An in Silico Study Anti-Inflammatory Activity of Active Compounds Bouea macrophylla Griff Against Toll Like Receptors-4

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ABSTRACT

Active compounds of ramania leaf extract (Bouea macrophylla Griff) have potential as an anti-inflammatory. It was found that the active compounds contained in Bouea macrophylla Griff consisted of humulene, caryophyllene, phytol, 2-methyl-cis-7,8-epoxynonadecane, squalene, vitamin E, retinol acetate, ethyl palmitate, gamma-himachalene, and methyl ricinoleate. This study aims to determine whether the active compounds of B. Macrophylla Griff have a good interaction with Toll Like Receptors-4 as an anti-inflammatory drug candidate. This type of research is in silico using the molecular docking method. The docking process is carried out using Autodock Vina which has been integrated into the PyRx application. All active compounds of Ramania leaf extract meet the requirements of Lipinski's five rules, pharmacokinetic test and toxicity test. The best compound obtained was vitamin e with a binding

affinity of -8.9 kcal/mol, had a hydrogen interaction and had the same seven amino acid residues as the inhibitor. The active compound of Ramania leaf extract has a good interaction with Toll Like Receptors-4, which means it has potential as an anti-inflammatory.

Keywords : Anti-inflammatory, binding affinity, ramania leaf, molecular docking, TLR4

1.0 Introduction

Inflammation is a physiological response of the immune system to damage to the body such as injury, invasion of pathogens, exposure to toxic compounds or irradiation [1]. Inflammatory responses occur through activation of coordinated signaling pathways that regulate levels of inflammatory mediators in host tissue cells and inflammatory cells recruited from the blood. . Cellular and molecular events and interactions efficiently minimize injury and contribute to the restoration of tissue homeostasis [2]

In the inflammatory phase, chemotactic agents such as bacterial products Pathogen Specific Associated Molecules Pattern (PAMP), Histamine, Damage Associated Molecules Pattern (DAMP) Prostaglandi, complement factor and leukotriene which will later be captured by Toll Like Receptors (TLR) and stimulate the activation of intracellular signaling pathways, namely NF-kB [3-5].

Toll Like Receptors (TLR) have an important role in the inflammatory process because they can recognize patterns and become the first line of defense against pathogens and affect the innate and adaptive immune systems [6]. Toll Like Receptors-4 is a member of the TLR family that is widely expressed on the surface of cells present in the oral cavity such as epithelium, neutrophils, macrophages, dendritic, endothelial, natural killer, stroma, whose signaling pathways are important for inducing the inflammatory phase [7].

Optimal conditions are needed for the wound healing process so drugs containing antibacterial, anti-inflammatory and antiseptic are often given [8-9]. People are starting to use medicinal plants for traditional medicine because medicinal plants have several advantages, namely lower side effects compared to drugs made from synthetic materials [10-12].

One of the plants used as traditional medicine is ramania (Bouea macrophylla Griff) which spreads to grow in the Southeast Asian region, especially Indonesia, Vietnam, Malaysia and Thailand [13-15]. Ramania leaves contain secondary metabolites such as triterpenoids, phenols, alkaloids, saponins, flavonoids, steroids and terpenoids [16-18].

In the GC-MS test, as many as 14 active compounds were found in B. macrophylla. These compounds consist of the two highest identified main compounds, namely caryophyllene (27.48%) and Squalene (32.11%) and other main compounds are humulene, caryophyllene oxide, ethyl hexadecanoate, Phytol, diisooctyl phthalate, vitamin E, retinol acetate. , and gamma-himachalene which has potential as anticancer, antibacterial, and anti-inflammatory [14, 19].

