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IN SILICO EXPLORATION OF PHOSPHOLIPASE A_2 INHIBITOR COMPOUNDS FROM *Lufariella variabilis* AS ANTIVENOM OF *Ophiophagus hannah*

EKSPLORASI SENYAWA INHIBITOR FOSFOLIPASE A_2 DARI Lufariella variabilis SEBAGAI ANTIVENOM Ophiophagus hannah SECARA IN SILICO

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ABSTRACT

The King Cobra (Ophiophagus hannah) is a venomous snake found in Southeast Asia and South Asia. Globally, it is estimated that there are 81,000 to 138,000 cases of snakebite deaths from 1.8 million to 2 million snakebite cases. The limited availability of antivenom is a problem in handling snake venom poisoning. Exploration of natural ingredients is needed as a preventive measurement from the spread of toxins when they are inside the body. Exploration could be carried out by utilizing natural metabolite compounds that can be inhibitors of the phospholipase A_2 (PLA_2) enzyme. Luffariella variabilis is known as a marine organism that can produce sesterterpenoid compounds and has the potential as an inhibitor of the phospholipase A_2 enzyme. This study aims to explore the potential of sesterterpenoid compounds produced by Luffariella variabilis as an in silico inhibitor of phospholipase A_2. Several methods used in this research are molecular docking simulation, toxicity test using pkCSM and Toxtree, and chemical bond analysis using Discovery Studio. The results showed that the manoalide compound was the most potent compound of the other sesterterpenoid compounds in its ability to become a snake antivenom candidate.

Keywords: *King Cobra, Lufariella variabilis, Phospholipase A_2*

ABSTRAK

King Cobra (Ophiophagus hannah) merupakan salah satu ular berbisa yang ditemukan di Asia Tenggara dan Asia Selatan. Kasus kematian akibat gigitan ular secara global diestimasikan sekitar 81.000 hingga 138.000 kasus dari 1,8 juta hingga 2 juta kasus gigitan ular. Ketersediaan antivenom yang terbatas menjadi permasalahan penanganan dalam keracunan bisa ular. Eksplorasi bahan alam diperlukan sebagai tindakan preventif dari menyebarnya racun ketika berada dalam tubuh. Eksplorasi dapat dilakukan dengan memanfaatkan senyawa metabolit alami yang dapat menjadi inhibitor terhadap enzim fosfolipase A_2 (PLA_2). Luffariella variabilis diketahui sebagai organisme laut yang memiliki kemampuan menghasilkan senyawa golongan sesterterpenoid serta berpotensi sebagai inhibitor enzim fosfolipase A_2. Penelitian ini bertujuan untuk mengeksplorasi potensi senyawa sesterterpenoid yang dihasilkan oleh Luffariella variabilis sebagai inhibitor fosfolipase A_2 secara in silico. Beberapa metode yang dilakukan dalam penelitian ini adalah simulasi penambatan molekuler (molecular docking), uji toksisitas dengan pkCSM dan Toxtree serta analisis ikatan kimia menggunakan Discovery Studio. Hasil penelitian menunjukan senyawa manoalide adalah senyawa yang paling potensial dari pada senyawa sesterterpenoid lainnya dalam kemampuanya menjadi kandidat antivenom ular.

Kata kunci: *fosfolipase A_2, King Cobra, Lufariella variabilis*

INTRODUCTION

Snakes are reptiles whose existence is feared by humans. Many species of snakes are known to be able to attack humans with venomous bites that have the potential to cause some damage, such as bleeding, impaired kidney function, and local tissue damage [1]. Global snakebite deaths are estimated to be around 81,000 to 138,000 cases, from 1.8 to 2 million snakebite cases [2]. In Indonesia, snakebite cases were estimated in 2007 as many as 12,739 – 214,883 cases with 2000 – 11,581 deaths [3]. Snakebite sufferers experience high morbidity and mortality due to inadequate access to health services and a low supply of antivenom, which is the only specific therapy [4]. In addition, the price of antivenom is very expensive, so that ordinary people tend to underestimate the case of snake bites and choose to use local medicines that have not been proven to be effective in healing snake bites. One of the snakes that have a venomous bite is the King Cobra (*Ophiophagus hannah*). King Cobra is known as a species of snake that spreads in South Asia and Southeast Asia [5].

