Computational analysis of ethyl acetate extract of *Nauclea subdita* (Korth.) Steud. leaves as peptidoglycan glycosyltransferase inhibitor in *Aeromonas hydrophila*

S Aisiah\(^1\), Olga\(^1\), W A Tanod\(^2\), Y Salosso\(^3\), Bambang\(^4\), and P H Riyadi\(^5\)

\(^1\) Study Program of Aquaculture, Faculty of Fisheries and Marine, Lambung Mangkurat University, Banjarbaru, South Kalimantan 70714, Indonesia
\(^2\) Institute of Fisheries and Marine (Sekolah Tinggi Perikanan dan Kelautan), Palu 94118, Central Sulawesi, Indonesia
\(^3\) Study Program of Aquaculture, Faculty of Marine Sciences and Fisheries, University of Nusa Cendana, Kupang, East Nusa Tenggara, Indonesia
\(^4\) Fisheries Instructor Banjar District Satminkal BPPP Banyuwangi, South Kalimantan, Indonesia
\(^5\) Department of Fish Product Technology, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Central Java 50275, Indonesia

E-mail: sitiaisiah@ulm.ac.id

Abstract. *Aeromonas hydrophila* is a cause of Motile Aeromonad Septicemia (MAS) disease in freshwater fish, which is often endemic and causes significant losses in a freshwater fish farming business. *Aeromonas hydrophila* belongs to the group of gram-negative bacteria and has a complex cell wall consisting of three layers, namely the outer layer in the form of lipoprotein, the middle layer in the form lipopolysaccharide and the inner layer in the form of peptidoglycan. GC-MS analysis of ethyl acetate extract of *Nauclea subdita* leaves was assayed in silico. Seven compounds from GC-MS analysis have potential as glycosyltransferase peptidoglycan inhibitors with highest probability of being active. Peptidoglycan glycosyltransferases play a role in the cell cycle, cell shape, and cell wall. In this study, we conducted a molecular docking of seven compounds for peptidoglycan glycosyltransferase. Binding Affinity Energy (Kcal/mol) of seven compounds was better than beta-lactam as a comparative control. The seven of fourteen compounds found also adhere to Lipinski’s rules on five criteria to indicate these compounds being safely used as oral drugs.

1. Introduction

Motile aeromonad septicemia (MAS) is a disease that often plagues a fish farming unit. MAS disease is caused by *Aeromonas hydrophila* infection that attacks freshwater fish in South Kalimantan, Indonesia. *A. hydrophila* is one of the most common bacteria that infect freshwater fish globally [1]. The characteristic of *A. hydrophila*, which is metropolitan in aquatic environments, allows contact with fish and amphibians. This contact can cause infection depending on the species and its virulence level [2]. *A. hydrophila* can cause mortality rates up to 100% of a fish culture population. The National
Fish and Environmental Health Commission has designated the disease as one of Indonesia's major fish diseases [3].

*A. hydrophila* is a gram-negative bacterium that has a layer of lipopolysaccharides and peptidoglycan in the cell wall. Peptidoglycan is a net-like macromolecule that envelops Gram-negative bacteria, plays a role in giving bacteria form, and protects bacteria from high osmotic pressure [4]. Glycosyltransferases are linked to the cytoplasmic portion of the membranes [5]. By inhibiting the formation of peptidoglycan glycosyltransferase, antibacterial activity ensures that the bacteria can not preserve their shape and defend themselves against osmotic strain.

Bangkal plants classified in the *Nauclea* genus, one of the swamp plants on Kalimantan, Indonesia. Bangkal leaves can function as a natural antibacterial for the treatment of *A. hydrophila* infection in freshwater fish farming. Literature studies show that the Bangkal plants produce bioactives from the tannins, alkaloids, terpenoids, saponins, and phenolic groups. *N. latifolia* leaf extract reported producing bioactive alkaloids, flavonoids, and saponins [6]. The leaves, bark, and roots of *N. latifolia, N. subdita*, and *N. officianalis* reported containing alkaloids, tannins, and phenolics, terpenoids, and saponins [7, 8, 9, 10]. Bioactive substances such as tannins, alkaloids, and flavonoids have shown antibacterial activity in fish [11].

