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In Silico Study of Betaine, Isoleucine, and DL-Stachydrine Compounds in Shipworm (*Spathoteredo obtusa*) Extract as an Antibacterial Agent of *Aeromonas hydrophila*

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Abstract

The rapid increase in freshwater commodity production is accompanied by various obstacles that pose challenges for farmers, namely, disease infections. Bacterial infection by *Aeromonas hydrophila* is the pathogenic agent causing Motile Aeromonas Septicemia (MAS), which can result in 100% mortality within a short period. The AhlC toxin protein in *A. hydrophila* bacteria plays the most critical role in the Ahl tripartite toxin, as AhIC acts as a protomer and inserts itself into one membrane layer, then binds to AhlB and AhlA to form pores in both membrane layers. Active compounds found in marine mussel extracts (*Spathoteredo obtusa*), particularly betaine, isoleucine, and DL-stachydrine, have the potential to inhibit the AhlC toxin protein produced by *A. hydrophila* bacteria. This study aims to predict the interaction between the AhlC receptor protein in *A. hydrophila* bacteria and the active compounds identified from the extract of shipworms (*S. obtusa*) using molecular docking methods. The test results showed that all three compounds met all ADME predictions, with the best binding affinity value of -4.2 kcal/mol for isoleucine and DL-stachydrine, followed by -3.5 kcal/mol for betaine. Based on the test results, there are appropriate, stable, and effective hydrogen and electrostatic charge interactions with the ligand-receptor complex (ASN:32, GLN:35, ARG:112, and ASP:116), which play a crucial role as active sites in ligand binding to the receptor.

INTRODUCTION

The production of superior freshwater commodities in Indonesia, such as catfish (*Pangasius* sp.), catfish (*Claridae* sp.), tilapia (*Oreochromis* sp.), and carp (*Cyprinus* sp.), has increased up to 9.26% over the last 3 years from 2020-2023, from the initial production of only 3.054.216 to 3.336.905 tons (Statistics Indonesia, 2025). The rapidly increasing production of freshwater commodities aligns with the high demand from national and foreign consumers. However, along with the increasing production of freshwater commodities in Indonesia, there is one major obstacle that

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becomes an obstacle for farmers, namely disease infection. One type of disease that is often found in freshwater commodities in tropical waters is bacterial disease infection caused by *Aeromonas hydrophila* bacteria, which is a pathogenic agent that causes Motile Aeromonas Septicemia (MAS) disease (Ramona *et al*., 2024).

Freshwater commodities infected with *A. hydrophila* bacteria have clinical symptoms in the form of skin lesions, pale liver color, organ swelling, and ulcers on the body. In addition, fish will swim slowly, appetite decreases, eyes become prominent, and bleeding occurs in several parts of the body (Nhinh *et al*., 2021). Fish that have been acutely infected with *A. hydrophila* bacteria, which do not have a good body defense system, will experience mass mortality of up to 80-100% within 1-2 weeks (Pereira *et al*., 2022). This adversely affects the economy of aquaculture due to the high mortality rate caused by *A. hydrophila* infection. Prevalence rates of *A. hydrophila* infection ranged from 12,5-47,3% in O. Niloticus species (Ammar *et al*., 2023), and the prevalence of *A. hydrophila* infection associated with freshwater fish disease was 46.4% (Nhinh *et al*., 2021). Currently, the treatment of *A. hydrophila* bacterial infections in freshwater commodities still relies heavily on antibiotics. However, the continuous use of antibiotics makes the aquatic environment polluted to damage the quality of fishery products. In addition, bacteria will accelerate the process of resistance formation, making it more difficult to handle (Okeke *et al*., 2022).

A. hydrophila bacteria that infect freshwater aquaculture commodities have a level of pathogenicity and virulence caused by the ability of bacteria to produce several components that intersect directly with gastroenteritis, endotoxins, exotoxins, siderophores, cytotoxins, adhesins, invasins, S-layers, and flagella (Semwal *et al*., 2023). The molecule with the most toxic and lethal properties produced by *A. hydrophila* bacteria is are exotoxin, in the form of hemolysin, which can lyse erythrocytes in infected commodities, causing a decrease in erythrocytes and hematocrit (Hasna *et al*., 2024). Hemolysin itself consists of three interconnected components, namely AhlA, AhlB, and AhlC, which have their respective roles and mechanisms in lysing cells. In addition, hemolysin also belongs to the tripartite alpha-Pore Forming Toxins (α-PFT) group, which is a collection of toxic proteins that can cause pore formation in the membrane of infected target cells (Wilson *et al*., 2019). Efforts can be made to minimize the impact of *A. hydrophila* bacterial infection by utilizing phytochemical compounds from natural materials that are proven to inhibit toxin production and kill bacteria (Affandi and Setyono, 2025).

