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Bioactive compounds characterization of *Anredera cordifolia* leaf and its in silico antibacterial mechanisms against *Aeromonas hydrophila*

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Abstract. *Dadiono MS, Prayitno SB, Sukardi P, Desrina, Sarjito, Riyadi PH. 2025. Bioactive compounds characterization of* Anredera cordifolia *leaf and its in silico antibacterial mechanisms against* Aeromonas hydrophila*. Biodiversitas 26: 3561-3570. Aeromonas hydrophila* is a major pathogenic bacterium in aquaculture, causing severe disease outbreaks and significant economic losses. Due to rising antibiotic resistance, this study aimed to identify bioactive compounds from *Anredera cordifolia* (*binahong*) leaf extract and evaluate their antibacterial mechanisms against *A. hydrophila* through an in silico approach. The compounds were identified using UV-Vis spectrophotometry, Fourier Transform Infrared (FT-IR), and Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analysis revealed six dominant compounds: Butylated Hydroxytoluene (BHT), Hexanedioic acid bis (2-ethylhexyl) ester, Diisooctyl adipate, Dimethylpyrroline, Cyclohexane, and 1-Ethyl-1-methylcyclohexane. Among these, BHT demonstrated the strongest predicted antibacterial activity, with a Pa (probability of activity) of 0.789 and Pi (probability of inactivity) of 0.004. In silico docking analysis indicated that BHT effectively targets two key bacterial enzymes, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and Carbonic Anhydrase (CAH), suggesting interference in bacterial metabolism and survival. These findings indicate that *A. cordifolia* leaf extract, particularly BHT, holds promise as a natural antibacterial agent to control *A. hydrophila* infections in aquaculture. Further in vivo validation is recommended to confirm its therapeutic potential.

Keywords: *Aeromonas hydrophila*, *Anredera cordifolia,* antibacterial, *binahong*, in silico

INTRODUCTION

Aeromonas hydrophila, a Gram-negative bacterium is known to primarily infect freshwater fish as well as mammals, birds, amphibians, and reptiles (Rasmussen-Ivey et al. 2016; Venkatasamy et al. 2021; Mulia et al. 2023b). This pathogen can cause gastroenteritis, necrotizing fasciitis, and septicemia in infected organisms (Assefa and Abunna 2018; Semwal et al. 2023; Ye et al. 2025). The ability of this pathogen to cause disease is very complex, as supported by various virulence factors, including cytotoxins, adhesins, hemolysins, proteases, lipases, and biofilm formation (Sheikh et al. 2023). These properties make *A. hydrophila* a highly adaptive and difficult-to-control threat in aquaculture environments (Nurhalisa et al. 2022; Pereira et al. 2022).

To address *A. hydrophila*, the use of Tetramycin and Romet-30 is among the most commonly used antimicrobial agents(Dolmans 2022). The main factors that favor the pathogenicity of *Aeromonas* species include surface polysaccharides, extracellular proteins, iron-binding systems, and exotoxins, which play a role in the disease progression process (Yassen et al. 2021). However, the widespread and uncontrolled use of antibiotics has contributed to the rise of antimicrobial resistance in aquaculture (Pereira et al. 2022).

Aquaculture systems are increasingly recognized as centers of biological activity in the process of transduction, conjugation, and transformation of bacteria that have antibiotic resistance (Watts et al. 2017). Searching for effective antibiotic alternatives is critical to reducing the risk of antimicrobial resistance and strengthening the fish immune system (Ahmadifar et al. 2021; Mulia et al. 2023a). In response to the growing concern regarding antibiotic resistance, various methods have been introduced, such as using plant-based metabolites to enhance and stimulate immune responses in aquaculture species (Pangastuti et al. 2021). In history, plants have been the basis for the discovery and development of various types of drugs (Van Hai 2015; Tadese et al. 2022). The therapeutic efficacy of these medicinal plants is mainly due to the presence of secondary metabolites, which are classified into three main groups, namely terpenes, nitrogenous, and phenolic compounds (Islam et al. 2023). Various natural compounds from plants and their secondary metabolites have shown

promising potential in inhibiting disease virulence factors, both through in vivo and in vitro tests (Devi et al. 2016).