King Cobra venom has been studied since the early 1970s. Various components identified in the snake venom are L-amino acid oxidase enzymes,

metalloproteinases, Three Finger Toxins (3FTxs), and phospholipase A_2 (PLA_2) [6]. A study of the genome of the King Cobra from Indonesia (Bali Specimen) indicated the presence of phospholipase A_2 in the snake's venom [7].

PLA_2 enzyme is a multi-toxic enzyme that systematically induces various pathophysiological changes in snakebite sufferers. PLA_2 with Asp49 amino acid on the active site will work in hydrolyzing ester bonds in phospholipids found in plasma membranes of various cells, including erythrocytes, to induce plasma membrane lesions and hemolysis [8]. In addition, these compounds can damage mitochondria, erythrocytes, leukocytes, platelets, peripheral nerves, striated muscles, endothelium of blood vessels, and cause the release of histamine and anticoagulants [9].

Phospholipase A_2 (PLA_2) enzyme isolated from snake venom showed inflammatory symptoms such as acute pain, edema, hypotension, bleeding until the termination of Neuromuscular Junction (NMJ) blockade [10]. The PLA_2 enzyme has the potential to be an inhibitory target that can be used in exploring metabolites found in nature that have potential as antivenom. The content of PLA2 in snake venom can be neutralized with natural inhibitors derived from marine organisms, which are antivenom reservoirs. One of the marine organisms that have the potential to produce snake venom neutralizing compounds is *Luffariella variabilis*.

Luffariella variabilis is a species of marine sponge that produces metabolites that have the potential as antivenom. In addition, this sponge contains compounds that have anti-inflammatory properties. Several sesterterpenoid compounds were found in *L. variabilis*, including manoalide, secomanoalide, luffariellolide, luffariellin A and B and luffolide [11]. A study showed that manoalide isolated from *L. variabilis* could inhibit the extracellular PLA_2 activity of cobras and rattlesnakes with IC50 values of 1.9 and 0.7 M, respectively [10].

An in silico exploration approach using the software can be an effective approach as an initial preparation before the in vivo testing phase in the laboratory. In addition, the use of the in silico method can minimize the risks that will occur when testing in the laboratory.

Exploration of compounds from these sponges is a major concern for researchers in realizing the use and conservation of marine sponge diversity in Indonesia. Therefore, this study aims to explore potential compounds from *Luffariella variabilis* which can be inhibitors of the PLA_2 enzyme in silico.

METHODS

Preparation of phospolipase A_2 enzyme 3D structure. Acidic Phospholipase A_2 from the venom of *Ophiophagus hannah* (code 1GP7) was downloaded from *RCSB Protein Data Bank* (*www.rcsb.org*) in pdb format. The 3D structure was opened by using *PyMol*. Water molecule as residue in the enzyme was removed and also saved in pdb format.

Ligand preparation. The compounds produced by *Luffariella variabilis* were used as ligands in inhibiting the action of the phospholipase A_2 enzyme. Ligands, which were metabolites produced by these species, were available on the PubChem website (*www.pubchem.ncbi.nlm.nih.gov*). The structure of the ligands was downloaded in sdf format. Some of the ligands downloaded on the Pubchem website were Luffariellolide (5387248), Luffolide (3081204), Luffariellin A (6091135), Luffariellin B (6391100), Manoalide (5942250), Secomanoalide (5387263). The selection of the sesterterpenoid compounds from *L. variabilis* was based on data obtained from Folmer [10]. In addition, the native ligand used as a comparison is Vitamin E (14985) [12]. Vitamin E had known as an effective inhibitor for the PLA_2 enzyme [13]. Each compounds further information could be seen in Table 1. The ligands were opened using the PyMol software and saved in pdb format.

