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ACTIVE COMPOUND OF RED BETEL (Piper crocatum Ruiz & Pav) AS ACTIVATOR of Cu/Zn SUPEROXIDE DISMUTASE IN SILICO

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ABSTRACT

Cancer degenerative disease is a serious problem in Indonesia, caused by free radicals, and can be prevented by antioxidant compounds. Superoxide dismutase (SOD) is the strongest enzyme that acts as a protective organism against oxidative stress. The active compound of red betel can be studied in silico as an activator of the SOD enzyme. Molecular docking visualization, physicochemistry, toxicity of red betel active compounds were studied and continued with molecular docking. The studied parameters were Gibbs free energy (ΔG) and receptor-ligand chemical bonding analysis. The test results show that the active compounds of red betel 6-Amino-2-(3-aminopropylamino)-9-(cyclohexylmethyl)- 2,3,4,5,6,7-hexahydro-1H-purin-8-one was well-absorbed dan safe for consumption with Gibbs free energy value of -9.1490 kcal/mol. Hydrogen and hydrophobic interactions of these compounds were similar to the control ligand, namely β-amyrin and Trehalose. This study concluded that the active compound of red betel 6-Amino-2-(3-aminopropylamino)-9-(cyclohexylmethyl)- 2,3,4,5,6,7-hexahydro-1H-purin-8-one has the best potential to increase the work of Cu/Zn-SOD enzymes. This compound also meets the requirements of Lipinski regulations so that it can be well absorbed orally by the body and is not carcinogenic.

1. INTRODUCTION

Cancer is a disease that ranks second as the leading cause of death worldwide. Based on data from the Global Cancer Observatory (GCO), there were 19.3 million new cases of cancer (GLOBOCAN, 2020). Cancer is a non-infectious disease usually caused by oxidative stress as an effect of free radical excess in almost all body parts that can cause cancer. Therefore it is necessary to increase the enzyme SOD before free radicals enter the mitochondria. This SOD enzyme is in the cytoplasm and is the main enzyme in preventing the formation of free radicals. Free radicals are reactive compounds because they have one or more unpaired electrons in their outer orbits, so they tend to be unstable. Therefore, compounds that can stabilize free radicals are needed, namely antioxidants.

Antioxidants are compounds that can neutralize or stabilize free radicals by completing the electron deficiency in free radicals. These antioxidant compounds can donate electrons to free radicals so that free radical molecules become unreactive and stable (Mokoginta, 2013). Based on the source, antioxidants can come from within the body (endogenous) and outside the body (exogenous). Endogenous antioxidants can overcome oxidative damage and are caused by free radicals in the body and catalase enzymes, glutathione peroxidase, and superoxide dismutase (SOD) (Murray et al., 2014). Superoxide dismutase (SOD) is the strongest enzyme in the body. Also, an enzymatic antioxidant that can catalyze superoxide into hydrogen peroxide. SOD is classified into three forms based on the metal ions usually bound, namely (i) Fe-SOD, (ii) Mn-SOD, dan (iii) Cu/Zn-SOD. CU/Zn-SOD is the dominant enzyme localized in the cytosol, chloroplasts, and peroxisomes (Ighodaro & Akinloye, 2018).

Red betel (*Piper crocatum Ruiz & Pav*) is one of the medicinal plants that has previously been studied and shows that it contains flavonoid compounds, alkaloids, and tannins (Safithri & Kurniawati, 2016). In vitro and in vivo studies on rats explain that red betel leaf extract can reduce diabetes complications because it acts as an activator of the SOD enzyme, which plays a critical role in reducing superoxide anion radicals produced in the body of people with diabetes (Safithri et al., 2012). According to Zaelani et al. (2021), molecular binding of red betel bioactive compound with HMG-CoA reductase receptor, atorvastatin control ligand. It showed that catechins, schisandrin B, and CHEMBL216163 had the highest inhibitory power with affinity energies of -7.9 kcal/mol and -8.2 kcal/mol, respectively -8.3 kcal/mol with the amino acid residues involved are Ser684, Asp690, Lys691, Lys692. Red betel from Palu is a powerful antioxidant with an IC50 value of 47.46 ppm using the DPPH method (Tonahi et al., 2014).