Shipworm is one of the natural materials with great potential that is still rarely utilized and is very abundant in the coastal waters of Indonesia. Previous research analyzing the content of compounds in the shipworm species Bactronophorus sp. Obtained the results of compound content in the form of eight types of essential amino acids and eight types of non-essential amino acids (Wairara *et al*., 2020). Other studies have also found other compounds such as fatty acids, vitamins B12, B6, minerals calcium, phosphorus, iron, zinc, selenium, magnesium, and contain alkaloids, flavonoids, steroids, triterpenoids, and saponins identified from shipworm species *Bactronophorus* sp. (Wiralis *et al*., 2024). In the shipworm digestive tract (in the gut), there are carbohydrate-active enzymes (CAZymes) that have antimicrobial properties, especially in gram-negative bacteria, and contribute to the degradation of lignocellulose (Borges *et al*., 2021). The number of potential compounds that have been identified has encouraged researchers to continue developing the potential of other shipworm species such as *Spathoteredo obtusa*, which is commonly found in Indonesian estuarine waters and plays an important ecological role as a biodegrader of dead wood in mangrove ecosystems. However, research examining its potential as an antibacterial agent in aquaculture remains limited (Hendy *et al*., 2022). This statement reinforces the importance of

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examining the potential compound content possessed by *S. obtusa* as an antibacterial against *A. hydrophila* in this study.

The in silico method is used to provide an overview of the interaction between the dominant active compounds contained in the shipworm extract (*S. obtusa*) against the target toxin protein in the form of AhlC produced by *A. hydrophila* bacteria, with the help of computers (Najih *et al*., 2023). The AhlC toxin protein in *A. hydrophila* bacteria has the most crucial role in the tripartite Ahl toxin, because AhIC becomes a protomer and inserts itself into one membrane leaflet, then binds AhlB and AhlA to form a pore in both leaflets (Herrera *et al*., 2022). This pore-forming mechanism is central to the toxicity and virulence of *A. hydrophila*, making AhlC a strategic target for anti-virulence interventions. Based on this premise, this study aims to predict interactions between the AhlC receptor protein and a series of bioactive ligands derived from shipworm extract (*S. obtusa*) using a molecular docking approach.

The novelty of this study lies in the exploration of *S. obtusa*, a marine bivalve mollusk commonly known for its wood-boring capabilities, as a previously unreported source of potential anti-virulence compounds against *A. hydrophila*. While *S. obtusa* has been studied primarily in the context of marine ecology and bioerosion, its secondary metabolites remain largely uncharacterized for biomedical or antimicrobial applications. This research is the first to investigate the potential inhibitory interaction between compounds from *S. obtusa* and the AhlC toxin, aiming to disrupt the early stage of pore formation in the bacterial membrane. Therefore, this study not only contributes to the search for new natural antibacterial agents but also opens new avenues for the utilization of marine invertebrates in combating aquaculture pathogens through anti-virulence strategies.

METHODOLOGY

Ethical Approval

Ethical approval is not required. The authors declare that this study does not involve experiments with human subjects or live animals directly.

Place and Time

This research was conducted at the Laboratory of Fish Disease and Health, Faculty of Fisheries and Marine Science, and the Integrated Research Laboratory (LRT) of Universitas Brawijaya. This research was conducted from September 2024 to April 2025.

Research Materials

The tools and materials used in this study were shipworm (*S. obtusa*) extract, 96% PA ethanol, vortex, centrifuge, 0.22 μm syringe filter, vial bottle, LC-HRMS machine (Thermo Scientific Dionex Ultimate 3000 RSLCnano-Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer) (Waltham, Massachusetts, USA), and laptop with Intel(R) Celeron(R) CPU N2840 @ 2.16GHz, 4 GB RAM Microsoft Windows 10 Pro 64-bit operating system.