Medicinal plants are known for their diverse bioactive compounds, which show antimicrobial, antifungal, anticancer, anti-inflammatory, and antioxidant activities (Reverter et al. 2017). Although plants contain many bioactive compounds, only a tiny fraction have been successfully identified (Van Hai 2015). The development of extraction technologies, supported by molecular spectroscopy methods such as Gas Chromatography-Mass Spectrophotometry (GC-MS) and Fourier Transform Infrared (FT-IR), has contributed to uncovering and characterizing new bioactive compounds. Recently, in silico testing has received increasing attention, offering efficient and cost-effective methods in the drug discovery process (Altayb et al. 2022).

Anredera cordifolia (Ten.) Steenis (*binahong*), has attracted attention due to its broad pharmacological properties, including antibacterial, anti-inflammatory, antioxidant, and wound-healing activities (Alba et al. 2020). Phytochemical studies have confirmed the presence of diverse bioactive constituents such as flavonoids, glycosides, saponins, and steroids in various parts of the plant (Ghosh and Mukherjee 2019). Although its therapeutic effects have been investigated in general medical contexts, there is a clear lack of research on its potential against *A. hydrophila*, especially using molecular docking and other in-silico approaches.

In-silico methods, such as molecular docking and pharmacokinetic modeling, offer a rapid, cost-effective, and mechanism-focused approach to screen plant metabolites and predict their interaction with bacterial targets (Altayb et al. 2022). These computational techniques help bridge the gap between compound identification and functional validation, guiding downstream in vitro and in vivo studies. While some preliminary efforts have examined endophytic bacteria from *A. cordifolia* (Nxumalo et al. 2020) or its effects on fish physiology (Noor et al. 2024), no published studies to date have assessed the antibacterial mechanisms of its leaf-derived metabolites against *A. hydrophila* in silico. Therefore, the present study aims to characterize the bioactive compounds in *A. cordifolia* leaf extract and evaluate their antibacterial potential and binding mechanisms against key *A. hydrophila* targets through in-silico analysis. This research contributes to the development of alternative antimicrobial strategies in aquaculture and addresses a critical knowledge gap in the exploration of *A. cordifolia* as a source of therapeutic agents against bacterial fish pathogens.

MATERIALS AND METHODS

This study was conducted from January to December 2024 at the Fish Pests and Diseases Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Jenderal Soedirman, and Processing Technology Laboratory, Faculty of Agriculture, Universitas Jenderal Soedirman, Purwokerto, Indonesia. Samples of *A. cordifolia* (Figure 1) were collected from community-owned plantations in Purwokerto area, while *A. hydrophila* isolates were obtained from the Laboratory Microbiology, Universitas Brawijaya, Malang, Indonesia. Antibacterial active compound content of the best fraction of *A. cordifolia* leaf extract was identified using three stages, namely UV-Vis (Ultra Violet-Visible) spectrophotometer, FT-IR, and GC-MS analysis, respectively. Then continued with in silico analysis using Pubchem, PassOnline, Stitch 5.0 and Uniprot.

Authenticated *A. cordifolia* samples based on character descriptions and identification keys in the book "Flora of Java (Spermatophytes only)" Volume 1 (Backer and Van Den Brink 1968). Determination of the best fraction of *A. cordifolia* leaf extract based on the results of fraction separation through column chromatography (fraction separation based on color) and antibacterial tests of all fractions obtained from previous studies that have not been published.

Anredera cordifolia leaf extract

A. cordifolia leaf extraction was carried out using the maceration method. This method utilizes volatile organic solvents, with the main focus on the length of contact time between the solvent and the extracted plant tissue. In this process, *A. cordifolia* leaves were extracted using methanol solvent, which is a polar solvent, with a ratio of 1:8 (Prameswari and Widjanarko 2014; Dadiono et al. 2017). The use of methanol was chosen because of its higher ability to dissolve active compounds compared to other solvents, considering that methanol is able to extract both polar and nonpolar compounds (Kusumaningtyas et al. 2008).