The antioxidant activity of red betel has been tested previously, but the active compounds that play a role are unknown. Therefore, it is necessary to identify the active compound of red betel that plays an active role in activating the superoxide dismutase enzyme using the in silico method. This research uses three methods: in silico, in vitro, and in vivo. In silico is a method that should be used before carrying out the other two methods because this in silico method does not involve living organisms. One of these in silico methods can be done by the docking method, which is a computational method to see whether a compound is toxic or not (Agistia et al., 2013). According to Gaba et al. (2010), molecular docking can predict the activity and ability of a compound as a drug. Docking also predicts the compound's active site and can determine whether the compound is toxic and a carcinogen by computation. This study aims to determine the active compound of red betel that has the potential to increase the activity of the superoxide dismutase enzyme.

2. METHODS

This research uses PubChem, YASARA Structure, PyMol, LigPlot+ (1.5.4), Discovery Studio Visualizer, RSCB, SMILES, PyRx, AdmetSAR, PROCHECK, and PDB. The material used in this study was the chemical structure of 69 red betel compounds obtained from PubChem. The chemical structure of superoxide dismutase enzyme with PDB ID 1CB4. Also, the chemical structure of β-amyrin and trehalose ligands as comparison ligands. The test and comparison ligands were prepared by optimizing their geometry using the energy minimization method in the em_runclean.mcr program on the YASARA Structure software. The energy minimization produces the minimum interatomic energy values.

Moreover, it forms a more stable and optimal molecular conformation. Receptor preparation is carried out using the same software as the docking process, which can remove water molecules, negative ligands, or residues. Then hydrogen is added. The prepared target protein is saved in PDB format.

Validation is done using YASARA Structure with a targeted docking technique. The ligand docking zone is bounded by a grid box around the active side of the Cu/Zn-SOD protein. Gridbox validation is done by determining the best grid box size and getting the RMSD (root mean square distance) value. Virtual scanning was carried out on the comparison ligand and 50 test ligands that had passed the physicochemical and ligand toxicity tests based on the screening results at the Lipinski and AdmetSAR sites. The test and comparison ligands were saved in PDB format, and their binding affinity energy was analyzed using pyRx. Prediction results are selected based on affinity and Gibbs free energy (ΔG).

Molecular docking of the test and comparison ligands was carried out using the optimum grid box obtained at the grid box validation stage. The receptor preparation file is opened on the YASARA Structure software in the '_receptor.sce' format. All test ligands and comparison ligands are opened individually in PDB format. Then the file was prepared by forming a ligand-receptor complex based on a predetermined grid box, then saved in the '_complex.sce' format. This step is carried out like the grid box validation step. Docking results can be read on the notepad software in '.log' format. Other docking results are binding energy values stored in '.txt' format and non-covalent interactions of ligand-receptor complexes stored in '.yob' format.

The analysis of molecular docking results can be determined by looking at the Gibbs free energy (ΔG^o/binding affinity). The Gibbs free energy value was used to analyze the binding affinity between the receptor and the ligand. The analysis of further molecular docking is in the form of two- and three-dimensional visualization of the ligand-receptor complex. The visualization in the form of analysis of hydrogen bonds, hydrophobic interactions, and bond distances was carried out using LigPlot+ (2D) software and Discovery Studio Visualizer (3D). Visualization begins by converting the molecular docking result file into a PDB file in '.yob' format. The test ligand in PDB format is then opened in the software used to view their interactions; the results will be visualized in 2D and 3D.

3. RESULTS AND DISCUSSION

The test ligands used in this study are a total of 69 compound ligands of the active compound of red betel and two comparison ligands, β-Amyrin, and Trehalose. The first test was carried out by looking at the structure and stability of the receptor used. A physicochemical test of 71 ligands was carried out, and seven were eliminated. The ligand toxicity test was carried out with the remaining 64 ligands, and 14 ligands were declared eliminated so that the remaining 50 ligands continued to the next test stage. In virtual screening, 50 ligands produced five red betel compounds with the best Gibbs free energy values. The five ligands proceed to the final test, molecular docking with β-Amyrin and

Trehalose as comparisons. The molecular docking result showed that one ligand has the most potential to increase the Cu/Zn-SOD enzyme.