In Silico is described as the use of high-performance computational computational computation to analyze a database of many chemical compounds to identify drug candidate probabilities [20-21]. This computational process works to find ligands that show a geometric match and an energy match by

predicting the bond affinity. Through molecular docking, an overview of compound activity can be seen without the need to synthesize compounds first [22-23]

The reference ligand used in this study was TAK-242 or Resatorvid which is a small molecule that inhibits TLR4 signaling. This study predicts the anti-inflammatory effect of 10 active compounds from Bouea macrophylla Griff which can inhibit inflammation through inhibition of Toll Like Receptors-4 and provides researchers with information about compounds that may be effective.

2.0 EXPERIMENTATION

2.1 MATERIALS& METHODOLOGY

Ligand and Protein Data Sampling

Molecular docking preparation of the 3D Toll Like Receptors-4 (TLR4) structure obtained from the Protein Data Bank website (https://www.rcsb.org/) with PDB ID: 4G8A. The 3D structure of the comparison ligand (resatorvid) was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The protein structures are downloaded in .pdb format while the ligands are downloaded in SDF format.

Protein and Ligand Preparation

The B. macrophylla Griff compound used was caryophyllene (CID5281515), humulene (CID 5281520), 2-Methyl-cis-7,8- epoxynonadecane (CID537313), ethyl palmitate (CID21859),*phytol*(CID5280435), squalene (CID 638072), vitamin E (CID 14985), retinol acetate (CID638034),*γ-himachalene*(CID577062), methyl ricinoleate (CID22821244) and a resatorvid comparator ligand (CID 11703255). Protein molecular docking was prepared usingBiovia Discovery Studio (Dassault Systèmes BIOVIA, 2021) to remove naturally attached ligands. Meanwhile, the ligands were prepared using Open Babel which was integrated into the PyRx 8.0 application (Dallakyan & Olson, 2015) to minimize energy and convert to pdb format.t.

Docking Method Validation

The way to validate the docking method is by re-docking between the natural ligand 2-acetamido-2-deoxy-beta-D-glucopyranose (CID 24139) and the TLR4 receptor that has been prepared. Root Mean Square Deviation (RMSD) is a parameter in the docking validation process.Validation results between natural ligands*2-acetamido-2-deoxy-beta-D-glucopyranose*(NAG)with TLR4 the RMSD value is 1.6 Å. If the RMSD value is ≤ 2 Å then the docking method is valid. The results of the validation show that the parameter settings used meet the validation criteria, so that these parameters can be used for the docking of the test compounds.

Protein-Ligan Docking Process and Visualization of Docking Results

*Docking*performed using Autodock Vina integrated on PyRx 8.0. Analysis of virtual prediction results and visualization of protein-ligand complexes from docking results was visualized using the Biovia Discovery Studio which consists of types of interactions, bond distances, and amino acid

residues. TLR4 was also docked with a resatorvid comparator ligand to compare the binding affinities of the 10 active compounds used in this study.

Prediction of Drug-Likeness, Pharmacokinetics, and Toxicity of Compounds.

*Lipinski's Five Rules*used to predict drug-likeness. The drug similarity test is carried out through the websitewww.molinspiration.com. Toxicity and pharmacokinetic predictions were performed via pkCSM (http://biosig.unimelb.edu.au/pkcsm/) and the Protox Online Tools website with the site addresshttps://tox-new.charite.de/protox_II/.

Pharmacokinetic parameters used include Caco-2, Volume Distribution at steady state (VDSs), Human Intestinal Absorption (HIA), CYP2D6, and Total Clearance. Parameters used for safety or toxicity tests include ames toxicity, Oral Rat Chronic Toxicity (LOAEL), Oral Rat Acute Toxicity (LD50), and hepatotoxicity.