Molecular docking simulation. Molecular docking simulations were performed using PyRx. The simulation aimed to determine affinity energy between a ligand and a protein, showing the strength of the bond between the two molecules through a calculation. The selected tab in PyRx is Vina Wizard. The simulation was begun by adding a macromolecule in the form of a protein pdb file for the phospholipase A_2 enzyme. The simulation was continued by adding ligands in pdb format. The macromolecule and one of the ligands were blocked, then the forward button was clicked. After that, the entire surface of the protein was tested by selecting maximize. The forward button was clicked again. The docking process ran until it brought up an affinity energy number from the docking simulation between the protein and the ligand. The docking simulation was repeated for the next ligands. All docking simulation results between all ligands and proteins in the form of affinity values are arranged in tabular form.

Molecular docking result analysis. All molecular docking simulation results between all ligands and proteins in the form of affinity values were arranged in tabular form. The analysis was carried out by determining the ligand that had the most negative affinity value. The most negative affinity value indicated the strongest ligand that

Table 1. Ligand name from *Lufariella variabilis* based on PubChem website

Compound Name	IUPAC Name	Compound ID
Manoalide	2-hydroxy-3-[6-hydroxy-5-[(E)-4-methyl-6-(2,6,6-trimethylcyclohexen-1-yl)hex-3-enyl]-3,6-dihydro-2H-pyran-2-yl]-2H-furan-5-one	5942250
Secomanoalide	(E,2E)-2-[3-hydroxy-3-(2-hydroxy-5-oxo-2H-furan-3-yl)propylidene]-6-methyl-8-(2,6,6-trimethylcyclohexen-1-yl)oct-5-enal	5387263
Luffariellolide	3-[(3E,7E)-4,8-dimethyl-10-(2,6,6-trimethylcyclohexen-1-yl)deca-3,7-dienyl]-2-hydroxy-2H-furan-5-one	5387248
Luffariellin A	2-hydroxy-3-[6-hydroxy-5-[(E)-4-methyl-6-(2-methyl-1-prop-1-en-2-ylcyclopentyl)hex-3-enyl]-3,6-dihydro-2H-pyran-2-yl]-2H-furan-5-one	6091135
Luffariellin B	(E,2Z)-2-[3-hydroxy-3-(2-hydroxy-5-oxo-2H-furan-3-yl)propylidene]-6-methyl-8-(2-methyl-1-prop-1-en-2-ylcyclopentyl)oct-5-enal	6391100
Luffolide	[(1R)-2-[(1R,2S,4aS,4bS,8aS,10aS)-2-formyl-4b,8,8,10a-tetramethyl-2,3,4,4a,5,6,7,8a,9,10-decahydro-1H-phenanthren-1-yl]-1-[(2R)-2-hydroxy-5-oxo-2H-furan-3-yl]ethyl] acetate	3081204
Vitamin E	(2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol	14985

bonds with the protein. The binding affinity between ligand and protein was referred to as Gibbs Free Energy (ΔG) [14]. Gibbs free energy was the amount of energy used to carry out the chemical reaction. The bond interaction between protein and ligand was stable if the Gibbs free energy value (ΔG) was negative [15].

Enzyme and ligand complex visualization. Visualization of the interaction between the ligand and the enzyme after the enzyme-ligand complex was performed using *PyMol* software for visualization in three-dimensional (3D) and software for visualization in two-dimensional (2D) using *BIOVIA Discovery Studio*. This interaction analysis aimed to understand the structure and binding of ligands to proteins.

Pharmacology characteristics prediction. Prediction of pharmacological properties was carried out to determine the bioavailability of a compound to become a drug. Pharmacological prediction used Lipinski's five rules. Analysis of pharmacological properties was carried out using *SCFBio* (*http://www.scfbio-iitd.res.in/*). The analysis began by entering the pdb file of a ligand then clicking the submit button. The results of the analysis of the pharmacological properties of several ligands were arranged in tabular form.

Toxicity test. Toxicity tests were carried out by using Toxtree and pkCSM (*www.biosig.unimelb.edu.au/pkcsm/prediction*).