3.1. Results

Receptor stability analysis was carried out using PROCHECK, which produces output from a Ramachandran plot diagram and plot statistic. The Ramachandran plot diagram contains four different quadrants with different colors showing the distribution of residues: the most favored region, additionally allowed region, generously allowed region, and disallowed region. The Cu/Zn-SOD receptor analysis shows that amino acid residues occupy 210 preferred area residues (89%). The amino acid residue in the additional region is preferably 24 residues (10.2%). Residues located in the area of consideration are two residues (0.8%), and there are no residues in areas that are not permitted (Figure 1). Ramachandran plot is used to see the distribution of amino acids and what percentage of the preferred and disliked areas.

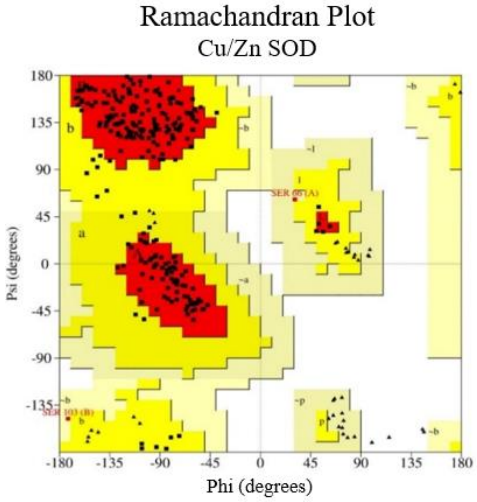


Figure 1 Ramachandran Plot of Cu/Zn Superoxide Dismutase

Table 1 shows Lipinski's rules; test ligands and comparison ligands that will be converted into drugs must pass physicochemical tests to determine certain levels of solubility and permeability, which can be carried out by referring to Lipinski's rules. Based on the Lipinski parameters, the test and comparison ligands must pass three or more of these rules. The result of the physicochemical test obtained seven test ligands that did not meet the three to four available rules, so they could not proceed to the next stage and could not be used as drug candidates.

Table 1 Ligand's Physicochemistry

Ligands	Molecular Mass (Da)	Hydrogen Bond Donors	Hydrogen Bond Acceptors	LogP	Molar refractivity
9985480	570	2	10	2.6818	143.3291
SCHEMBL6997934	578	10	17	0.9754	143.8961
LMPK12050438	698	8	18	-1.954	160.7210
CHEMBL2398181	599	4	6	6.3839	172.3723
ZINC103074353	610	0	10	6.4478	163.0609

N-(hexadecanoyl)-sphinganine	539	3	3	9.4710	191.1987
131733858	958	5	11	5.0243	240.5657

Description: Values do not meet Lipinski's rules

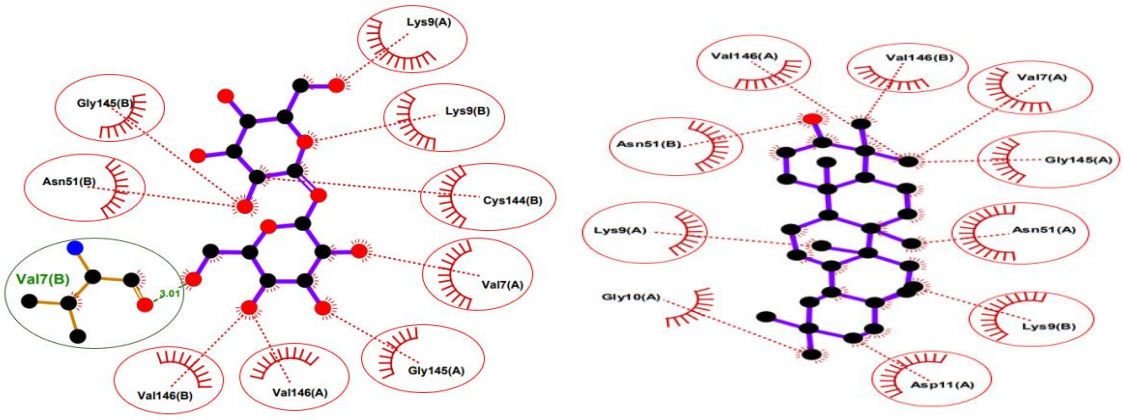
Ligands that meet the requirement of Lipinski's rule then proceed with a toxicity test using three parameters. The results of the ligand toxicity test showed that 14 ligands did not pass the test. Ligands that did not pass are classified as carcinogenic, strong inhibitory, and acute oral toxicity, falling into categories I and II. Test ligands declared carcinogenic and fell into acute oral categories I and II are declared toxic and cannot be used as drugs (Table 2).

