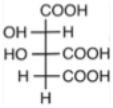
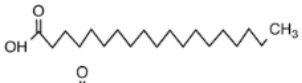
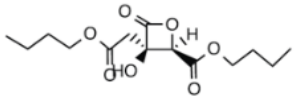
The absorption and distribution profile is predicted by PreADMET software which is accessed on the https://preadmet.bmdrc.kr/ site with the parameters of human intestinal absorption (HIA), Caco-2 cells permeability (CCP), and plasma protein binding (PPB). Prediction of mutagenicity and carcinogenicity profiles using Toxtree software with Benigni / Bossa rule-base parameters.

3. Result and Discussion

Molecular Docking Simulation

The 3D crystal structure of the macromolecules used in this study is the H7N9 influenza neuraminidase receptor complex with oseltamivir downloaded from the Protein Data with PDB ID: 5L15 obtained

Table 1. 2D chemical structure of asam gelugur fruit compounds

No	Senyawa	Struktur
1	Citric Acid	
2	Tartaric Acid	
3	Malic Acid	
4	Ascorbic Acid	
5	Hydroxycitric Acid	
6	Pentadecanoic Acid	
7	Nonadecanoic Acid	
8	Dodecanoic Acid	
9	2-(butoxycarbonylmethyl) -3-butoxycarbonyl-2-hydroxy-3-propanolide	

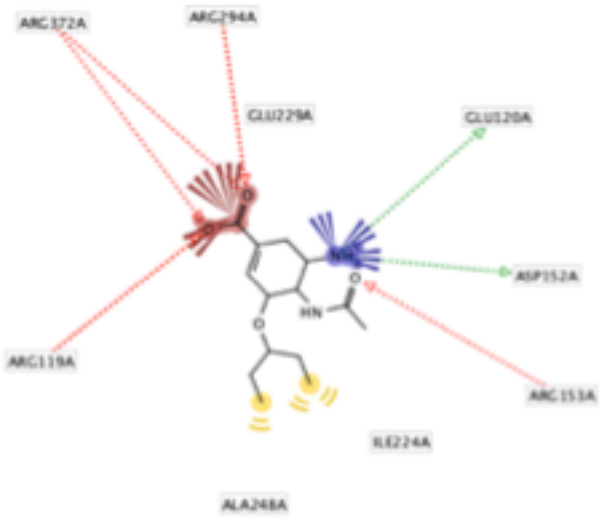


Figure 1. 2D structure-based pharmacophore of oseltamivir with H7N9 neuraminidase

Table 2. The docking simulation results of asam gelugur compounds with H7N9 neuraminidase

No	Molecule Name	Chemical Formula	Ei (Kcal/mol)	Ki (μM)	Amino Acid Interactions	
					Hydrogen Bond	Hydrophobic Bond
	Oseltamivir	$C_{16}H_{28}N_2O_4$	-6,10	3,34	Arg119, Arg294, Arg372, Tyr406, Asp152	Ile224, Ala248
1	Citric Acid	$C_6H_8O_7$	-5,10	1,81	Arg294, Arg372	-
2	Tartaric Acid	$C_4H_{12}N_2O_6$	-4,70	3.56	Arg119, Arg294, Arg372, Tyr406, Asp152	-
3	Malic Acid	$C_4H_6O_5$	-4,70	3.56	Arg119, Arg294, Arg372, Tyr406, Asp152	-
4	Ascorbic Acid	$C_6H_8O_6$	-4,70	3.56	Arg119, Arg294, Arg372, Tyr406, Asp152	-
5	Hydroxycitric Acid	$C_6H_8O_8$	-5,40	1,09	Arg119, Arg157, Tyr406, Trp180, Glu 229	-
6	Pentadecanoic Acid	$C_{15}H_{30}O_2$	-5,00	2,16	Arg119, Arg294, Arg372	Ile429
7	Nonadecanoic Acid	$C_{19}H_{38}O_2$	-4,90	2,54	Arg119, Arg294, Arg372	Ile429
8	Dodecanoic Acid	$C_{12}H_{24}O_2$	-4,60	4,22	Arg294	Tyr406
9	2-(butoxycarbonylmethyl) - 3-butoxycarbonyl-	$C_{14}H_{22}O_7$	-5,80	5,56	Arg119, Arg372	Ile224, Ala248
10	2-hydroxy-3-propanolide 1',1''-dibutyl methyl hydroxycitrate	$C_{15}H_{26}O_8$	-5,60	7,86	Arg294, Arg372, Tyr406, Asp152	Ile224, Trp180, Ala248
11	(-)-β-caryophyllene	$C_{15}H_{24}$	-6,40	2,01	-	Ile224, Trp180, Ala248
12	β-caryophyllene alcohol	$C_{15}H_{26}O$	-7,10	6,19	Glu229, Glu279	Ile224, Ala248
13	α-humulene	$C_{15}H_{24}$	-6,30	2,39	-	Ile224, Trp180, Ala248

through X-ray diffraction with a resolution value of 2,4Å.