RESULTS AND DISCUSSION

Results of binding affinity values from various ligands to PLA_2 enzyme after molecular docking simulation. Molecular docking simulation was a method used to determine the conformation of the interaction of the test compound on the active site of the target protein receptors and which test compound had the best affinity values for the target protein receptor [16]. The best ligand parameter obtained after molecular docking simulation is shown by the binding affinity results. According to Muttaqin [16], binding affinity was an important aspect that must be considered in the interaction between ligands and receptors. A lower binding affinity value indicated that a compound required less energy to bind or interact with the receptor. In other words, a lower binding affinity value had a greater potential to interact with the target protein [17]. The PLA_2 enzyme produced by the King Cobra snake is classified as Secreted PLA_2 Enzymes. Secreted PLA_2 had the characteristics of low molecular weight, had six disulfides bonds, required histidine on the active site, and required Ca^{2+} as a cofactor [18]. Thus, the enzyme used was classified as a holoenzyme.

The results were obtained from the binding affinity value of molecular docking simulation using PyRx with phospholipase A_2 enzyme as a macromolecule and metabolites that came from of Five. There were five things in Lipinski's rule, namely, the molecular weight was less than 500 Da, the LogP value was less than 5, the donor hydrogen bond was less than 5. The acceptor hydrogen bond was less than 10, and the molar refractivity was between 40-130 [21, 22]. The binding affinity value used was at RMSD 0.0 Å. Susanti and colleagues stated that the molecular docking method was valid if it had an RMSD value ≤ 3 Å [19].

Based on Table 2, Manoalide had the lowest binding affinity value of -9.2, followed by Secomanoalide with a binding affinity value of -8.9. It could be concluded that Manoalide is the most potent compound as an inhibitor of the phospholipase A_2 enzyme by binding affinity test. This was consistent with a study conducted by

Table 2. Binding affinity value from various ligand compounds to PLA_2

Ligand	Binding Affinity	RMSD/ub	RMSD/lb
Luffariellin A	-8.8	0	0
Luffariellin B	-8	0	0
Manoalide	-9.2	0	0
Secomanoalide	-8.9	0	0
Luffariellolide	-8.7	0	0
Luffolide	-8.7	0	0
Vitamin E (*Native*)	-8.2	0	0

Yarla and colleagues in which phospholipase A_2 (PLA_2) was a group of enzymes that break down phospholipids specifically at the sn-2 position to release free fatty acids, mostly arachidonic acid (AA) and lysophospholipids (LPL). PLA_2 inhibition prevented the release of AA and LPL. Therefore, the researchers had considered that PLA_2 could be a better therapeutic target than the downstream enzymes of cyclooxygenase and lipoxygenase [20]. The study also attached a table of various compounds capable of being inhibitors of the PLA_2 group, one of them was Manoalide that came from *Luffariella variabilis*. The second-order after Manoalide was Luffarielline A, which has a binding affinity value of -8.8, followed by Luffariellolide and Luffolide with a binding affinity value of -8.7.

Pharmacological test results. The search for drug candidates was carried out by in silico drug feasibility testing using the "Lipinski Rule of Five" principle. Planning an oral drug follows Lipinski's rules. The molecular weight of a compound was related to the permeability of a drug in the body. The increase in molecular weight could be correlated with a decrease in the permeability of a compound in the digestive and central nervous systems. The decrease in the rate of permeability of a compound to the lipid bilayer could also be associated with an increase in the molecular weight of a compound so that compounds with higher molecular weights tend to be orally inactive compared to compounds with lower molecular weights [23].

Log P was expressed as the logarithm of the ratio of the drug that was divided into the organic phase with that in the aqueous phase or commonly referred to as lipophilicity. Lipophilicity was described as the ability of a molecule to partition with octanol or water. Lipophilicity was a chemical property that was generally considered to be very relevant to the absorption rate [24]. As many as 90% of compounds showing a Log P value of less than 5 had bioavailability for oral consumption [23].

The hydrogen bonding of donors that was more than the standard set by Lipinski's rule can reduce the ability of a molecule to penetrate the multiple membrane layers (membrane bilayer). A particular compound that exhibits a large number of donor hydrogen bonds tended to partition with a solvent with strong hydrogen bonds such as water rather than the lipophilic environment generally found in cell membranes. A large number of acceptor hydrogen bonds could also reduce the ability of a molecule to penetrate the lipophilic environment because it tends to interact with water [23]. Lipinski and other researchers [25] found that adding up the nitrogen and oxygen atoms in a molecule can provide a good representation of the correlation to the bioavailability of these compounds as drug candidates.