Table 2 Ligan's Toxicity

Ligands	Inhibition of *Human Ether-A-Go-Go Related Gene* (herG)	Carcinogenicity	Acute Oral Toxicity
Furaldehyde	Weak inhibitor	Non-carcinogenic	II
4-Isoprophiltoluena	Weak inhibitor	Carcinogenic	III
CHEMBL350028	Weak inhibitor	Carcinogenic	III
Thiosulfuric acid	Weak inhibitor	Carcinogenic	III
Chavicol	Weak inhibitor	Non-carcinogenic	II
N-methyl octadecyl amine	Weak inhibitor	Non-carcinogenic	II
Tinosporin	Weak inhibitor	Non-carcinogenic	I
Allylpyrocatechol	Weak inhibitor	Non-carcinogenic	II
4202426	Weak inhibitor	Non-carcinogenic	I
Trans-beta-farnesene	Weak inhibitor	Carcinogenic	III
Z-11-Tetradecen-1-OI trifluoroacetate	Weak inhibitor	Carcinogenic	III
10104370	Weak inhibitor	Non-karsinogenik	II
SCHEMBL10902683	Weak inhibitor	Non-carcinogenic	II
CHEMBL3311447	Strong inhibitor	Non-carcinogenic	III

Description: Values do not meet ligand toxicity

Molecular docking or grid box validation was carried out to obtain the right grid box size or cover the active site of the Cu/Zn superoxide dismutase enzyme. The validation results show that Trehalose and β-amyrin are tethered to the allosteric side of the receptor with a total interaction of 1 hydrogen bond and nine hydrophobic bonds (figure 2a), and ten hydrophobic bonds in β-amyrin (figure 2b). the results of this study also obtained an RMSD value of 0 in both comparison ligands and affinity energy of 8.6520 kcal/mol for Trehalose and 4.1590 kcal/mol for the β-amyrin comparison ligand. In addition, the interacting residues in this study are the same as the previous study, namely Val7, Lys9, Asp11, Asn51, Gly54, Cys144, Gly145, and Val146 (Hatai & Banerjee, 2019).



(a) (b)

Figure 2 Hydrogen bond and hydrophobic bond. (a) Trehalose; (b) β-Amyrin

Molecular docking was carried out to observe the interaction between the receptor and the ligand. The test ligands and receptors used are the results of screening in the previous test. Molecular docking in this study was carried out with a targeted docking approach which was carried out on certain active sites. The results of molecular docking of the active compound of red betel with the Cu/Zn superoxide dismutase enzyme showed different affinity energy values. One best red betel ligand could increase the activity of the Cu/Zn-SOD enzyme with the most negative affinity energy and the smallest dissociation constant compared to the comparison ligand (Table 3). The best red betel ligand has the same hydrogen bonding and hydrophobic interactions as Trehalose and β-amyrin comparison ligands (Figure 4).

Table 3 Affinity energy and ligand's chemical bonding

	Ligands		
	1	2	3
Val 7(B)	H (3.31)	H(3.01)	
Val7(A)	√	√	√
Lys9(A)	√	√	√
Lys9(B)	√	√	√
Gly10(A)	-	-	√
Asp11(B)	-	-	-
Asn51(A)	√	-	√
Asn51(B)	H (2.92)	√	√
Gly54(B)	√	-	-
Gly145(A)	-	√	√
Val146(A)	H (2.65)	√	√
Val146(B)	H (2.47)	√	√
Val5(A)	H (3.31)	-	-
Gly145(B)	√	√	-
Cys6(A)	√	-	-
Cys144(B)	-	√	-
Asp11(A)	-	-	√
Gly49(B)	H(3.30)	-	-
Affinity Energy (kkal/mol)	-9.1490	-6.8320	-7.4780
Dissociation constant (μM)	1.967	98.190	33000
∑ Amino acid	13	10	10

Description: 1. 6-Amino-2-(3-aminopropylamino)-9-(cyclohexylmethyl)- 2,3,4,5,6,7-hexahydro-1H-purin-8-one, 2. Trehalose (comparison), 3. β-Amyrin (comparison). √ = hydrophobic bond, H = hydrogen bond.

