WCEJ

WORLD CHEMICAL ENGINEERING JOURNAL

Journal homepage: http://jurnal.untirta.ac.id/index.php/WCEJ

In Silico Study and Bioactivity of Flavonoid Extract *Syzygium polyanthum (Wight) Walp*. Leaves Against *Salmonella typhi*

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ARTICLE HISTORY

Received June 19, 2020
Received in revised form July 8, 2020
Accepted August 15, 2020
Available online August 16, 2020

ABSTRACT

Salmonella typhi bacteria caused complications of bleeding in the intestine or intestinal perforation. However, *Salmonella typhi* bacteria would become resistant to chloramphenicol due to the formation of plasmids that produce the chloramphenicol acetyl transferase (CAT) enzyme which worked to activate chloramphenicol. So, the search for active groups of natural compounds which were expected to have the work of deactivating the CAT enzyme. One of them is a flavonoid compound. The flavonoid group was widely used as an antimicrobial. This research was aimed at the extraction of bay leaf flavonoids. The presence of flavonoids was proven by phytochemical tests, namely a yellow color change with a slightly acidic amyl alcohol solvent - Mg metal and strengthened by the scanning results of the maximum wavelength on the band 1: 407 nm and band 2: 338 nm which was a special feature of flavonoid backbone. The results of GC-MS analysis obtained 4 flavonoid compounds from bay leaf extract which had an abundance above 1% and qualification above 90%: 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, alpha- tocopherol, beta-tocopherol, and pyragallol. The correlation between the results of the inhibitory test against Salmonella typhi at a concentration of 100 ppm with the results of docking tocopherol - 3CLA receptors which had an affinity bond energy / $\Delta G_{binding}$ = 7.40 kcal / mol, indicates that the bay leaf flavonoid extract could inhibit the formation of chloramphenicol acetyl transferase (CAT).

Keywords: ***c****hloramphenicol acetyl transferase (CAT), beta-tocopherol, docking in silico, 3CLA*

1. INTRODUCTION

Typhoid or Typhoid fever was a disease that often affects people in Indonesia. This disease caused abnormalities in the small intestine mucosa and could also lead to complications of bleeding in the intestine or intestinal perforation if you did not get proper medication, diet, and care (Paul and Bandyopadhyay 2017). The cause of this fever was the *Salmonella typhi* bacteria that sticks to food or drinks when snacking carelessly.

One of the medical treatments provided was chloramphenicol. The use of chloramphenicol was aimed at inhibiting the synthesis of germ protein in the peptidyl transferase enzyme (Ugboko and De 2014), although it often acts as a catalyst to form peptide

bonds in the process of germ protein synthesis (Wain et al. 2015). From the study of molecular biology, it was described that *Salmonella typhi* bacteria would become resistant to chloramphenicol due to the formation of plasmids that produced the chloramphenicol acetyl transferase (CAT) enzyme which worked to activate chloramphenicol (Cita 2011).

Treatment with synthetic antimicrobial drugs was always limited by high drug prices and the incidence of drug side effects (Maulana 2009). The unavailability of antibiotics, the high cost of antibiotics, and the resistance made by these antibiotics have resulted in increased morbidity and mortality. Of course, the use of traditional medicines which were inherited from our ancestors is considered safer, cheaper, and easier to obtain in Indonesia than modern medicine.

Indonesia had many plants that were used as food preservatives, such as bay leaves. Some people had traditionally used bay leaves to treat high cholesterol, hypertension, diabetes, ulcers, and diarrhea (Kusuma, Kuspradini, and Arung 2011). Meanwhile, the anti-bacterial ability of bay leaf extract against Salmonella typhi bacteria has been carried out in several solvents: 96% ethanol (Trisharyanti et al. 2017), and methanol pa (Evendi 2017) with clear zone values of 10-13 mm and suspected active compounds from the phenolic group.

Flavonoids were secondary metabolites of plants with polyphenolic structure, and a part of phenolic. Where flavonoids and phenolic acids has been reported by several studies. Increases bile secretion, reduces blood. cholesterol and lipid levels and antimicrobial activity against some strains of bacteria such as staphylococcus aureus were some of biological activities (Ghasemzadeh and Ghasemzadeh 2011). Phenolics and flavonoids possed diverse biological activities, likes: antiulcer, anti-inflammatory, antioxidant, cytotoxic and antitumor, antispasmodic, and antidepressant activities (Ghasemzadeh and Ghasemzadeh 2011).