3.0 Conclusions

Drug-Likeness Test

Drug-likeness analysis used Lipinski's Rule of Five [23-24]. Named Lipinski's 5th law because the parameters involved use multiples of 5 [26]. The lipinski rule is used to determine the physicochemical properties of a ligand in determining the hydrophilic or hydrophobic character of a compound to pass through the cell membrane by passive diffusion. Compounds have properties similar to drugs if their molecular weight is <500 dalton, the log partition coefficient value is P <5, the number of hydrogen bond donors is <5, and the number of hydrogen bond acceptors is <10 and the TPSA value is between 0-140 [27-29]. Table 1 shows that there are several active compounds of Bouea macrophylla Griff that do not meet the parameters of the log partition coefficient value P>5 of these compounds, namely 2-methyl-cis-7,8-epoxynonadecane,*ethyl palmitate*, Phytol, Squalene and Vitamin E. If the log P value is large, then the molecule is hydrophobic. The nature of the molecule that is too hydrophobic will have a high level of toxicity because it will stay longer in the lipid bilayer and will be distributed more widely in the body so that the selectivity of the binding to the target enzyme is reduced. Molecules that have a log p value that is too negative are also not good because they will not be able to penetrate the lipid bilayer membrane [30-32].

According to the lipinski rule of molecular weight <500 Dalton, all active compounds meet the requirements. Active compounds that are <500 Daltons more easily penetrate cell membranes [33-34]. The number of hydrogen bond donors and acceptors indicates that the higher the hydrogen bond capacity, the energy required for the absorption process is also higher. Based on this theory, all active compounds and reference ligands fulfill the requirements for the number of hydrogen bond donors and acceptors, meaning that the active compounds do not require high energy so that the absorption process will occur more easily [35-36].

Table 1. Prediction of the Physicochemical Properties of the Active Compound Bouea macrophylla Griff using the Five Rules Of Lipinski's

Name	*Molecular Weight*	Log P	*Hydrogen Donor*	*Hydrogen Acceptor*	TPSA	Ket
Caryophyllene	204.35	4,4	0	0	0 Å²	Fulfil
Humulene	204.35	4,7	0	0	0 Å²	Fulfil
2-Methyl-cis-7,8-epoxynonadecane	296.5	7.2*	0	1	12.5 Å²	Fulfil
Ethyl Palmitate	284.5	6.52*	0	2	37.3 Å²	Fulfil
Phytol	296.5	6.9*	1	1	20.2 Å²	Fulfil
Squalene	410.7	9.6*	0	0	0 Å²	Fulfil
Vitamin E	430.7	9.9*	1	2	0 Å²	Fulfil
Retinol Acetate	328.5	4,9	0	2	26.3 Å²	Fulfil
γ-himachalene	204.35	4,4	0	0	0 Å²	Fulfil
Methyl Ricinoleate	312.5	5.3*	1	3	46.5Å²	Fulfil
Resatorvid	361.8	3,1	1	6	0 Å²	Fulfil

The analysis in this study aims to evaluate the toxicity of the tested plant compounds and to select which active compounds have the best potential as drug ingredient candidates. Based on the toxicity test of the active compound Bouea macrophylla Griff with the parameters AMES Toxicity, Hepatotoxicity, LD50, and LOAEL (Table 2) it was found that all active compounds did not cause mutagenic or carcinogenic effects, while the results of the hepatotoxicity test showed that Retinol Acetate can cause liver damage . Toxicity tests were carried out using a web-based program, namely pkCSM and Protox Online Tool to determine whether there is a toxic effect of the test compound [37-38]. Mutagenicity test based on the Ames test is a test used to determine levels of reverse mutation of a chemical compound that can cause genetic damage and trigger gene mutations [39-40]. To determine the toxicity of a chemical, you can do the Oral Rat Acute Toxicity (LD50) Test [40] LD50 is the amount of compound given that can cause 50% of the death of the experimental group of animals. Based on the classification of Hodge and Sterner, there are six classes of toxicity. Class one with an LD50 value of ≤1mg/kg is classified as very toxic, class two with an LD50 value of 1-50 mg/kg which is classified as toxic, class three with a value of 50-500 mg/kg and classified as moderately toxic, class four is mildly toxic with the LD50 value is 500-5000 mg/kg, the fifth grade is practically non-toxic with an LD50 value of 5-15 g and the last is class 6 which is relatively harmless with an LD50 value ≥15 g [42-45]. Table 3 shows the results of the oral toxicity test in rats (LD50). It can be seen that the active compound B. macrophylla Griff has an LD50 value ranging from 2,000 to 16,000 mg/kg. Based on the theory of toxicity class hodge and sterner, there are 6 classes of toxicity where the active compound that has the highest LD50 level is methyl ricinoleate with a toxicity prediction value of 16,000 mg/kg and belongs to class 6 toxicity which is relatively harmless with a prediction accuracy of 72.9%.