Research Design

The design of this research is the interaction between ligand and receptor (target protein) using molecular docking. The ligands in the study refer to three dominant compounds from shipworm (*S. obtusa*) extracts in the form of betaine, DL-stachydrine, and isoleucine, while the receptor refers to the AhlC toxin in *A. hydrophila* bacteria.

Work Procedure

Sample Preparation of Shipworm Extract (*S. obtusa*)

Shipworm samples were obtained in mangrove areas located in Sedati District, Sidoarjo Regency, East Java. The extraction process in this study used the maceration method with 96% ethanol solvent at a ratio of 1:5 and left at room temperature for 3×24 hours, with filtration performed every 1×24

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hours. After that, filtration was performed and evaporation was carried out using a rotary evaporator to remove the solvent (Wairara *et al*., 2020).

Compound Screening

Screening of shipworm extract compounds was carried out through the LC-HRMS method using 100 μL extract samples dissolved in 1.5 mL of 96% PA ethanol. The sample was then homogenized using a vortex at a speed of 2,000 rpm for 2 minutes, followed by a spindown at a speed of 6,000 rpm for 2 minutes. Next, the supernatant from the spindown, filtered using a 0.22 μm syringe filter, was taken and put into a vial bottle. The sample in the vial was then inserted into the autosampler and injected into the LC-HRMS machine (Thermo Scientific Dionex Ultimate 3000 RSLCnano-Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer). The LC-HRMS process was carried out for 30-60 minutes, and continued with the software data process using Compound Discover with mzCloud MS/MS Library (Klijnstra *et al*., 2021).

Ligand Structure Molecular Preparation

The structures of betaine, isoleucine, and dl-stachydrine, were obtained from the PubChem database website (https://pubchem.ncbi.nlm.nih.gov/) using the following PubChem CID numbers: 247 for betaine, 6306 for isoleucine, and 555 for dl-stachydrine. The structures of the active compounds were downloaded in 3-dimensional form in SDF format. The file was converted into PDB using the OpenBabel GUI application.

Protein Receptor Preparation

The structure of the AhlC target protein of *A. hydrophila* bacteria was obtained through the Protein Data Bank website (https://www.rcsb.org/) with PDB ID: 6H2D. The protein structure was downloaded in PDB format and entered into the Discovery Studio 2021 application to remove H2O groups (if any) and other unnecessary groups. The ready protein receptor structure was then saved in the same folder in the PDB format.

Molecular Docking

The tools used are the PyRx application to determine the interaction between the ligand (active compound) and receptor (target protein). The results are presented in terms of binding affinity (BA) and Root Mean Square Deviation (RMSD). RMSD values have different types depending on the software used, because in this test, using PyRx software to obtain two RMSD results, namely RMSD/UB (RMSD upper bound) and RMSD/LB (RMSD lower bound).

Data Analysis

Data obtained in the form of screening results of active compounds of shipworm extract (*S. obtusa*) and the results of interactions between ligands (active compounds) with receptors (target proteins) were visualized using molecular docking. The results were then analyzed descriptively and compared with the literature.

RESULTS AND DISCUSSIONS

Compound Screening

The content of active compounds that have been identified from shipworm (*S. obtusa*) extracts, three types of compounds that are very dominant with great potential as antibacterial agents of *A. hydrophila* are presented in Table 1 below.

Table 1. Content of Active Compounds Shipworm (*S. obtusa*).

Compound	Chemical Formula	Retention Time (Minute)	Area	Percentage (%)
Betaine	C5H11NO2	1.285	1.03E+10	62.27
DL-Stachydrine	C7H13NO2	1.316	3.44E+09	20.80
Isoleucine	C6H13NO2	1.346	1.01E+09	6.11

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Based on the identification of active compounds shown in Table 1, it can be seen that there are three dominant compounds in shipworm (*S. obtusa*) extract, namely betaine, DL-stachydrine, and isoleucine. The content of the dominant compound from a collection of compounds in a material can be determined by comparing the area of the compound and the overall area of the material. Of the three compounds, it is known that the most dominant betaine compound with the largest area is 62.27% of the total area of the entire compound, followed by DL-Stachydrine by 20.80%, and Isoleucine by 6.11%. Research by Birnie *et al*. (2000) states that betaine compounds have antimicrobial activity against Staphylococcus aureus and Escherichia coli bacteria. In addition to betaine, two other compounds, such as dl-stachydrine, also exhibit antibacterial activity against S. Aureus and E. Coli (Abouzeid *et al*., 2023), as well as isoleucine, which can combat both Gram-negative and Gram-positive bacteria through membrane interactions (Hu *et al*., 2013).