Another efficacy, bay leaves have been proven by previous studies and the results were that bay leaves have substances that were useful for anti-cholesterol, antihypertensive, anti-glycemic, and antibiotics (Rizki, AR, and Amalia 2016). This research was aimed at the content of flavonoid compounds that had inhibition of *Salmonella typhi* in vitro and silico.

2. METHODS

2.1 Materials

This research was conducted in the Basic Chemistry Laboratory and Chemical Computing Laboratory, Faculty of Engineering, University of Sultan Ageng Tirtayasa, and the Microbiology Laboratory of PT Samco Farma. GC-MS analysis was carried out in the DKI Jakarta Regional Health Laboratory. The research took place from May 2019 to January 2020. The research design was described schematically in Figure 1. This research included qualitative testing of flavonoids, antibacterial testing, GCMS testing and bioinformatics studies through docking in silico.

2.2 Experimental method

Simplicia Preparation (Rochmat 2015)

Fresh bay leaves were sorted and washed then dried in the air to minimize the amount of water contained in the leaves. The leaves were crushed using a mixer to form a dry powder with a size of 50-100 mesh and then weighed.

Bay Leaf Extract Preparation (Rochmat 2015)

Bay leaf powder was macerated with ethanol 96% solvent for 3 days. Every day, the solvent was replaced with a new solvent. The filtrate was separated from the waste, then partitioned using slightly acidic amyl alcohol and water. Then, the filtrate was separated into two, let stand for a few minutes, and the top was taken. Furthermore, the filtrate was concentrated using a rotary evaporator until the solvent content residue of 10-15%. The concentrated filtrate was proceeded to dry in a vacuum oven for 18 hours at a temperature of 40^{o}C. The result was a thick extract formed. This thick extract was thought to be a bay leaf phenolic extract.

Identification of flavonoids as active compounds (Balamurugan, Sheerin, and Velurajan 2019; Harborne 1998; Rochmat 2018)

The extract was taken about 0.5 grams and dissolved in 5 - 10 ml of water, sonicated, and heated in a water bath for 5 minutes. The filtrate was filtered to obtain 5 mL of extract solution. Then, magnesium powder, 0.5 ml of concentrated hydrochloric acid, and 2 mL of amyl alcohol respectively was added to the extract solution, followed by sonication for 2 minutes and allowed to separate. The presence of flavonoids could be seen by the formation of a yellow, red, or orange colour on the amyl alcohol layer.

Agar Diffusion Method (Somia Gu, Asma Eraj 2015)

Inoculum was prepared by dissolving 3 to 5 colonies of *Salmonella typhi* ATCC bacteria from 24-hours agar culture in sterile saline. Turbidity was adjusted until 0.5 Mc Farl was obtained. Muller Hinton Agar (20ml) was poured into each of the 90 mm Petri dishes. The bacterial suspension (100 ul) was evenly spread on the MH media using a Pasteur pipette and allowed to dry. Using a sterile borer, the well with a diameter of 6 mm was swabbed into the agar. 60 ul, each of the test solution, and negative control (60 ul salina) and positive control (chloramphenicol) were added carefully into the well designated on the agar surface containing bacteria. The plates were stored for 30 minutes and then incubated at 37 ± 1 ∘ C for 24 hours. The plates were checked for growth and the zone of resistance measured using a ruler. The endpoint of inhibition is where growth begins.

Docking In Silico Testing (Herdiawan et al. 2018; Samykannu, Vijayababu, and Natarajan 2019; Suresh 2014)

Protein Preparation

Chloramphenicol acetyl transferase (CAT) enzyme molecules via http://www.rscb.org with code 3CLA

was downloaded and saved in PDB format (* .pdb). Next to preparation using the Discovery Studio software, unused ligands or molecules such as water molecules were removed. Then the protein preparation was stored in pdb (*. Pdb) format.

Ligand Preparation

In this test, the ligands were compounded from the fractionation tested by GC-MS with an abundance of more than 1% and chloramphenicol as the original ligand as well as positive control. The structure of the compound was confirmed via PubChem http://pubChem.ncbi.nml.nih.gov and downloaded in the SDF format (*.sdf). The next step was using Chem3D to minimize the steric energy and storing the structure in * .pdb form.