The antibacterial action mechanism of each metabolite has a specific character to inhibit the growth of bacteria. The different activities that develop due to bioactive compounds have various synergistic effects based on the characteristics and morphology of the bacteria [12]. Ideally, an antimicrobial agent, including an antibacterial agent, would have selective toxicity, meaning that the antimicrobial substance is detrimental to specific pathogens but does not endanger the host. The mechanism of action of most antimicrobial substances divided into 1) inhibition of cell wall synthesis, 2) inhibition of cell membrane function, 3) inhibition of protein synthesis, and 4) inhibition of nucleic acid synthesis. 5) disrupting the structure of the cell membrane [13].

The use of antibiotics and synthetic chemicals to treat infected fish has now begun to be abandoned. The use of natural products using plant extracts is being used to control *A. hydrophila* infection. Previous research has analyzed ethyl acetate extract from *Nauclea subdita* leaves with GC-MS and found that 14 compounds and 12 of them were terpenoids [14]. Terpenoid compounds play a variety of roles in milk, medicine, cosmetics, hormones, and vitamins [15]. The terpenoid compounds react with the active side of the membrane, shed lipid constituents, and increase permeability [16]. As a drug terpenoid compounds work as antibacterial by damaging bacterial cell membranes.

In this study, a computational analysis of GC-MS compounds was performed by observing the molecular docking and antimicrobial mechanism of the ethyl acetate extract of *Nauclea subdita* (Korth.) Steud. as an inhibitor of peptidoglycan formation on the cell wall of *A. hydrophila*. This study aimed to determine the potential and action mechanism of compounds contained in the ethyl acetate extract of *N. subdita* leaves (GC-MS analysis) used computational study as peptidoglycan glycosyltransferase inhibitor. In this study, molecular docking between compounds (ligands) with peptidoglycan glycosyltransferase receptor proteins was visualized. The position of protein receptor adhesion, the types of bonds between ligands-receptors, and the pathway of the compounds as peptidoglycan glycosyltransferase inhibitor are known.

2. Material and methods

Computational analysis (in silico) is a method used to design a drug by computerization by selecting target proteins, visualizing the structure of target proteins, and developing drug molecules or chemical compounds based on target proteins. The analysis carried out on 14 compounds resulting from Gas Chromatography Mass Spectrometry (GC-MS) analysis of Bangkal leaves extract. Steps were taken in this study:

- Analyzing the prediction of potential compound activity in the Bangkal leaf ethyl acetate extract using WAY2DRUG PASS prediction [17]. Search for probability to be active (Pa) values for the prediction of antibacterial-related activities. Pa value is a value that describes the potential activity of a compound. If the Pa value is more than 0.7, it indicates that the compound predicted
to have high activity potential as a computational or laboratory test. If the Pa value is more than 0.3 but less than 0.7, the compound has the computational ability as an antibacterial, but it has not been proven in laboratory assays.

- The peptidoglycan glycosyltransferase protein sequence used in this study was downloaded from Uniprot (https://www.uniprot.org/) with entry number A0A081UYK9. Peptidoglycan glycosyltransferase protein was selected based on an analysis of the potential activity of the ethyl acetate extract of Bangkal leaves.
- Protein sequences are modeled using the homology modeling method using a web server (https://swissmodel.expasy.org/).
- The compounds (GC-MS analysis) were simulated molecular docking using PyRx 0.8 software to determine the energy of the bond's affinity and the ability of the compound to bind with the peptidoglycan glycosyltransferase protein [18].
- Bond interactions visualized using Discovery Studio Visualizer software.
- The Compounds (GC-MS analysis) analyzed as drug-likeness according to Lipinski’s rules with SwissAdme Software [19].
- The compounds were analyzed for Toxicity predictions with Protox II software [20].