Table 2. Prediction of the Toxicity of the Active Compound Bouea macrophylla Griff Using the pkCSM Website

Ligand	*AMES Toxicity*	*Hepatotoxicity*	*LOAEL*(log mg/kg_bw/day)
Caryophyllene	No	No	1.416
Humulene	No	No	1,336
2-Methyl-cis-7,8-epoxynonadecane	No	No	0.991
Ethyl Palmitate	No	No	3,313
Phytol	No	No	1,043
Squalene	No	No	0.946
Vitamin E	No	No	1,987
Retinol Acetate	No	Yes	2,276
γ-himachalene	No	No	1,346
Methyl Ricinoleate	No	No	2,701
Resatorvid	No	No	1,043

Table 3. Prediction of the LD50 Toxicity of the Active Compound Bouea macrophylla Griff Using the Protox Online Tools Website

Ligand	LD50 (mg/kg)	*Toxicity Class*	*Average Similarity(%)*	*Prediction Accuracy (%)*
Caryophyllene	5,300	5	86.96	70.97
Humulene	3,650	5	86,36	70.97
2-Methyl-cis-7,8-epoxynonadecane	5,000	5	100	100
Ethyl Palmitate	900	4	100	100
Phytol	5,000	5	100	100
Squalene	5,000	5	100	10 0
Vitamin E	5,000	5	82.25	70.97
Retinol Acetate	4,100	5	100	100
γ-himachalene	4,400	5	95.65	72,9
Methyl Ricinoleate	16,000	6	91.39	72,9
Resatorvid	2,000	4	35,36	23

Pharmacokinetic Analysis

Pharmacokinetic analysis consisting of Caco-2, Human Intestinal Absorption, VDss, CYP2D6, and Total Clearance in this study aims to evaluate the toxicity and pharmacokinetic activity of the test compounds and to select which active compounds have the best potential as drug candidate materials [46-47]. Caco-2 cells are a parameter of permeability ability used to determine the process of drug transfer through epithelial cells in the intestine derived from human colon adenocarcinoma with multiple transport pathways with an in vitro model. There

are three categories of Caco-2 cell parameters. The category that has a high permeability of compounds is that which has a value of >70 nm/s, the medium category has a value of 4-70 nm/s while the third category is the one with a low permeability ability which has a value of <4 nm/s [48-49]. Based on these categories, almost all of the active compounds and ligands tested had a value of <4 nm/s, which means their permeability is low.

The Human Intestinal Absorption (HIA) parameter aims to predict the absorption process that occurs in the intestine. The results of the HIA test are the result of the sum of bioavailability with absorption which is evaluated from the results of the ratio of excretion through bile, urine and feces. HIA has three categories of parameters, namely good, medium and low. The good category has an HIA value of 70-100%. The moderate category ranges from 20-70% and the low category has a presentation of 0-20% [48, 50]. In Table 4 it can be seen that all active compounds have HIA values between 70-100% which are included in the good category.