Docking Molecular

The prepared receptors were opened in Autodock software and the format was converted into PDBQT. Then the ligand was converted into its torque formation and stored in * .pdbqt format. After the ligands and receptors were ready, a working area (grid box) was made following the positive control grid box and followed by the docking process. After the docking process was completed, the binding affinity was stored and the docking ligand was analyzed for the binding site via the Discovery Studio Visualizer device.

The schematical research flow was described in Figure 1.

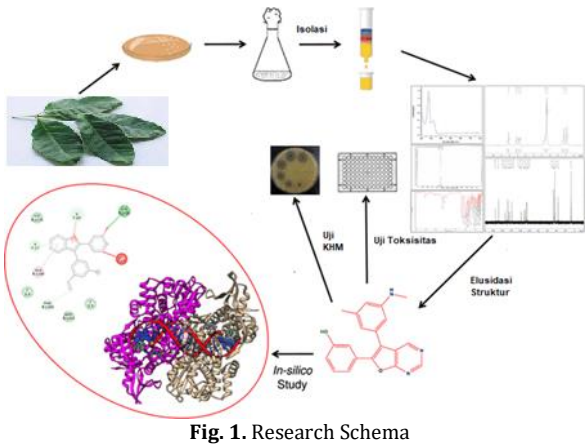


Fig. 1. Research Schema

3. RESULT AND DISCUSSION

3.1. Flavonoid Extract Fractionation

The phytochemical test's results of the partition fraction using ethyl acetate as a solvent has showed a change in dark brown to yellow. This change indicated the presence of flavonoids in the filtrate of the ethyl acetate fraction of bay leaves (Balamurugan, Sheerin, and Velurajan 2019).

The presence of flavonoids in the ethyl acetate fraction filtrate was strengthened by scanning wavelengths using a UV-Vis spectrophotometer.

The scanning results has showed a characteristic flavonoid group backbone where the adsorption results of band 1 was 407 nm and band 2 was 338 nm. This pattern was identical to the flavonoid compounds of the anthocyanidin compound group having an adsorption range of 445-560 in band 1 that these compounds (Harborne 1998; Ayyanar and Subash-Babu 2012; Jadhav, Kamble, and Kadam 2009)

Fig. 2. Flavonoid Testing

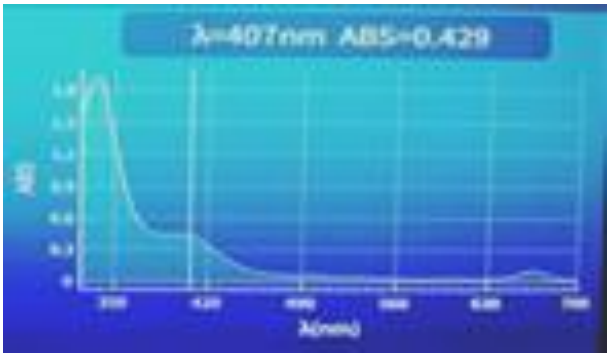


Fig. 3. Spectra Visible of Flavonoid Sample

3.2. Inhibition Testing Against to Salmonella Typhi

Treatment of *Salmonella typhi* antibacterial activity test fraction was used a concentration range of 20-100 ppm and two control groups, namely positive control and negative control. The positive control used 5 ppm chloramphenicol antibiotics. Chloramphenicol was used as a positive control because it was the antibiotic of choice that was sensitive to *Salmonella typhi* (Kesehatan 2018). Where, chloramphenicol was a potent bactericidal compound against Salmonella species which was still used as a first-line antibiotic drug with a sensitivity value of 99.05% and a resistance effect of 0.95% (Sandika and Suwandi 2017; Sidabutar and Satari 2016). The negative control used was Tween 20 which was a compound of 5% Polyethylene glycol sorbitan monolaurate. The concentration of Tween 20 as a negative control was usually around 2% -8%, and most importantly it has no antibacterial activity (Sugijanto, Anggraeny, and Zaini 2011).

Table 1. *Inhibition Testing Against to Salmonella Typhi*

Solvent	Concentration ppm				Positif Control*	Negative Negative **
	20	50	80	100		
Ethyl Acetic	9.89	12.36	17.21	24.49	35.22	6.00

Source : primary data
*) Kloramfenikol, 5 ppm
**) Tweet 20, 5 %

The antibacterial test results obtained were expressed in terms of the length of the white zone diameter by units of millimeters (mm). Based on the antibacterial test table above, it showed an increase in the clear zone at each extract concentration tested. The minimum clear zone occured at the lowest concentration of 20 ppm in each solvent. An increase in the diameter of each concentration was due to an increase in the concentration of active compounds in the sample fluid which results in higher activity of antibacterial compounds contained in bay leaves.