3. Results and discussion

3.1 Results
The bioactive profile of the ethyl acetate extracts of *N. subdita* leaves using GC-MS found fourteen compounds. The results of the GC-MS analysis can be seen in Table 1.

| No | Compounds           | Formula       | PubChem (CID) | Metabolites Group |
|----|---------------------|---------------|---------------|------------------|
| 1  | Beta-caryophyllene  | C_{15}H_{24}  | 5281515       | Terpenoids       |
| 2  | Alpha guaiane       | C_{15}H_{24}  | 5317844       | Terpenoids       |
| 3  | Seychellene         | C_{15}H_{24}  | 519743        | Terpenoids       |
| 4  | Selina -3,7(11)-diene | C_{20}H_{24} | 522296        | Terpenoids       |
| 5  | Methandrostenolon   | C_{20}H_{24}O_2 | 6300         | Steroids         |
| 6  | Alpha panasinsen    | C_{15}H_{24}  | 578929        | Terpenoids       |
| 7  | Alpha-patchoulene   | C_{15}H_{24}  | 521710        | Terpenoids       |
| 8  | E germacrene D      | C_{15}H_{24}  | 5317570       | Terpenoids       |
| 9  | (+)-aromadendrene   | C_{15}H_{24}  | 11095734      | Terpenoids       |
| 10 | Allo-aromadendrene  | C_{15}H_{24}  | 42608158      | Terpenoids       |
| 11 | Longifolen          | C_{15}H_{24}  | 1796220       | Terpenoids       |
| 12 | Alpha-Bulnesene     | C_{15}H_{24}  | 94275         | Terpenoids       |
| 13 | Phthalic acid       | C_{4}H_{7}O_{4} | 1017         | Terpenoids       |
| 14 | Patchouli alcohol   | C_{15}H_{26}O | 10955174      | Terpenoids       |

Bioactive compounds on the leaves of Bangkal (*N. subdita*) predicted to be potential as antibacterial using WAY2DRUG PASS prediction, compared to comparative control of beta-lactam. The value of Pa (Probability to be active) is a value that describes a compound's potential. Bioactive compounds in the ethyl acetate extract of Bangkal leaf (*N. subdita*) having different Pa values presented in Figure 1.

Figure 1 showed only seven of fourteen compounds that have a Pa value above 0.5. Then the seven compounds computed by molecular docking using PyRx 0.8 software to determine the binding affinity energy and the ability of the compounds to bind peptidoglycan glycosyltransferase receptor protein. The binding affinity energy of compounds in the ethyl acetate extracts of Bangkal leaves could be seen in Figure 2.
Figure 1. Potential of bangkal leaves extract with WAY2DRUG PASS as peptidoglycan glycosyltransferase inhibitor
Figure 2. Binding affinity energy (Kcal/mol) of the seven compounds with peptidoglycan glycosyltransferase receptor protein

Furthermore, the molecular docking simulation between seven compounds with peptidoglycan glycosyltransferase receptor proteins visualized with Discover Studio Visualizer software. Visualization of molecular docking can be seen in Figure 3. Figure 3 showed seven compounds (GC-MS analysis) of the ethyl acetate extracts of Bangkal leaves (N. subdita), which can bind to the active site of the peptidoglycan glycosyltransferase receptor protein. Then the binding position visualized in 2 dimensions so that the position of the amino acid receptor protein binding to the ligand (compound) can be known. 2-dimensional visualization can be seen in Figure 4.

Figure 4 showed seven compounds (GC-MS analysis), there are interactions of hydrophobic bonds and hydrogen bonds with receptor proteins. Hydrophobic bonding interactions occur between Seychellene and Longifolen binding to the receptor protein in Lysine (A:350) with distances of 4.10 and 3.79 Å (respectively). Selina-3,7 (11)-diene interacts with receptor proteins with hydrophobic bonds, on Proline (A:272) with a distance of 4.06 Å. Methandrostenolone interacts with hydrophobic bonds in Leucine (A:216) at a distance of 4.34 Å. Alpha panasinsen interacts with receptor proteins with hydrophobic bonds, in Tyrosine (A:24) with a distance of 3.48 Å. Hydrogen bonding occurs between phthalic acid interacting with the receptor protein on Proline (A:176) with a distance of 2.14 Å. Patchouli alcohol interacts with receptor proteins through hydrogen bonds, in Serine (A:76) at a distance of 2.09 Å. In comparison control, beta-lactam interacts with receptor proteins through hydrogen bonds in Glutamine (A:127) at a distance of 2.40 Å.