The extraction process using the maceration method was carried out by dissolving 125 g of *A. cordifolia* leaf powder into a 2000 mL Erlenmeyer flask containing 1000 mL of methanol solvent, following a ratio of 1:8. The mixture was then homogenized using an incubator shaker for 60 minutes at a speed of 150 rpm, then left for 48 hours. After that, the filtrate was separated from the dregs by filtration using Whatman No. 42 filter paper. Furthermore, the filtrate obtained was evaporated using a rotary vacuum evaporator at a temperature of 50°C until all the solvent evaporated (Dadiono 2017).

Figure 1. *Anredera cordifolia*

UV-Vis (Ultra Violet-Visible) Spectrophotometer analysis

UV-Vis spectrophotometer method measured the absorbance wavelength spectra of the best fraction of ultraviolet radiation with a 1 nm resolution spectrophotometer (from 200-800 nm) on a standard quartz bowl with a long trace. After placing the best fraction of 3 mL column chromatography results into the cuvet, then a sample of 1 µL was taken into the spectrophotometer and scanned with a wavelength starting at 200 nm (Rizkia et al. 2014).

FT-IR Spectrophotometer analysis (Fourier Transform Infrared)

The FT-IR model used is the FTS 1000 Schimitar made by Agilent Tehnologies. For the specifications of the pellet-making press tool, Pike Technologies uses a capacity of 9 tons with a pressing time of 1-3 minutes. Functional groups of the best fraction of *A. cordifolia* leaf extract were characterized using FT-IR analysis with spectra observed in the 4000-400 cm^{-1} range with a resolution of 4 cm^{-1}. FT-IR uses thin samples 10-50 µm thick to allow sunlight to pass through. The scanned sample will be passed through infrared light, where the waves transmitted by the sample will be detected by a sensor device connected to a computer. This detector then produces a spectrum as a representation of the sample being analyzed. The spectrum reflects the chemical structure, type of molecular bond, and the presence of certain functional groups in the sample being tested.

Samples to be analyzed for functional group identification using FT-IR spectrophotometer must be in solid form, while liquid samples are placed in glass containers. The sample preparation process is relatively simple and does not require the use of solvents. Solid samples are simply crushed or blended, while liquid samples only need to be homogenized before analysis (Dadiono 2017).

GC-MS (Gas Chromatography-Mass Spectrophotometry) analysis

GC-MS analysis determined the dominant compounds in the best fraction of *A. cordifolia* leaf extract. This analysis combines Gas Chromatography (GC) to quantitatively analyze the number of compounds indicated by relative percent and Mass Spectrophotometry (MS) to analyze the molecular structure of compounds. The sample fraction of *A. cordifolia* extract used was 1 µL, then injected into GC-MS with the following conditions: GC-MS specification used was Gas Chromatogram-HP 6890, equipped with Agilent 1909 model capillary 1 S-433 HP-5 MS (5% Phenyl Methyl Siloxane), 250 µm diameter, 0.25 µm thickness, and 30 µm capillary length at a flow rate of 1 ml/min. Oven temperature was 80°C/minute to 325°C/15 minutes, with helium as carrier gas, 19.9 ml/min flow rate at a pressure of 9.32 psi, and 300°C injector temperature.

The sample was inserted into the instrument using the split injection method at a ratio of 20:1, with an injection volume of 1 µL. The gas chromatography-mass spectrometry data were obtained using an HP 6890 instrument equipped with an Agilent 19091S-433 HP-5MS capillary column (containing 5% phenyl methyl siloxane), with the same temperature and programming as in the gas chromatography process. Compound identification was carried out by matching it to the data library available in the GC-MS system, namely the Wiley 275.L database (Dadiono 2017).