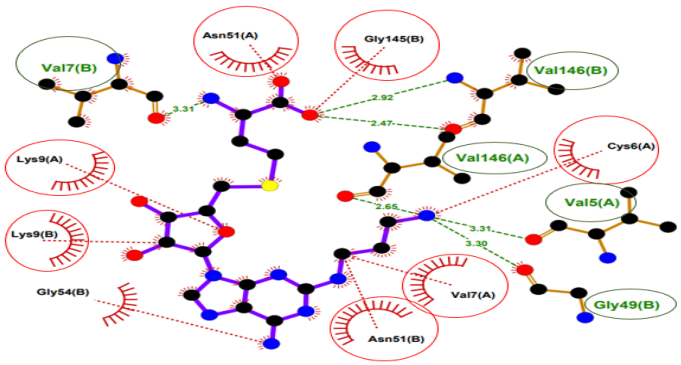


Figure 4 Receptor interactions with the best ligand

3.2. Discussion

The Ramachandran plot provides an overview of the two-dimensional distribution of the protein chain at phi (φ) and psi (ψ) torsion angles (Sobolev et al., 2020). Cu/Zn superoxide dismutase has an amino acid distribution in 0, and 89% disallowed regions. The most favored region indicates that the enzyme has high quality and structural stability because a protein or receptor is said to be good and stable if the amino acid distribution in the favored region is more than 85% (Rao et al., 2020). The poor quality protein can be seen if the preferred area is less than 80% and the non-glycine residue in the restricted area is more than 15% (Suhadi et al., 2019). In Hasan et al. (2022), Cu/Zn superoxide dismutase (PBD ID:1CB4) receptor has a high structural quality. It is stable because the distribution of amino acids in the most favored region is more than 80% and in the disallowed region is less than 15%. Rahman (2020) also stated that the protein structure is said to be good if the distribution of amino acids in the most favored region area is higher than in the disallowed region.

The manufacture of new drug candidates in silico must begin with a physicochemical test of the ligand. This test aims for drugs that will be taken orally, easily absorbed by the digestive organs, metabolized by the body, and eliminated without causing any harm to the body (Zheng & Polli, 2010). Ligand physicochemistry was carried out according to Lipinski's five rules which guide the criteria for new drugs, including absorption, distribution, metabolism, and excretion of a compound in the body (Singh et al., 2013). The analysis results of 69 tests and two comparison ligands were seven ligands that did not meet Lipinski's rules. A ligand's level of absorption and permeability is said to be good if three of the five parameters of Lipinski's rules are met, so the ligands that do not pass are not included in the ligand toxicity test. Schisdandrin B compound is one of the compounds that passed the physicochemical test, where the compound is one of the best compounds of red betel in inhibiting HMG-CoA reductase (Zaelani, 2021).

Ligand toxicity analysis was carried out after physicochemical analysis. The parameters used included carcinogenicity, human a-go-go-related gene (HERG) inhibition analysis, and acute oral toxicity (Pannindriya et al., 2021). Of all the red betel test ligands, one ligand is classified as a strong inhibitor of herG, so it is said to be toxic. Ligands that fall into the category of strong inhibitors cannot be used because if they enter the body, they can cause inhibition of the channel work resulting in loss of consciousness and an increased risk of sudden death (Lamothe et al., 2016). A compound is classified into four groups based on the International Agency for Research on Cancer (IARC), namely carcinogenic to humans (group 1), possibly carcinogenic (group 2), unidentifiable carcinogenic (group 3), and non-

carcinogenic (group 4) (Fakhruri & Rahmayanti, 2021). The prediction results from the remaining 62 compounds contained five carcinogenic ligands that can cause tumors and cancer (Table 2). Acute oral toxicity testing resulted in the ligands being categorized I (LD50 ≤ 50 mg/kg) and category II (50 < LD50 ≤ 50 mg/kg). The acute oral toxicity test results provide information related to the dangers a chemical poses to the body when it enters the gastrointestinal tract directly (Astri et al., 2012). Ligands with acute oral toxicity categories I and II are toxic and dangerous compounds when they enter the human body (Guan et al., 2019). The test ligands which were declared carcinogenic and included in the acute oral category I and II were declared toxic and could not be used as drugs (Table 1).