Ligand Molecule Structure

Information related to the ligand used in this study, along with the 2D structure, is presented in Table 2, which includes the compound name, PubChem ID information, molecular weight, Hydrogen Bond Donor (HbD), Hydrogen Bond Acceptor (HbA), Rotatable Bond Count (nRB), and 2D structure.

Table 2. Ligand Structure Information Compound.

Compound	PubChem CID	MW (g/mol)	HbD	HbA	nRB	logP	Number of Violations	2D Structure
Betaine	247	117.15	0	2	1	0.5	0	
Isoleucine	6306	131.17	2	3	3	-1.7	0	
Dl Stachydrine	555	144.19	1	2	1	0.4	0	

The ligands shown in Table 2, obtained data from the prediction of ADME properties (Absorption, Distribution, Metabolism, and Excretion) referring to the five Lipinski rules, namely, molecular weight (MW) ≤ 500 g/mol, Lipophilicity (logP) ≤ 5, hydrogen bond donor (nHBD) ≤ 5, hydrogen bond acceptor (nHBA) ≤ 10, and the number of violations of Lipinski's rules ≤ 2. The Lipinski rules help in evaluating the potential of compounds for pharmacokinetic and physicochemical suitability. It also supports the Lipinski rule in determining the potential of the compound to be absorbed more through the digestive tract and reach the target (Haritha *et al*., 2024). In addition to Lipinski's rule, there is also data on the number of rotated bonds (nRB), which describes the flexibility of a molecule, thus affecting the ability of compounds to absorb and bioavailability. The lower the nRB value, or ideally has the number of turns ≤ 7, the better the effectiveness of the compound (Tarko, 2011).

Molecular Docking

The results of molecular docking in this study are in the form of binding affinity values ranging from -2.9 to -4.2 kcal.mol. The results of molecular docking are presented in Table 3, which includes the binding affinity score and RMSD to determine the strength of the bond between the three compounds used with the target

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protein AhlC, so that it can describe the antibacterial activity against *A. hydrophila* bacteria.

Table 3. Molecular Docking Results: Compound.

Compound	Mode	Binding Affinity	RMSDUB	RMSDLB
Isoleucine	1	-3.7	22.337	21.367
	2	-3.8	3.553	2.578
	3	-3.8	10.31	9.003
	4	-3.8	2.135	1.667
	5	-3.8	17.659	16.558
	6	-3.9	17.268	16.195
	7	-3.9	23.053	22.032
	8	-4.1	3.373	2.393
	9	-4.2	0.0	0.0
DL Strachydrine	1	-3.8	30.518	29.377
	2	-3.8	10.237	9.289
	3	-3.8	18.196	17.667
	4	-3.9	17.526	16.727
	5	-4.0	2.206	1.888
	6	-4.0	17.489	16.684
	7	-4.1	17.66	16.882
	8	-4.2	2.638	2.078
	9	-4.2	0.0	0.0
Betaine	1	-2.9	25.469	24.766
	2	-3.0	2.427	1.601
	3	-3.0	25.077	24.492
	4	-3.0	27.775	26.49
	5	-3.0	21.731	20.811
	6	-3.1	37.405	36.46
	7	-3.4	18.054	17.145
	8	-3.4	17.484	16.517
	9	-3.5	0.0	0.0

Based on the results shown in Table 2, it is known that the three active compounds used have relatively equivalent potential, with the comparison of the average binding affinity values of the 9 repetition modes obtaining close results. However, of all the modes displayed on the three compounds, only mode 9 has the best results, namely -4.2 kcal/mol for isoleucine; -4.2 kcal/mol for DL-stachydrine; and -3.5 kcal/mol for betaine. These results are said to be the best because they have the lowest binding affinity values compared to other modes. Thus, it can be said that the lower or more negative the binding affinity value is directly proportional to the higher the stability when binding to the receptor (Nurlailiyah *et al*., 2023) and the stronger the ability to bind (Malaisamy *et al*., 2024).