Bioinformatics analysis (in silico)

The identification results showed the molecular structure and pre-predict the possibility of antibacterial properties against *A. hydrophila* and the mechanism with Bioinformatics analysis (in silico) divided into four stages of analysis. The first stage was analyzed with PubChem (https://pubchem.ncbi.nlm.nih.gov/), while the second, third, and fourth were analyzed with PassOnline (https://www.way2drug.com/passonline/) (Riyadi et al. 2023), Stitch 5.0 (https://bio.tools/stitch), and Uniprot (https://www.uniprot.org/), respectively (Aisiah et al. 2020, 2022).

RESULTS AND DISCUSSION

UV-Vis (Ultra Violet-Visible) spectrophotometer analysis

UV-Vis spectrophotometer analysis of the best fraction of *A. cordifolia* leaf extract produced 18 absorbance wavelengths. The highest wavelengths were at 249 nm, 222 nm, 226 nm, and 228.9 nm, with absorbance values of 3,556, 3,669, 4,091, and 4,177, respectively (Figure 2).

Based on the UV-Vis analysis results at 249 nm and 226 nm, flavonoid compounds are the largest phenol group. Flavonoids are found in vascular plants as a mixture and rarely in single flavonoids (Aisiah et al. 2022). According to Harborne (2006), flavonoids contained conjugated aromatic systems, causing strong absorption bands in UV-Vis spectrophotometer regions, which varied depending on the type of flavonoid. For example, anthocyanins have a central wavelength maximum between 475-560 nm, while aurones are in the 390-430 nm range. Chalcones absorb at 365-390 nm, while flavonols show two absorption bands at 250-270 nm and 350-390 nm. Moreover, flavones and flavonols can absorb at approximately 225 nm and 275-290 nm. Regarding isoflavones, there are central maximum wavelengths of 255-265 nm.

FT-IR spectrophotometer analysis (fourier transform infrared)

FT-IR spectrophotometer analysis of the best fraction obtained 10 frequency regions of absorption bands showing the functional groups of a type of compound (Figure 3).

Based on the number of absorption bands showing the existence of certain compound bonds, the best fraction obtained the most dominant functional groups at 3482.965 cm^{-1}, 1727.878 cm^{-1}, and 1265.962 cm^{-1}. The frequency can be observed to function based on the characterization of Silverstein et al. (2005) (Table 1).

Table 1 shows that the most dominant absorption band at a frequency of 3482.965 cm^{-1} is a group of phenols, alcohols (ROH), and carboxylic acids (RCO_2H) (Figure 4). Meanwhile, the frequency of 1727.878 cm^{-1} is an alkane group, and the 1265.962 cm^{-1} represents an alcohol (ROH), ether, and ester group.

The results show that the best fraction of *A. cordifolia* leaf extract has the most dominant group of active compounds,

namely the phenol and its derivatives. Similarly, Socrates (1994) stated that the functional groups forming the structure of phenol compounds were in the form of alcohol (ROH), aldehyde (CHO), and carboxylic acid (RCO_2H).

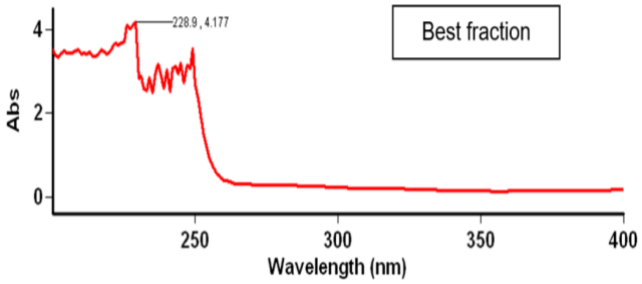

Figure 2. UV-Vis analysis results of the best fraction of *Anredera cordifolia* leaf extract

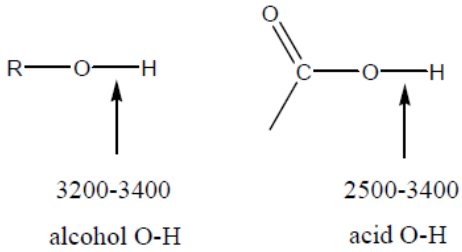

Figure 4. Alcohol (ROH) and Carboxy Acid (RCO_2H) groups

GC-MS (Gas Chromatography-Mass Spectrophotometry) analysis

GC-MS analysis of the best fraction of *A. cordifolia* leaf extract detected 34 types of active compounds, which were identified based on the comparison of retention time and mass spectrophotometry with the GC-MS tool library (Figure 5).