The volume that shows the value of a total drug dose that is distributed as a whole is the Volume Distribution at Steady States (VDSs). If the VDss value is high, then the distribution drug in tissues is more than plasma. A compound is categorized as having a high volume of distribution if the Log VDss value is> 0.45 and low if the Log VDss value is <-0.15 [51-52]. Based on Table 4, the VDss value of the Bouea macrophylla Griff compound ranges from -0.578 to 1.617. There are three active compounds that have a VDss value <-0.15 namely*ethyl palmitate,methyl ricinoleate*, and resatorvid which means low volume of distribution. Other active compounds that have a log volume distribution value of > 0.45 and are classified as high are caryophyllene, humulene, phytol, vitamin E, and γ-imachalene so that it can be concluded that the five compoundsThese actives can be distributed uniformly to provide the same concentration as in blood plasma.

The results of the pharmacokinetic analysis showed that all active compounds and reference ligands had no inhibitory effect on CYP2D6. Metabolic reactions in general involve oxidation processes. Important enzymes such as cytochrome P450 as detoxification enzymes are found in the liver and function to oxidize foreign organic compounds, including drugs. Many drugs are inactivated by cytochrome P450, and some drugs can also be activated by P450 [53-54]. This enzyme has inhibitors such as grapefruit, which can affect drug metabolism so that cytochrome P450 enzymes are contraindicated in this study represented by cytochrome P2D6 isoforms [55]. CYP2D6 is responsible for the metabolism of most drugs and chemical compounds [56-57]. *Total Clearances*(CLTOT) is used to predict the excretion process of the compound. The combination of hepatic clearance (metabolism in the liver and bile) and renal clearance (excretion through the kidneys). This is related to bioavailability, and it is important to determine the dose level to achieve steady state concentrations [57-59]. Table 4 shows that CLTOT values range from 0.11 to 1.791 log mL/min/kg.

Table 4. Pharmacokinetic Prediction of Active Compounds*Bouea macrophylla Griff*

Ligand	*Caco-2*(nm. sec-1)	*Human Intestinal Absorption*(%)	*VDSs*(logL/kg)	CYP2D6 Inhibitors	*Total Clearances*(log mL/min/kg)
Caryophyllene	1.423	94,845	1.617	No	1,088
Humulene	1,421	94,682	0.505	No	1,282
2-Methyl-cis-7,8-epoxynonadecane	1.226	91,808	0.406	No	1,631
Ethyl Palmitate	1,572	91,462	-0.578	No	1,678
Phytol	1.515	90.71	0.468	No	1,686
Squalene	1.216	90,341	0.411	No	1,791
Vitamin E	1.345	89,782	0.709	No	0.794
Retinol Acetate	1.188	94.33	0.408	No	1.503
γ-himachalene	1.418	94,556	0.648	No	1,093
Methyl Ricinoleate	1.57	91,956	-0.509	No	1.615
Resatorvid	1.133	91.51	-0.418	No	0.11

Molecular Docking Analysis of TLR4 with Active Compounds Bouea macrophylla Griff

Based on the results of molecular docking of the active compound Bouea macrophylla Griff with TLR4, there were six compounds whose binding affinity values were lower than the reference ligands. The six compounds are retinol acetate -9 kcal/mol, vitamin E -8.9 kcal/mol, squalene -8.8 kcal/mol, caryophyllene -8.1 kcal/mol, humulene -7.4 kcal/mol, and γ-himachalene -7.4 kcal/mol. The active compound whose binding affinity value is lower than that of the reference ligand has potential as a drug substance candidate because the binding affinity indicates the strength of the bond resulting from the interaction between the receptor and the ligand in the form of low energy in the formation of a drug complex. Binding affinity analysis was performed to determine the spontaneity of a reaction and the stability of the receptor-ligand interaction. Stability between the receptor-ligand is indicated by a low binding affinity value. The more negative the binding affinity value, the higher the tendency of the ligand and receptor to bond or join [60-62].