Asam gelugur compounds were docked to the H7N9 neuraminidase active pocket using AutoDock Vina. The result of molecular docking is several ligand positions with molecular belay scores in the form of free binding energy (Ei) for each position. The selection of the best ligand position is usually taken based on the smallest Ei value [27].

Pharmacophore Modeling

Validation was carried out by screening 200 active sets and 6000 decoys sets of neuraminidase obtained from Directory of Useful Decoys enhanced (DUDe). Validation

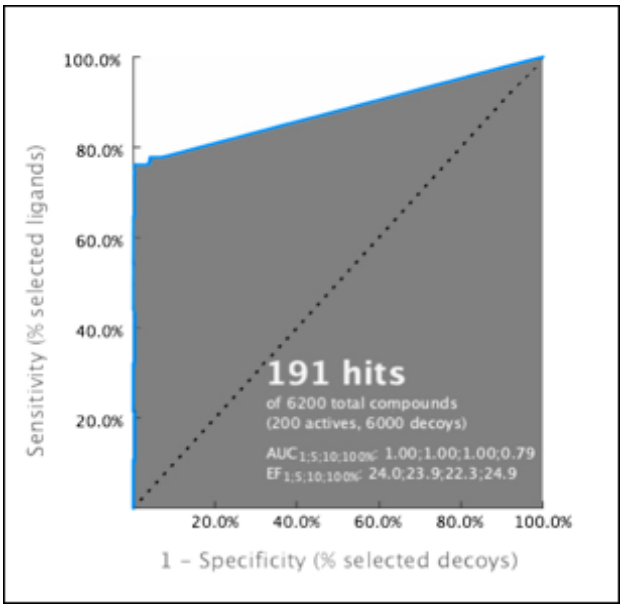


Figure 2. ROC validation curve of the pharmacophore model

Table 3. The pharmacophore fit-score of asam gelugur compounds

No	Molecule Name	Pharmacophore-Fit Score (%)	Docking Score (Kcal/mol)
1	1',1"-dibutyl methyl hydroxycitrate	78,88	-5,60
2	β-caryophyllene alcohol	78,87	-7,10
3	2-(butoxycarbonylmethyl) -3-butoxycarbonyl-2-hydroxy-3-propanolide	78,84	-5,80
4	Malic Acid	78,84	-4,70
5	Citric Acid	76,50	-5,10
6	Tartaric Acid	76,06	-4,70
7	Hydroxycitric Acid	75,91	-5,40

results show enrichment factor ($EF_{100\%}$) is 24.0 and $AUC_{100\%}$ of 1.00 on the Receiver Operating Characteristic (ROC) curve which indicates that the pharmacophores feature model tested is very efficient for the screening process of active compounds. Enrichment factor (EF) and Area Under Curve (AUC) values reflect the ability of mooring to distinguish native ligands in many decoy compounds. The higher the EF and AUC values, the better the performance generated by virtual screening [28].

Pharmacophore modeling can also determine the value of pharmacophore or pharmacophore-fit of the compounds in the fruit of Asam Gelugur as seen in Table 3. The pharmacophore suitability is a percentage measure of the geometric similarity of chemical features compared to the active pharmacophore ligand model oseltamivir.

The results of 3D structure-based pharmacophore modeling showed that 7 of the 13 compounds in the asam gelugur fruit had a high pharmacophore match value (≥ 50%). The compound that has the highest pharmacophore match value is 1 ', 1 "-dibutyl methyl hydroxycitrate followed by β-caryophyllene alcohol, 2-(butoxycarbonylmethyl) -3-butoxycarbonyl-2-hydroxy-3-propanolide, malic acid, citric acid, tartaric acid, and hydroxycitric acid.

Prediction of Absorption and Distribution Profiles, Mutagenicity and Carcinogenicity

From the results of the Lipinski's Rule of Five predictions using Ligandscout, it shows that 11 of the 13 compounds contained in asam gelugur fruit were fulfilled the parameters of

Table 4. Computed properties of asam gelugur compound on Lipinski's Rule of Five

No	Molecule Name	Lipinski's *Rule of Five*			
		Molecular Weight (<500 Da)	Number of Hydrogen Bond Acceptors (<10)	Number of Hydrogen Bond Donors (<5)	LogP (<5)
1	Citric Acid	192,12	7	1	-1,24
2	Tartaric Acid	150,08	9	2	-2,12
3	Malic Acid	150,08	6	2	-2,12
4	Ascorbic Acid	150,08	7	2	-2,12
5	Hydroxycitric Acid	208,12	8	2	-2,12
6	Pentadecanoic Acid	242,40	5	0	3,99
7	Nonadecanoic Acid	298,51	5	0	6,72
8	Dodecanoic Acid	201,31	2	0	3,99
9	2-(butoxycarbonylmethyl) -3-butoxycarbonyl-2-hydroxy-3-propanolide	302,32	4	1	0,72
10	1',1"-dibutyl methyl hydroxycitrate	334,365	5	2	0,32
11	(-)-β-caryophyllene	204,35	0	0	4,72
12	β-caryophyllene alcohol	222,37	1	1	3,75
13	α-humulene	204,35	0	0	5,03

the Lipinski's Rule of Five. Nonadecanoic acid and α-humulene compounds have LogP values above 5 which make them not meet the parameters.