In literature data, the ethanol extract of bay leaves had an antibacterial effect against *Salmonella sp*. bacteria with a clear zone in the range of 21 - 27 mm (Nafsa, Mutmaina, and Sopinah 2018).

Some concentrations had antibacterial effects which were classified as strong because they had a diameter range between 10 - 20 mm (Evendi 2017). The concentration of the extract which was included in the strong group was in the hexane solvent, among others 80% and 100% as well as 50ppm, 80%, and 100% isopropyl acetate.

Although it was inhibitory ability against *Salmonella typhi* was still below the ability of chloramphenicol, this was generally influenced by several factors, namely technical factors, biological factors, extract concentration, antibacterial compound content, extract diffusion power and the type of bacteria used (Clinical and Laboratory Standards Institute 2015).

3.3. GC-MS Testing Result of Syzygium polyanthum (Wight) Walp

From the GC-MS results, it was confirmed that only 4 of the 18 compounds had a flavonoid backbone with a % quantity of more than 1% and quality more than 85. These 4 compounds will be followed by docking in silico to the chloramphenicol acetyl transferase (CAT) enzyme to prove the affinity bond interaction of flavonoid compounds - CAT.

Table 2. GC-MS test results of phenolic extract's of Syzygium polyanthum

No	Retention Time	Quantity, %	Compound	MW	Qual
1	80,891	1,437	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144,13	94,4
2	83,782	4.933	Alpha-Tocopherol	430,71	89,7
3	83,081	0,935	Beta - Tocopherol	430,71	91,2
4	203.18	5,326	Pyragallol (1,2,3, benzenetriol)	126,11	90,1

*) Source: primary data of GC-MS chromatogram

3.4. Results of Docking Molecular Phenolic Compounds against Salmonella typhi Bacteria

Macromolecular information on proteins could be seen in Table 3. The method for determining the structure of macromolecules chosen was X-Ray Diffraction due to this method could be applied to large macromolecules (> 100 kDa) and more precise.

Table 3. Original Macromolecular and Ligand Information (Ali et al. 2020)

Parameter	Protein Bakteri
PDB ID	3CLA
Organism	Escherichia coli
Method	X-Ray Diffraction
Resolution	1,7 A
Ligan	CLM

3CLA was the crystallography result of the chloramphenicol acetyl transferase (CAT) enzyme formed by *Salmonella typhi* bacteria when activating chloramphenicol (Cita 2011; Leslie 1990). chloramphenicol acetyl transferase was a bacterial enzyme that detoxifies chloramphenicol antibiotics and is responsible for chloramphenicol resistance in bacteria. This enzyme covalently attached acetyl groups from acetyl-CoA to chloramphenicol, which prevented chloramphenicol from binding with ribosomes (Leslie 1990; Ugboko and De 2014).

The docking process was carried out using the Autodock Tools version 1.5.6 application, which aims to calculate the amount of affinity bonding between a biomolecular amino acid compound (such as a protein or enzyme) and a ligand (a medicinal compound as an inhibitor or catalyst) in a sterically stable conformation. This affinity bond was the energy value that a compound has at the best conformation when forming a ligand-protein bond.

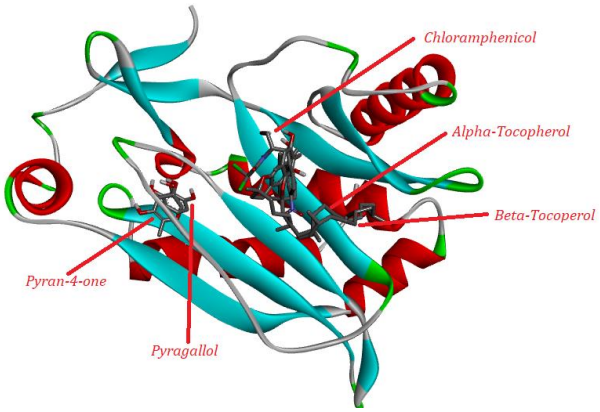


Fig. 4. 4-ligand confirmation to the 3CLA receptor

These results were confirmed by 3D visualization, in which alpha tocopherol and beta-tocopherol coincided with a positive control ligand, chloramphenicol. Meanwhile, pyran-4-one and pyrogallol were located far apart from chloramphenicol.