Based on Lipinski's rule of the five compounds (Table 3), only Secomanolide did not meet the qualifying standard. Secomanoalide had a molecular mass, hydrogen bond donor, and acceptor that exceeded the limit. The same applies to the level of high lipophilicity, and molar refractivity exceeded the limit as well. Manolide, which was based on the value of binding affinity as the most potent compound, had met the requirements of the five Lipinski rules for drug candidates. Likewise with Luffarielline A was the second most potent candidate compound as the same antivenom. The Lipinski rule was used as a consideration of whether the drug candidate can be used orally or not.

Table 3. Pharmacological tests of compounds based on Lipinski's Rule

Ligand	Molecular Weight	H Donor	H Acceptor	Lipophilicity	Molar Refractivity
Luffariellin A	416	2	5	4.710900	116.248566
Luffariellin B	416	2	5	4.553499	117.439568
Luffariellolide	386	1	3	5.686361	124.420761
Luffolide	460	1	6	4.583799	122.690758
Manoalide	416	2	5	4.855000	116.318558
Secomanoalide	1448	12	24	7.247049	343.823456
Vitamin E (*native*)	430	1	2	8.840264	134.390778

Drug molecules that did not comply with Lipinski's regulations were not recommended for use orally but were recommended for injection, and zopolrestat was a commercial drug used as a comparison ligand was a drug that was not used orally [26].

Toxicological analysis of compounds of *Luffariella variabilis*. Based on Table 4, the results showed that manoalide, which was the best compound in the previous test, had carcinogenicity values of 8 and 9, which means its chemical ability was negative to cause mutations in harmful genes. Based on ISS for in vitro test against *Salmonella typhimurium* bacteria, Manoalide compound had a value of 2 in Table 4,which means that there was no indication of mutagenicity of *S. typhimurium* bacteria. To test the corrosive/irritating properties of the skin, all compounds were safe because they had point 1, which was non-corrosive to the skin. Even in vivo studies with the micronucleus in rodents such as mice/guinea pigs also showed the number 1, which means at least there was a positive warning for the micronucleus assay.

According to Barbezan et al. [27], the mutagenicity test of a compound administered to the bacterium *S. typhimurium* was based on the knowledge that mutagenic substances in bacteria might subsequently be carcinogenic in laboratory animal tests and present a cancer risk to humans.

Based on the test with pkCSM (Table 5) for Manoalide and Luffarielline A compounds, both had their respective advantages and disadvantages. For the AMES test, Manoalide was positive but Luffarielline A was negative. The AMES test played an important role in drug safety. As explained by Kesuma et al. [28], determining the toxicity of compounds could be done with the AMES Toxicity test. AMES Toxicity Test was a widely used method to assess the mutagenic potential of compounds using bacteria. A positive test result indicated that the compound was mutagenic and therefore could act as a carcinogen.

It was different with the hepatotoxicity test, where Manoalide had a negative result while Luffarielline A had a positive result. If there were toxic compounds in liver cells, there would be a change in the permeability of the cell membrane. So that the enzymes that should be in the cell finally came out of the cell and were in the blood [29].

The two potential compound candidates as antivenom did not meet the required criteria. However, the Luffolide compound had the same binding affinity value as Luffariellolide, which was -8.7. In Luffolide, the AMES Toxicity and Hepatotoxicity Test on pkCSM was negative. So, this compound was more potential to be used as a drug candidate compound than Manoalide and Luffarielline A when viewed from the results of pkCSM. Based on the exploration of candidate antivenom compounds, both Luffarielline A and Manoalide had advantages and disadvantages. Likewise, Luffolide even though in the AMES test, hepatotoxicity and the response to allergies was negative/none, but in terms of the maximum dose in the human body based on the test with pkCSM, the number showed below 0 or negative, namely -0.25 as well as in Luffarielline A the result was -1.01.