Furthermore, seven compounds from the ethyl acetate extract of N. subdita leaves analyzed for the pathways as peptidoglycan glycosyltransferase inhibitors. Pathway analysis performed with STITCH software. From the STITCH database, only four compounds obtained, which could be described as pathways, namely logifolene, phthalic acid, patchouli alcohol, and seychellene. Pathway analysis can be seen in Figure 5.

Seven compounds were then analyzed with SwissAdme software. The analysis aims to determine the similarity of the compound as a medicinal compound. Analysis of drug-likeness with SwissAdme presented in Table 2.
Figure 3. Visualization of molecular docking seven compounds with peptidoglycan glycosyltransferase receptor protein.
Figure 4. Visualization of 2 dimensions of seven compounds with peptidoglycan glycosyltransferase receptor protein.
Figure 5. Pathway mechanism as peptidoglycan glycosyltransferase inhibitor

Then the seven compounds were analyzed to predict their toxicity with Pro-Tox II software to determine the prediction of LD$_{50}$. Toxicity assessed in LD$_{50}$ in units of mg/kg body weight. LD$_{50}$ is the median lethal dose, where 50% of test subjects die after exposure to a compound. Toxicity classes defined according to a globally harmonized chemical labeling (GHS) classification system. LD$_{50}$ class system, namely Class I: fatal (LD$_{50}$ ≤ 5); Class II: highly toxic (5 < LD$_{50}$ ≤ 50); Class III: toxic (50 < LD$_{50}$ ≤ 300); Class IV: dangerous (300 < LD$_{50}$ ≤ 2000); Class V: may be dangerous (2000 < LD$_{50}$ ≤ 5000); and Class VI: non-toxic (LD$_{50}$ > 5000). LD$_{50}$ analysis results from 7 active compounds GC-MS analysis with Pro-Tox II presented in Table 3.
Table 2. Analysis of drug-likeness

| Compounds            | Molecular Weight < 500 Dalton (g/mol) | High Lipophilicity (expressed as Log P < 5) | Hydrogen Bond Donors < 5 | Hydrogen Bond Acceptors < 10 | Molar Refractivity 40-130 |
|----------------------|---------------------------------------|---------------------------------------------|--------------------------|------------------------------|-----------------------------|
| Alpha panasinsen     | 204.35                                | 5.65                                        | 0                        | 0                            | 66.62                       |
| Methandrostenolon    | 300.44                                | 3.73                                        | 1                        | 2                            | 89.73                       |
| Selina -3,7(11)-diene| 204.35                                | 4.63                                        | 0                        | 0                            | 68.78                       |
| Longifolen           | 204.35                                | 5.65                                        | 0                        | 0                            | 66.88                       |
| Phthalic acid        | 166.13                                | 1.20                                        | 2                        | 4                            | 40.36                       |
| Patchouli alcohol    | 222.37                                | 3.81                                        | 1                        | 1                            | 68.56                       |
| Seychellene          | 204.35                                | 5.65                                        | 0                        | 0                            | 66.88                       |
| Beta-Lactam          | 188.18                                | -3.28                                       | 4                        | 5                            | 46.24                       |

Table 3. LD₅₀ prediction with Pro-Tox II

| Compounds            | Prediction of LD₅₀ (mg/kg) | Class |
|----------------------|---------------------------|-------|
| Seychellene          | 5000                      | V     |
| Selina -3,7(11)-diene| 4400                      | V     |
| Methandrostenolon    | 1000                      | IV    |
| Alpha panasinsen     | 3700                      | V     |
| Longifolen           | 5000                      | V     |
| Phthalic acid        | 2530                      | V     |
| Patchouli alcohol    | 940                       | IV    |
| Beta-Lactam          | 5000                      | V     |