The position of a ligand structure confirmation was highly dependent on the interaction of hydrogen bonds, electrostatic interactions, hydrophobic and Van der Waals forces with the amino acid residues of the target protein. The interaction with this bond was quantified in the form of affinity bond energy, where the magnitude of the affinity bond energy was strongly influenced by the partition function - Q, free energy - A, Internal energy - U and entropy - S when a ligand binds to its receptor (Antao 2015). The value of the affinity bond was expressed in kcal / mol.

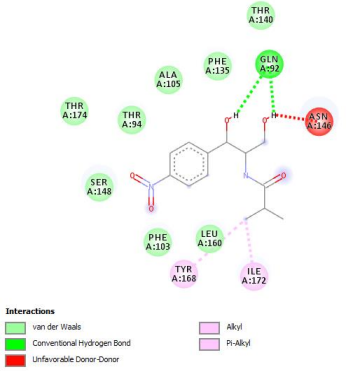
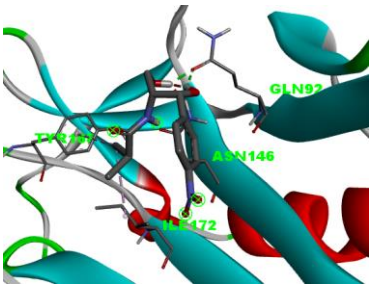
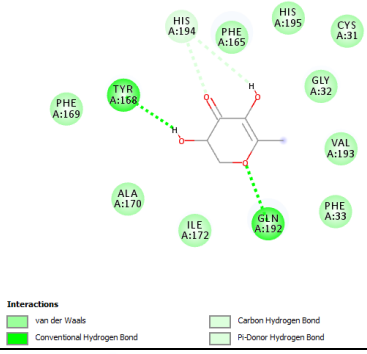
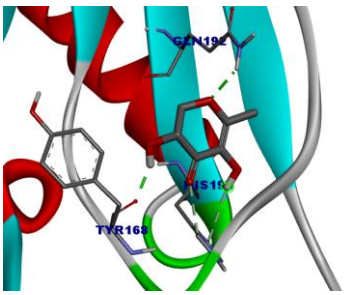
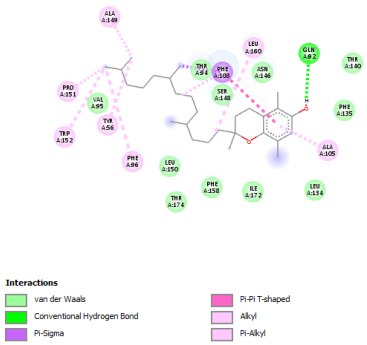
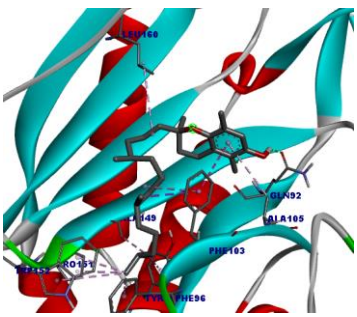
Table 4. Results of Docking of Bay Leaf Flavonoid Extract Compounds against 3CLA Receptors