Table 4. Toxicity test by Toxtree

Decision Tree	Luffarielin A	Luffarielin B	Luffariellolide	Manoalide	Secomanoalide
Carcinogenicity & Mutagenicity based on ISS	8,9	1,4,9	8,9	8,9	1,3,9
AMES Test	2	1,4	2	2	1,4
Skin irritation/skin corrosion	1	1	1	1	1
Micronucleus assay	1	1	1	1	1

Table 5. Toxicological test by pkCSM

Parameters	Manoalide	Luffarielin A	Luffolide
AMES Toxicity	Yes	No	No
Maximum dose (Human)	0,878	-1,01	-0,25
h ERG I Inhibitor	No	No	No
h ERG II Inhibitor	No	No	No
LD50	2,482	2,222	2,515
LOAEL	0,172	1,896	0,092
Hepatotoxicity	No	Yes	No
Skin Sensitivity	No	No	No
Toxic to *T. pyriformis*	0,285	0,33	0,312
Minnow Toxicity	3,707	-0,135	-1,617

Visualization of the interaction between ligands and PLA$_2$. Interaction visualization was done by looking at the binding site representing the location of amino acid residues in forming various interactions with ligands [30] (Figure 1). Manoalide had a bond length of 0.356 nm with the carbon-hydrogen bond with CYS29. Luffariellin A had a bond length of 0.258 nm with the type of bond being donor-donor with GLY30, and for Luffolide itself, it had a bond length of 0.196 nm, with the type of bond being conventional hydrogen bond with ASP49 (Figure 1 and 2). Based on length bond visualization, the most potential result as an antivenom candidate was Luffolide which had the shortest bond distance. The shorter the bond distance indicated that the intermolecular bonds were getting stronger. As explained by Kilo [31] that because the distance between the ions of a small radius was shorter, the electrostatic attraction between the ions was stronger.

Based on these results, the most potent antivenom compound was considered and selected were Manoalide and Luffolide. Manoalide was selected with the highest consideration in terms of binding affinity value, passing the Lipinski and Toxtree rules criteria. But, the effect of the AMES test on testing with pkCSM was considered a side effect and was also dosed in the safest dose. When viewed from the results of 2D analysis, Luffolide itself had the shortest length bond with the PLA$_2$.