3.2 Discussion

Peptidoglycan glycosyltransferase is an enzyme that plays a role in the biosynthesis of peptidoglycan in the formation of bacterial cell walls [21]. By inhibiting the action of the peptidoglycan glycosyltransferase enzyme, the bacteria cannot synthesize peptidoglycan, so that the bacteria cannot maintain their shape and protect themselves against osmotic pressure. Previous research reported that after incubation, 12 hours of treatment of Bangkal leaves ethyl acetate extract seen A. hydrophila cell walls disrupted. After 24 hours incubation saw the damage to A. hydrophila cell walls [22].

In this study, it was suspected that terpenoid derivatives from the ethyl acetate extracts of Bangkal leaves play a role in cell wall damage. This study supported the results of previous studies that reported essential oil compounds from the terpenoids group can disrupt bacterial cell walls [23]. Eucalyptol terpenoid compounds reported causing damage to gram-negative bacterial cells by making bacterial cell wall leakage [24]. Likewise, it reported that essential oil compounds from terpenoids could cause the release of gram-negative bacterial cell constituents [25]. Terpenoid compounds can affect the outer membrane permeability of bacterial cells [26]. The terpenoid compounds were known to interact with the phospholipid membranes making up bacterial cells, in terms of the effects of fulidation and the introduction of lipophilic molecules that act as intruders in the structure of bacterial lipid bilayer membrane structures [27]. Antibacterial compounds can react with the phospholipid component and cause cell lysis [28]. Cells that lysed will cause the release of cell walls in Gram-negative bacteria called spheroplasts. The terpenoid group compounds can inhibit cytoplasmic membranes, nucleic acid synthesis, damage cell walls. They can also inhibit oxygen consumption by interfering with the performance of the electron chain in pathogenic bacteria. The content of compounds in the ethyl acetate extracts of Bangkal leaves, generally classified in the terpenoids. Therefore, the ethyl acetate extracts of Bangkal leaves can disrupt the cell wall of A. hydrophila by inhibiting the action of the enzyme peptidoglycan glycosyltransferase, which plays a role in the biosynthesis of peptidoglycan.
The working principle of compounds in the ethyl acetate extracts of Bangka leaves is similar to Beta-lactam. Beta-lactam works to kill bacteria by inhibiting the synthesis of cell walls. In the cell wall formation process, a transpeptidation reaction occurs, catalyzed by the enzyme transpeptidase, and results in cross-linking between two peptidoglycan chains. The transpeptidase enzyme, which was located on the cytoplasmic membrane of bacteria, can bind beta-lactams so that this enzyme is unable to catalyze the transpeptidation reaction even though the cell wall continues to be formed. The formed cell wall does not have cross bonds, and the peptidoglycan formed is imperfect, so it is weaker and easily degraded. Under normal conditions, the difference in osmotic pressure in gram-negative bacterial cells can lead to cell lysis. In addition, the protein transpeptidase complex and beta-lactam antibiotics stimulate autolysin compounds, which can digitize the bacterial cell walls. Thus, bacterial cell walls undergo lysis and eventually die [29].

Figure 2 showed the compounds resulting from GC-MS analysis have lower binding affinity energy than beta-lactam. This showed that seven compounds (GC-MS analysis) could bind to the peptidoglycan glycosyltransferase receptor protein. This statement, reinforced by the visualization in Figure 3, showed the ability of compounds to bind well to the receptor protein's active site. The type of bond between seven compounds with receptor proteins, namely hydrophobic alkyl and hydrogen, shows the ability of seven compounds as inhibitors. Substances that act as inhibitors have many hydrophobic and hydrogen bonds [30].