In addition to the binding affinity value, there is also a Root Mean Square Deviation (RMSD) value, which is the value of the average shift distance between the atomic positions of a protein, to determine the stability of the protein structure (receptor) after interacting with the ligand (Ghahremanian *et al*., 2022). Thus, the RMSD results are also used to ensure the quality of the active compound when interacting. RMSD values have different types depending on the software used, because in this test, using PyRx software to obtain two RMSD results, namely rmsd/ub (RMSD upper bound) with values ranging from 8.6 to 2 À and rmsd/lb (RMSD lower bound) with values between 1 to 2 À (Iqbal and Matsabisa, 2024). However, the determination of the best results prioritizes the results of binding affinity, and the RMSD value is only an additional reference for the

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validity of the docking results (Rachmah and Nurhayati, 2024).

Based on the molecular docking score in the form of binding affinity and RMSD values between active compounds in shipworm (*S. obtusa*) extract and AhlC target protein in *A. hydrophila* bacteria, the results showed strong interactions to low interactions. The compounds with the strongest interaction are isoleucine and DL-stachydrine, while the compound with the lowest interaction is betaine. These results indicate that the lower the binding affinity value, the lower the energy required for a bond. In addition, the results of molecular docking tests can predict the interaction formed from the ability of antibacterial activity between the active compounds of shipworm extract (*S. obtusa*) with the target protein AhlC in *A. hydrophila* bacteria. The level of strength of the interaction formed can be seen from the binding affinity value, where the more negative binding energy indicates a stronger interaction because the active compound has access to the target protein, so that it is likely that the compound can inactivate bacteria by certain mechanisms. Therefore, it can be concluded that the more negative the binding affinity value, the greater the potential of a compound as an antibacterial candidate (Lailiyyah and Lisdiana, 2023).

Compound Interaction with AhlC Protein

Visualization of the interaction between each compound and the target protein AhlC owned by *A. hydrophila* bacteria was further analyzed using Biovia Discovery Studio 2021 software. The resulting interaction between betaine compounds and Ahlc target protein is presented in Figure 1, which shows the presence of 4 amino acid residues, including ASN 32, GLN 35, ARG 112, and ASP 116, as well as 3 interactions, namely attractive charge, conventional hydrogen bond, and carbon-hydrogen bond.

The interaction results of the isoleucine compound with the target protein AhlC are presented in Figure 2, which shows the presence of 2 amino acid residues and 2 interactions. The amino acid residues involved are GLU 39, forming a conventional hydrogen bond interaction, and ARG 112, forming an unfavorable donor-donor interaction. The results of interactions owned by DL stachydrine compounds with AhlC target protein are presented in Figure 3, which shows the presence of 4 interactions, including attractive charge involving amino acid residue GLU 39, conventional hydrogen bond interaction involving amino acid residue SER 108, carbon-hydrogen bond interaction involving amino acid residue ASN 36, and unfavorable donor-donor interaction involving amino acid residue ARG 112.

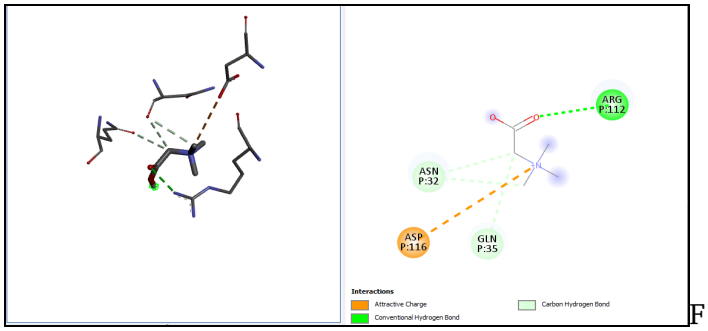

Figure 1. Interaction of Betaine Compound with AhlC Target Protein: (a) 3D Structure; (b) 2D Structure.

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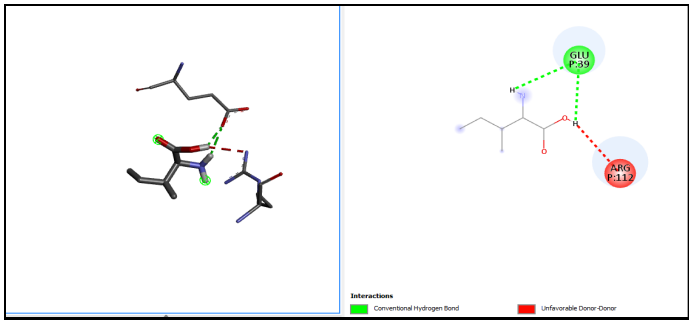

Figure 2. Interaction of Isoleucine Compound with AhlC Target Protein: (a) 3D Structure; (b) 2D Structure.