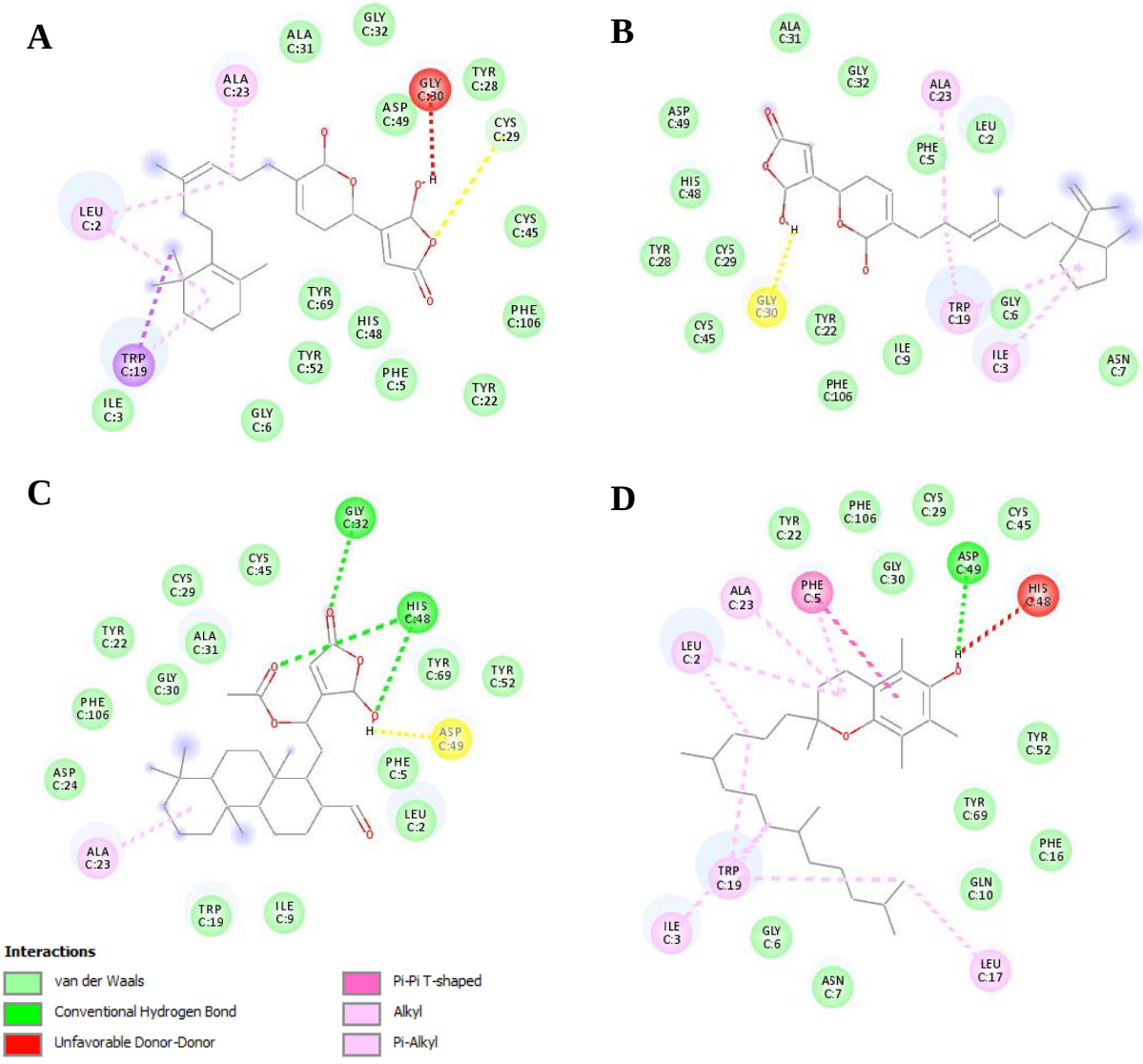

Figure 1. 2D Visualization of Interaction between PLA$_2$ with Several Ligand (a) Manoalide (b) Luffariellin A (c) Luffolide (d) Vitamin E. The yellow residue indicated the location of interaction between ligand with the PLA$_2$

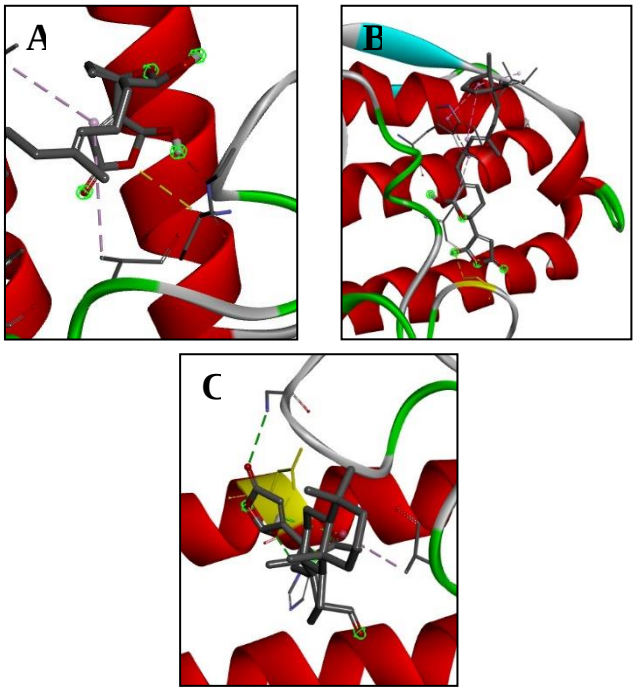

Figure 2. Bond length interaction between PLA_2 with several ligand (a) Manoalide (b) Luffariellin A (c) Luffolide. The yellow residue indicated the location of interaction between ligand with the PLA_2

CONCLUSION

Based on the results of molecular docking, Lipinski's five rules, 2D molecular interaction test, and toxicity test, it was found that Manoalide was the most potent compound to be used as an antivenom against cobra (*Ophiophagus hannah*) because it had the highest binding affinity value, passed the five Lipinski rules test, passed the test Toxtree toxicity. However, the Manoalide compound in the pkCSM toxicity test got a positive result against the AMES test, so it was considered ineligible and had the longest bond with the PLA_2.

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