In the interaction of compounds (ligands) with proteins (receptors), there are three main types of chemical bonds: covalent bonds, electrostatic bonds, and hydrophobic bonds. Covalent bonds were powerful bonds and are not reversible in biological conditions. Electrostatic bonds were more common than covalent bonds in the interaction of receptors and ligands, including hydrogen and van der Wals bonds. Hydrophobic bonds are relatively weak bonds but are very important in the interaction of receptors and ligands that are very fat-soluble with fats from cell membranes, including alkyl bonds.

Pathway analysis with STITCH in Figure 5 showed the mechanism of longifolene as peptidoglycan glycosyltransferase inhibitors, through the gpp protein (guanosine). The gpp protein catalyzes the conversion of pppGpp (guanosine pentaphosphatase) to ppGpp (guanosine phosphatase). The pppGpp protein was a cytoplasmic signaling molecule that plays a role in regulating bacteria's amino acid requirements. Longifolene inhibits the response of extracellular stimuli in bacterial cell membranes by inhibiting the need for amino acids in forming peptidoglycan membranes [31, 32, 33]. Peptidoglycan is a polymer consisting of sugars and amino acids that form a layer outside the plasma membrane in most bacteria and play a role in forming cell walls [34]. By inhibiting the amino acid requirements of bacteria, peptidoglycan membranes cannot be formed.

Figure 5 also showed that longifolene could also act as a peptidoglycan inhibitor via the dinB → dnaE → dnaN → polA pathway. This chain was a protein that plays a role in inhibiting pyrimidine metabolism. In peptidoglycan synthesis, the first stage of glutamine donates an amino group to the sugar, fructose 6-phosphate. This converts fructose 6-phosphate to glucosamine-6-phosphate. In the second step, the acetyl group was transferred from acetyl COA to the amino group in glucosamine-6-phosphate, which produces N-acetyl-glucosamine-6-phosphate. In the third stage of the synthesis process, N-acetyl-glucosamine-6-phosphate was isomerized, which will convert N-acetyl-glucosamine-6-phosphate to N-acetyl-glucosamine-1-phosphate [35]. N-acetyl-glucosamine-1-phosphate, which was now a monophosphate in the fourth stage, uses UTP (Uridine triphosphate). UTP was a pyrimidine nucleotide, can act as an energy source. In this particular reaction, after monophosphate uses UTP, an inorganic pyrophosphate was released and replaced by monophosphate, forming UDP-N-acetylglucosamine (When UDP used as an energy source, it releases inorganic phosphate) [36]. This initial stage used to make the NAG (N-acetyl-glucosamine) precursor in peptidoglycan [34]. Therefore, by inhibiting pyrimidine metabolism, the bacteria do not get the energy to synthesize peptidoglycan.

Figure 5 also showed the peptidoglycan inhibitor pathway mechanism by phthalic acid through the nadC → nadD → nadB → nadA pathway. This protein chain is a protein that plays a role in the process of biosynthesis of pyrimidine nucleotides. Pyrimidine nucleotides could act as an energy source [36].
The ability of phthalic acid inhibited the biosynthesis of pyrimidine nucleotides so that bacteria do not get energy in synthesizing peptidoglycan.

Figure 5 showed the peptidoglycan inhibitors' mechanism by patchouli alcohol through the farnesyl pyrophosphate (FPP) pathway as a catalyst for longifolene synthase (LgfS). LgfS catalyzes the biosynthesis of longifolene using FPP as a direct substrate through the polycyclization reaction. Gram-negative bacteria use part of FPP for the synthesis of octaprenyl pyrophosphate (OPP) and undecaprenyl pyrophosphate (UPP) as part of its primary metabolism [37]. OPP is responsible for synthesizing isoprenoid quinone side chains, ubiquinone-8, and demethylmenaquinone-8, which are essential for the respiratory chain [38]. UPP is an important component involved in the construction of peptidoglycan cell walls [39].