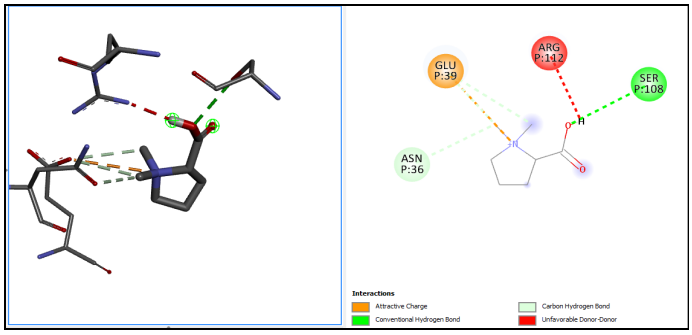

Figure 3. Interaction of DL Stachydrine Compound with AhlC Target Protein: (a) 3D Structure; (b) 2D Structure.

The results of the analysis of molecular interactions between ligands and receptors in Figure 1 have the highest bond stability compared to Figures 2 and 3. This is because there are three interactions identified, namely conventional hydrogen bonds with arginine residues (ARG: 112), which play an important role in stabilizing the ligand-receptor complex through directional electrostatic interactions between hydrogen donors and acceptors. In addition, there are two carbon-hydrogen bond interactions with asparagine (ASN:32) and glutamine (GLN:35) residues. These bonds are weaker than conventional hydrogen bonds, but they still contribute to specific ligand binding because they occur in the relatively polarized active binding pocket (Desiraju and Steiner, 2001). Furthermore, the presence of attractive charge interactions with negatively charged residues, namely aspartate (ASP:116), suggests that the ligand is likely to contain positively charged groups, which could strengthen affinity to the receptor through electrostatic attraction. Overall, the interaction pattern reflects the good affinity and potential effectiveness of the ligand as a bioactive candidate or target inhibitor.

In contrast, in Figures 2 and 3, although there are positive interactions such as hydrogen bonds with residues GLU:39 and SER:108, as well as electrostatic interactions with GLU:39 in Figure 3, these two complexes show unfavorable donor-donor interactions with residue ARG:112. These interactions can cause repulsion between donor atoms, potentially reducing the stability of the ligand-receptor complex (Meng *et al.*, 2011). Therefore, although binding occurs, this configuration is considered less optimal than that in Figure 1.

Overall, this analysis shows that the presence of appropriate hydrogen interactions and electrostatic charges, as well as the absence of unfavorable interactions, are key factors in forming stable and effective ligand-receptor complexes. Based on these data, it is concluded that residues ASN:32, GLN:35, ARG:112, and ASP:116 play an important role as active sites in ligand binding to the receptor in this model.

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So far, no other studies have addressed molecular docking testing on AhlC toxin in *A. hydrophila* bacteria, making it very difficult to compare the efficiency of active compounds in *S. obtusa* extracts against the toxin. Therefore, it is important to conduct further research using other compounds against AhlC toxin to determine the efficiency of which compounds have the most potential to suppress toxin production. However, in silico or molecular docking tests have limitations that can only interpret the bonds formed without real results.

CONCLUSION

Based on research that has been conducted on the three dominant active compounds obtained from shipworm (*S. obtusa*) extract in the form of betaine, DL-stachydrine, and isoleucine, it has the potential to be an antibacterial agent for *A. hydrophila*. The best binding results were obtained by DL-stachydrine and isoleucine with a binding affinity value of -4.2 kcal/mol. The interactions between the three compounds and the AhlC receptor indicate a sufficiently strong interaction. This is evidenced by the presence of conventional hydrogen bonds, which play a crucial role in stabilizing the ligand-receptor complex through electrostatic interactions between hydrogen donors and acceptors. Based on these results, it is recommended to conduct in vitro and in vivo testing to support new information regarding the efficacy of shipworm extract as an antibacterial agent.

CONFLICT OF INTEREST

The authors have no conflict of interest in writing and publishing the manuscript.

AUTHOR CONTRIBUTION

Andi Al Furqan played an important role in designing the research topic, collecting and processing data, and compiling the initial draft of the article, which was reviewed and revised by Mohamad Fadjar and Yunita Maimunah. All authors have read, reviewed, and approved the final manuscript.

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