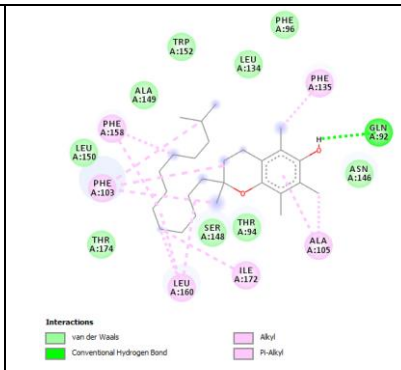
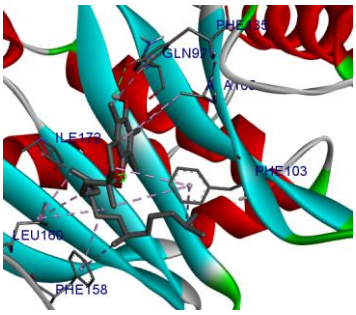
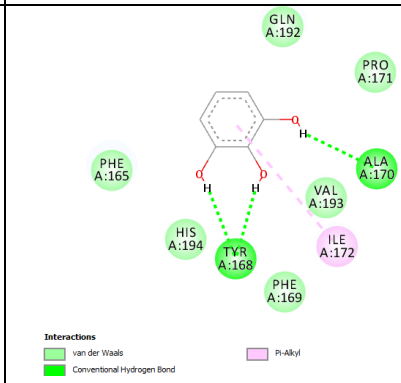
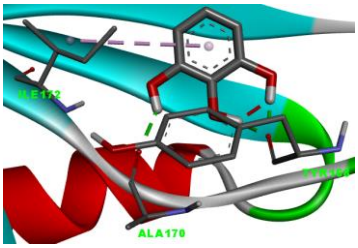
No	Ligan	$\Delta G_{binding}$, kcal/mol	RMSD	H-Bond Acceptor	H-Bond Donor
1	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-4.90	0,14	4	2
2	Alpha-Tocopherol	-7.10	0,00	2	1
3	Beta – Tocopherol	-7.40		2	1
4	Pyrogallol (1,2,3, benzene triol)	-5.30	0.08	3	3
5	Chloramphenicol*	-5.40	0,00	5	3

*) Chloramphenicol was native ligan and positive control

From Table 4, the RMSD results are below 2. This shows that all ligands were validated and following the general rules used. Where, the docking method is said to be valid if the RMSD value is below 2Å (Granchi et al. 2015). The smaller the RMSD value indicated that the ligand position is better because it is closer to its stable conformation (Deligkaris et al. 2014). Meanwhile, the affinity bond between the ligands of flavonoid compounds and their receptors was reflected in the $\Delta G_{binding}$ value (Table 4). If the smaller the value of ΔG binding of a ligand, the energy required to bind to the target receptor was required in smaller amounts, meaning that the easier this ligand binds to the receptor. Minimum $\Delta G_{binding}$ is good for binding form is –7.0 kcal / mol (Deligkaris et al. 2014). So, alpha tocopherol and beta-tocopherol are more likely and easily to bind to 3CLA receptor.

Table 5. 2D and 3D Interaction Receptor – Ligan visualization

No	Ligan	Amino Acid Residu	Binding Visualization	
			2D Image	3D Image
1	Chloramphenicol	**ILE172** **TYR168** **GLN92** LEU160 PHE103 SER148 THR174 THR94 ALA105 PHE135 THR140 ASN146		
2	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	**TYR168** **GLN192** HIS194 PHE169 ALA170 **ILE`172** PHE33 VAL193 GLY32 CYS31 HIS195 PHE165		
3	Beta – Tocopherol	PRO151 TRP152 VAL95 TYR56 PHE96 LEU150 **THR174** PHE158 ILE170 LEU134 **ALA105** PHE135 **THR140** **GLN92** ASN142 **LEU160** **PHE103** **SER148** **THR94** ALA149		

4	Alpha_Tocopherol	**THR174** **LEU160** ILE170 **SER148** **THR94** **ALA105** **ASN146** **GLN92** **PHE135** PHE96 LEU134 TRP152 ALA149 PHE158 LEU150 **PHE103**		
4	Pyragallol (1,2,3, benzenetriol)	**TYR168** **ILE172** ALA170 GLN192 PRO171 VAL193 PHE169 **HIS194** **PHE165**		

The ability to interact with the affinity bond of tocopherol with the 3CLA receptor was better than the positive control - chloramphenicol. This was evidenced by the number of amino acid residues that bind to tocopherol in the various types of bonds that occur. Both hydrogen bonds, hydrophobic, and van der Waals bonds.

4. CONCLUSION

Native test for the presence of flavonoids on bay leaf was indicated by a yellow color change with a slightly acidic amyl alcohol solvent - Mg metal. The flavonoid backbone was indentified by the maximum wavelength scanning results obtained in the first band at 407 nm and second band at 338 nm, which is a special feature of the flavonoid group. The results of GC-MS analysis showed that the flavonoid compounds of bay leaf extract are 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, alpha-tocopherol, beta-tocopherol, and pyrogallol. There is a correlation between the results of the inhibition test against *Salmonella typhi* at a concentration of 100 ppm with the docking results between tocopherol and 3CLA receptors which have affinity bond energy / $\Delta G_{binding}$ = 7.40 kcal /mol.

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