Prediction of compound absorption using HIA (Human Intestinal Absorption) parameters which are divided into 3 categories as follows: poorly absorbed (0-20%), moderately absorbed (20-70%) and well absorbed (70-100%) (Yee, 1997). Based on the prediction results, 7 of 13 compounds namely pentadecanoic acid, nonadecanoic acid, dodecanoic acid, 2- (butoxycarbonylmethyl) -3-butoxycarbonyl-2-hydroxy-3-propanolide, (-) - β-caryophyllene, β-caryophyllene alcohol, and α -humulene has an HIA value of 70-100%. Oseltamivir as control has an HIA value of 87.16%. HIA parameters are an overview of the bioavailability and absorption of drugs or compounds measured from the excretion or cumulative excretion ratio in urine, bile, and feces [29].

The permeability level of the asam gelugur fruit compounds was measured using CCP (Caco-2 Cells Permeability) parameters which were divided into low permeability compounds (<4), medium permeability compounds (4-70), and high permeability compounds (>70) (Yazdanian, 1998). Predicted results indicate that the compounds in the fruit are classified as compounds with moderate permeability (4-70) and low (<4). Oseltamivir as control is classified as moderate permeability with a CCP value of 14.12%. The CCP parameter is an *in vitro* model that is often used to determine the cycle of drug transport through intestinal epithelial cells. Cells used in CCP are colon adenocarcinoma cells, where this model is a model recommended *in vitro* tests to predict oral drug absorption [30].

Bonds of drugs with plasma proteins known as the distribution process are predicted using PPB (Plasma Protein Binding)

Table 5. The prediction result of absorption and distribution profile, mutagenicity and carcinogenicity of asam gelugur compounds

No	Molecule Name	Absorption & Distribution			Benigna/bossa	
		HIA (%)	CCP (%)	PPB (%)	Mutagencity	Carcinogenicity
	Oseltamivir	87,16	14,12	37,83	No Risk	No Risk
1	Citric Acid	11,83	8,67	4,22	No Risk	No Risk
2	Tartaric Acid	18,45	0,81	27,99	No Risk	No Risk
3	Malic Acid	33,62	5.63	62,52	No Risk	No Risk
4	Ascorbic Acid	33,15	2,48	5,30	No Risk	No Risk
5	Hydroxycitric Acid	6,23	3,49	3,68	No Risk	No Risk
6	Pentadecanoic Acid	98,11	25,04	100	No Risk	No Risk
7	Nonadecanoic Acid	98,44	29,46	100	No Risk	No Risk
8	Dodecanoic Acid	97,48	23,16	100	No Risk	No Risk
9	2-(butoxycarbonylmethyl) - 3-butoxycarbonyl-2-hydroxy-3-propanolide	76,01	2,78	79,72	No Risk	No Risk
10	1',1"-dibutyl methyl hydroxycitrate	53,06	2,85	70,12	No Risk	No Risk
11	(-)-β-caryophyllene	100	23,63	100	No Risk	No Risk
12	β-caryophyllene alcohol	100	55,45	100	No Risk	No Risk
13	α-humulene	100	24,53	100	No Risk	No Risk

HIA: *Human Intestinal Absorption*; CCP: *Caco-2 Cells Permeability*; PPB: *Plasma Protein Binding*.

parameters. PPB values can be classified into compounds that are strongly bound to plasma proteins (> 90%) and weakly bound compounds with plasma proteins (<90%). Based on the results of predictions, 7 of 13 has a weak protein bond. Oseltamivir also has a weak plasma protein bond value of 37.83%. In general, only free drugs or drugs that do not bind to plasma proteins can diffuse and penetrate membranes and bind to their pharmacological targets. Therefore, the number of plasma protein bonds from compounds or drugs can affect action, disposition, and efficacy [31].

In predicting the toxicity test, Benigni/ Bossa parameters were used to determine the mutagenicity and carcinogenicity of the asam gelugur fruit compounds. Based on the results of the prediction, all the compounds are thought to be not mutagenic and carcinogenic. This indicates that the structure of the compounds in the asam gelugur fruit is safe and not reactive which can cause damage to the DNA level [32]. Hydroxycitric acid which is the main component of asam gelugur fruit does not cause mutagenic activity in 5 strains of S. typhimurium bacteria based on *in vitro* (Ames test) with a dose of 0, 20,100, 500, 2500, 12500 µM/plate [34,35]

4. Conclusions

Interaction of bond mode formed between 13 asam gelugur fruit compounds with active influenza neuraminidase receptor sacs occurred through hydrogen bonds (Arg119, Arg294, Arg372, Asp152, Tyr406,

Glu229, and Glu279) and hydrophobic bonds (Ala248, Ile224, Ile429, Trp180, Leu135, and Tyr406). Hydroxycitric acid is a compound that is predicted to have the best inhibitory activity with the lowest Ki value of 1.09 µM. Hydroxycitric acid meets the Lipinski's Rule of Five parameters, with medium category of absorption and distribution profile and is predicted to be safe based on in silico mutagenicity and carcinogenicity parameters.

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