The docking results were visualized to see the type of interaction and amino acid residues formed between the test compound and the receptor. The interaction of the same amino acid residues allows contact between the ligand and the receptor so that it has the potential for inhibitory activity. The active site indicates that the amino acid residue has an important role in forming interactions between the ligand and the receptor such as hydrogen bonds, hydrophobic bonds and electrostatic bonds [63-65]. Test ligands with amino acid residues and hydrogen bonds that are close to natural ligands show similar types of interactions in this case describe similar activities [66-67]. Following are the results of the visualization of 2D, 3D and ligand interaction structures from the docking of the active compound Bouea macrophylla Griff with TLR4 which has hydrogen bonds.

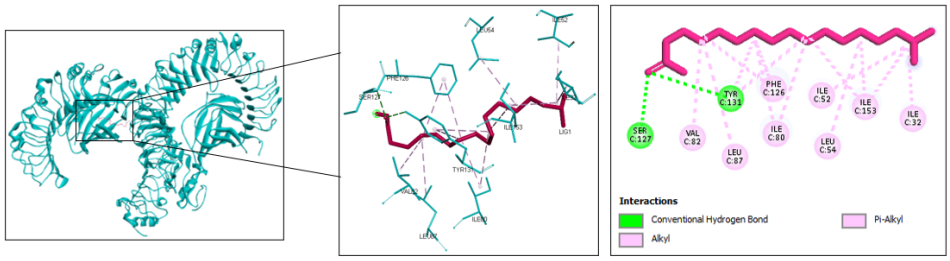

Figure 1.TLR4-Ethyl Palmitate

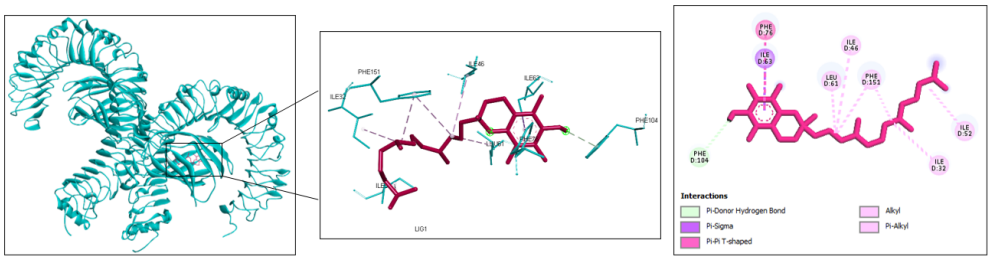

Figure 2.TLR4-Vitamin E

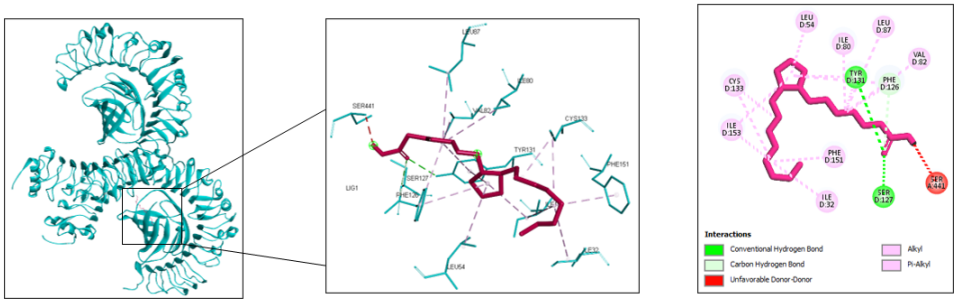

Figure 3.TLR4-Methyl Ricinoleate

Table 5. Binding Results of Bouea macrophylla Griff and Comparative Ligands against Toll Like Receptors-4