Trehalose, as a comparison ligand, is one of the carbohydrates that can activate the superoxide dismutase enzyme (Ma et al., 2017). β-amyrin is also a comparison ligand that has been shown to activate the Cu/Zn superoxide dismutase enzyme in CCL4-induced mice (Sunil et al., 2014). The two comparison ligands have the same binding interactions as in previous studies. The interactions that occur in Trehalose are hydrogen bonds in the form of Val7 (B), and residues that interact with hydrophobic bonds are Asn51(B), Gly145(B), Lys9(B), Cys144(B), Val7(A), Gly145(A), Val146(A), Val146(B). whereas β-Amyrin, residues that interact with hydrophobic bonds are Val146(A), Val146(B), Val7(A), Gly145(A), Asn51(A), Lys9(B), Asp11(A), Gly10(A), Lys9(A), Asn51(B). The research of Hatai & Benarjee (2019) shows interactions between two comparison ligands, where the interaction of β-Amyrin with Cu/Zn superoxide dismutase includes residues Val7, Lys9, Asp11, Asn51, Gly54, Cys144, Gly145, dan Val146.

Amino-2-(3-aminopropylamino)-9- (cyclohexylmethyl)- 2,3,4,5,6,7-hexahydro-1H-purin-8-one is an active compound of red betel that has the best potential as an activator of Cu/Zn-SOD enzymes based on affinity energy value -9.1490 kcal/mol, and dissociation constant 1.967 µM. The compound has the most negative Gibbs free energy value and the smallest dissociation constant. Affinity energy is the energy used to describe the energy of the interaction between the receptor and the ligand. The more negative resulting value indicates a better affinity of the ligand-receptor complex, so the activity is hoped to be better (Chairunnisa & Runadi, 2016). The dissociation constant is the binding kinetics of the ligand and receptor to form the stability of the ligand-receptor complex, which can be measured by the strength of its interaction with the dissociation constant (Kd) at the equilibrium point. According to Noviardi & Fachrurrazie (2015), the affinity value is directly proportional to the Kd value. The more negative the affinity energy and the smaller the resulting Kd value, the stronger the complex formed.

Red betel compound Amino-2-(3-aminopropylamino)-9-(cyclohexylmethyl)-2,3,4,5,6,7-hexahydro-1H-purin-8-one has a high similarity of binding sites compared to another red betel compound. The higher the similarity of the interacting residues, the higher the similarity of the type of interaction and the similarity of the test ligands and comparison ligands (Forli & Olson, 2012). The presence of hydrogen bonds with the amino acid Val7 can improve the quality of the compound complex test with the Cu/Zn, superoxide dismutase enzyme (Hatai & Benerjee, 2019). The active compound of red betel Amino-2-(3-aminopropylamino)-9-(cyclohexylmethyl)- 2,3,4,5,6,7-hexahydro-1H-purin-8-one is a compound from alkaloid group and the result of extract red betel water (Safithri et al., 2016). Research found that red betel contains flavonoid compounds, alkaloids, and tannins (Safithri et al., 2016). Research in vitro and in vivo red betel can activate the superoxide dismutase and catalase enzymes (Safithri, 2012).

4. CONCLUSION

Red betel compound Amino-2-(3-aminopropylamino)-9-(cyclohexylmethyl)-2,3,4,5,6,7-hexahydro-1H-purin-8-one has the best potential to increase the Cu/Zn-SOD enzyme based on the results of physicochemical tests, ligand toxicity, and molecular docking. These compounds are included in the category of weak inhibitors, non-carcinogenic and non-toxic, so they are safe for oral consumption.

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