Figure 5 also showed the mechanism of the peptidoglycan inhibitor pathway by seychellene through the farnesyl pyrophosphate (FPP) pathway. However, it is different from the pathway of patchouli alcohol. Seychellene, together with the alpha-guaiene, alpha-bulnesene, alpha-patchoulene, and patchouli alcohol, stimulates farnesyl pyrophosphate (FPP). FPP plays a role in the synthesis of undecaprenyl pyrophosphate (UPP) involved in constructing peptidoglycan cell walls [39].

Druglikeness analysis, according to Lipinski's rule, showed that seven compounds that have the potential as peptidoglycan glycosyltransferase inhibitors could function as drugs because they meet the standards of the Five Lipinski Rules. Comprehensive Medicinal Chemistry (CMC), Derwent World Drug Index (WDI) and Modern Drug Data Report (MDDR) collect a database of compounds that have similarities as drugs by oral administration, known as Rules of Five (RO5), namely molecular weight (<500 Da); High lipophilicity (expressed as LogP <5); less than five hydrogen bond donors; less than ten hydrogen bond acceptors; and molar refractivity between 40-130 [19]. In addition, the prediction of toxicity from seven compounds included in class V, which is a group with LD50 that is safe to use. Therefore, computationally ethyl acetate extract of N. subdita (Korth.) Steud. leaves, potentially as a safe antibacterial with a mechanism of action as a peptidoglycan glycosyltransferase inhibitor in gram-negative bacteria such as A. hydrophila.

4. Conclusion
Computational and molecular docking simulations of seven from fourteen compounds (GC-MS) have the strongest potential as peptidoglycan glycosyltransferase inhibitors, namely logifolene, phthalic acid, patchouli alcohol, selina-3,7(11)-diene, methandrostenolon, alpha panasinsen, dan seychellene. The seven compounds interact with receptor proteins through hydrophobic and hydrogen bonds. Seven compounds act as peptidoglycan glycosyltransferase inhibitors by inhibiting the need for A. hydrophila in obtaining amino acids and energy.