Compound	*Affinity Bindings*	Amino Acid Residues	Interaction Type
Caryophyllene	-8,1	ILE63, VAL135*,**ILE46***, ILE63, LEU74, ILE94, LEU71, LEU74, LEU146, ILE63, TYR65, PHE76*, PHE104, PHE147	*Hydrophobic*
Humulene	-7,4	**VAL135*, PHE151***	*Hydrophobic*
2-Methyl-cis-7,8-	-7,1	PHE104, ILE44, ILE46*, LEU61*, ILE63, VAL135*, ILE63, VAL113, ILE32*, VAL48*, ILE52*, TYR65, PHE147, PHE151*	*Hydrophobic*

epoxynonadecane			
Ethyl Palmitate	-6,7	SER127, TYR131	*Hydrogen Bonds*
		VAL82, ILE80, ILE153*, LEU54, ILE153*, ILE32*, ILE52*, LEU87, PHE126 and TYR131	*Hydrophobic*
Phytol	-7	**ILE46*,**VAL48*, LEU61*, VAL135*, ILE44, ILE46*, ILE63, TYR65, PHE147 and PHE151*	*Hydrophobic*
Squalene	-8,8	**LEU61***, LEU78, ILE44, ILE46*, ILE63, LEU74, VAL48*, LEU61*, ILE32*, LEU54, ILE153*, ILE152*, LEU54, VAL135*, TYR65, PHE76*, PHE119*, PHE147, PHE151*	*Hydrophobic*
Vitamin E	-8,9	PHE104	*Hydrogen Bonds*
		ILE63, PHE76*, ILE32*, ILE46*, ILE52*, LEU61*, PHE151*	*Hydrophobic*
Retinol Acetate	-9	SER441, SER441	*Hydrogen Bonds*
		TYR131, ILE32*, ILE52*, ILE153*, LEU54, PHE126, TYR131	*Hydrophobic*
γ-himachalene	-7,4	**PHE151*, ILE46***, LEU78, VAL135*, CYS133, PHE76*, PHE151*	*Hydrophobic*
Methyl Ricinoleate	-7	**ILE46***, ILE63, LEU71, ILE94, VAL113, ILE117, LEU61*, PHE76*, TYR102, PHE104, PHE147	*Hydrophobic*
Resatorvid (Inhibitor)	-7,1	**PHE76, PHE151, ILE32, ILE153, VAL48, ILE52, LEU61, PHE119, PHE151, ILE46 and VAL135.**	*Hydrophobic*

Information :

* : the same amino acid residue as the reference ligand

The interactions of amino acid residues can be seen in Table 5. Based on the molecular docking of the 10 active compounds and one reference ligand, hydrogen bonds and hydrophobic bonds are formed. Hydrogen bonds have the shortest distance and are the strongest bonds compared to other molecular bonds. This is in accordance with the theory that the bond distance between the ligand and the molecule has an important role in determining the strength of the bond. Bonds get stronger when the distance is short and also applies to hydrogen bonds where the more hydrogen bonds, the more stable the bonds are [64]. Hydrogen bonds can form even though the distance between the ligand and the receptor is quite far, therefore hydrogen bonds are strong bonds [68].

Based on the research results of molecular docking of the active compound of ramania leaf extract with TLR4, the hydrophobic bond is the most abundant bond. Hydrophobic bonds are bonds that have important strengths in the process of combining non-polar and polar regions of drug molecules with non-polar regions of biological receptors [69]. In addition, the presence of hydrophobic bonds can increase protein stability by changing hydrophilic amino acids in a hydrophobic environment and being able to determine amino acid residues that contribute significantly to maintaining protein stability [70]. The hydrophobic bond is a parameter of the strength of the interaction between the receptor and the ligand, which is useful in helping to maintain the conformation of the binding [71].

According to research results, all active compounds have several amino acid residues that are similar to inhibitory amino acid residues. The similarity of the amino acid residues from the binding of the test ligand to the receptor indicates a similarity in the type of interaction in this case describing the similarity of activity [66].

Conclusion

The active compound Bouea macrophylla Griff can bind to the TLR4 receptor in silico so that it has the potential as an anti-inflammatory drug candidate. Vitamin E is the best compound among other compounds because it has a lower binding affinity than the inhibitor, which is –8.9 kcal/mol, has hydrogen bonds at the PHE104 residue, and has the same seven amino acid residues as the inhibitor.

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