References
[1] Cipriano C R 2001 Aeromonas hydrophila and motile aeromonad septicemias of fish Fish Disease (Washington: United States Departement of the interior)
[2] Floyd R F 2002 Aeromonas Infection (Florida : University of Florida)
[3] Krom D A, Wira R, Perkasa B, Tiara R and Wasito 2014 J. Sain Vet. 32 105
[4] Vollmer W, Blanot D and de Pedro M A 2008 FEMS Microbiol. Rev. 32 149
[5] Van den Brink-van der Laan E, Boots J W P, Spelbrink R E J, Kool G M, Breukink E, Killian J A and de Kruijf B 2003 J. Bacteriol. 185 3773
[6] Ettebong E O, Edwin U P M, Edet E C, Samuel E U, Ezekiel A O and Dornu T V 2014 Asian J. Med. Sci. 6 6
[7] Amos S, Abbah J, Chindo B, Edmond I, Binda L, Adzu B, Buhari S, Odutola A A, Wambebe C and Gamaniel K 2005 J. Ethnopharmacol. 97 53
[8] Abbah J, Amos S, Chindo B, Ngazal I, Vongtau H O, Adzu B, Farida T, Odutola A A, Wambebe C and Gamaniel K S 2010 J. Ethnopharmacol. 127 85
[9] Liew S Y, Looi C Y, Paydar M, Cheah F K, Leong K H, Wong W F, Mustafa M R, Litaudon M and Awang K 2014 PLoS One 9 e87286
[10] Liew S Y, Khaw K Y, Murugaiyah V, Looi C Y, Wong Y L, Mustafa M R, Litaudon M and Awang K 2015 *Phytomedicine* **22** 45
[11] Haniffa M A and Kavitha K 2012 *J. Agric. Technol.* **8** 205
[12] Nohynek L J, Alakomi H, Käähkönen M P, Heimonen M, Helander I M, Oksman-Caldentey K-M and Puupponen-pimiä R H 2006 *Nutr. Cancer* **54** 18
[13] Jawetz E, Melnick J. and Adelberg E 2005 *Medical Microbiology* (Jakarta: Salemba Medika) Edition XXII pp 327-335
[14] Aisiah S, Prajitno A, Maftuch and Yuniarti A 2018 *Russ. J. Agric. Socio-Economic Sci.* **6** 488
[15] Paridah M, Moradbak A, Mohamed A, Owolabi F Abdulwahab Taiwo, Asniza M and Abdul Khalid S H 2016 *Terpenes and Terpenoids* (New York: IntechOpen)
[16] Cowan M M 1999 *Clin. Microbiol. Rev.* **12** 564
[17] Filimonov D A, Lagunin A A, Gloriozova T A, Rudik A V., Druzhilovskii D S, Pogodin P V and Porokov V V 2014 *Chem. Heterocycl. Compd.* **50** 444
[18] Olson A and Trott O 2011 *NIH Pubic Access* **31** 455
[19] Lipinski C A 2004 *Drug Discov. Today Technol.* **1** 337
[20] Banerjee P, Eckert A O, Schrey A K and Preisssner R 2018 *Nucleic Acids Res.* **46** W257
[21] Derouaux A, Sauvage E and Terrak M 2013 *Front. Immunol.* **4** 78
[22] Aisiah S, Prajitno A, Maftuch and Yuniarti A 2019 *AACL Bioflux* **12** 2093
[23] di Pasqua R, Ercolini D, Mauriello G, Betts G, Hoskins N and Edwards M 2007 *J. Agric. Food Chem.* **55** 4863
[24] Zengin H and Baysal A H 2014 *Molecules* **19** 17773
[25] Lv F, Liang H, Yuan Q and Li C 2011 *Food Res. Int.* **44** 3057
[26] Hirakura Y, Alvarez-Bravo J, Kurata S I, Natori S and Kirino Y 1996 *J. Biochem.* **120** 1130
[27] Trombetta D, Venuti V, Cristani M, Saija A, Bisignano G, Castelli F, Sarpietro M G, Daniele C and Mazzanti G 2005 *Antimicrob. Agents Chemother.* **49** 2474
[28] Davidson P and Branen A 1980 *Food Sci* **45** 1607
[29] Giguère S 2013 Therapy antimicrobial drug action and interaction: an introduction *Antimicrobial Therapy in Veterinary Medicine* (5th Edition) ed J F Prescott and P M Dowling (New Jersey: Wiley Blackwell) pp 3–10
[30] Yahmin Y, Faqih K and Subarti S 2019 *JC-T (Journal Cis-Trans) J. Kim. dan Ter.* **3** 34
[31] Artsimovitch I, Patlan V, Vassyleva M N, Hosaka T, Yokoyama S and Vassylev D G 2002 *Cell* **117** 299
[32] Magnusson L U, Farewell A and Nyström T 2005 *Trends Microbiol.* **13** 236
[33] Potrykus K and Cashel M 2008 *Annu. Rev. Microbiol.* **62** 35
[34] Demchick P and Koch A L 1996 *J. Bacteriol.* **178** 768
[35] White D, Drummond J and Fuqua C 2012 *Physiology and Biochemistry of Prokaryotes* (Oxford: Oxford University Press)
[36] Hogan M C 2010 *Bacteria The Encyclopedia of Earth* ed C J Cleveland and S Draggan (Washington D C: Environmental Information Coalition, National Council for Science and the Environment)
[37] Cao Y, Zhang R, Liu W, Zhao G, Niu W, Guo J, Xian M and Liu H 2019 *Sci. Rep.* **9** 95
[38] Okada K, Minehira M, Zhu X, Suzuki K, Nakagawa T, Matsuda H and Kawamukai M 1997 *J. Bacteriol.* **179** 3058
[39] Guan Z, Breazeale S D and Raetz C R H 2005 *Anal. Biochem.* **345** 336

**Acknowledgments**

All authors thanks to the Rector of Lambung Mangkurat University and the Dean of Faculty of Fisheries and Marine, Lambung Mangkurat University for providing research facilities. All authors also said thank you to Didik Wahyudi, S.Si., M.Si. from UIN Malang for guidance in using computing software.