Based on the GC-MS database library, the three peaks are known to contain different compounds. At the peak of 21.359 m/z, there are two compounds of Hexanedioic acid, bis(2-ethylhexyl) ester and Diisooctyl adipate. Peak 9.998 m/z contains three compounds: Dimethylpyrroline, Cyclohexane, and 1-Ethyl-1-methylcyclohexane. Meanwhile, peak 7.781 m/z contains only Butylated Hydroxytoluene (BHT). As presented in Table 2, the relative percentage shows the correlation between the compounds in the test sample and the GC-MS database library. Bioinformatics (in silico) analysis was carried out to identify compounds contained in the three GC-MS peak points further.

Table 1. Characterization of wavelengths of functional groups of the best fractions

Frequency of absorption bands	Functional group	Frequency of absorption bands of isolate (cm^{-1})	Functional group
3300-3500	OH	3482,965	Alcohol, Phenol, Carboxylic acid
2850-2970	C-H stretch	2860,085; 2927,347	Alkyl
1600-1750	C-H aliphatic	1638,751; 1727,878	Alkanes
1370-1465	$-CH_3$ (bending)	1383,573; 1458,277	Alkyl
800-1300	C-O alkohol	1098,539; 1265,962	Alcohols, Ethers, Esters
570-995	C-H	730	Aromatics

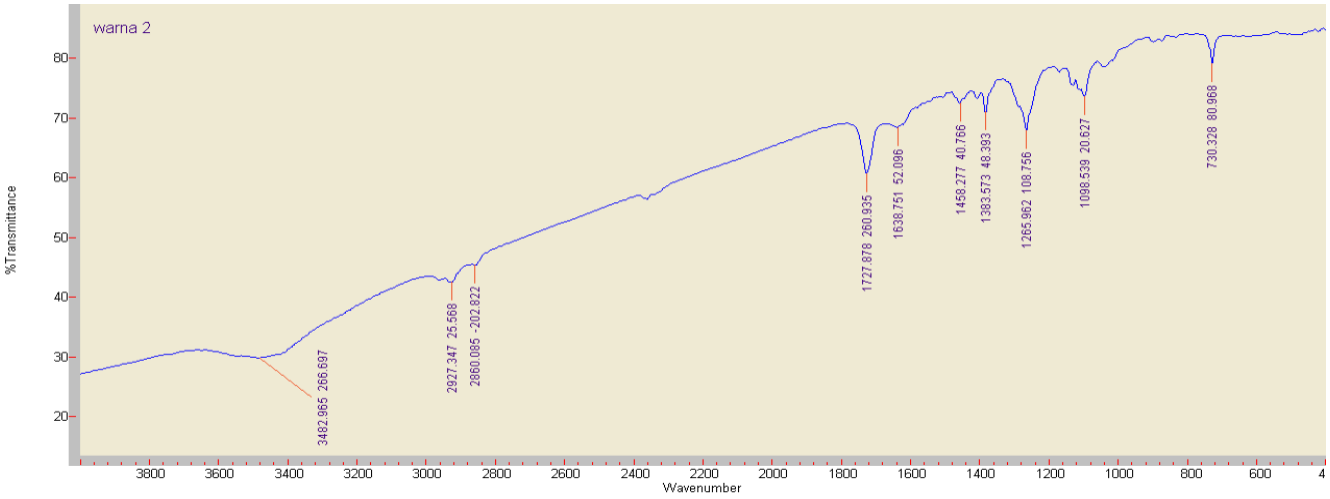

Figure 3. FT-IR analysis results of the best fraction of *Anredera cordifolia* leaf extract

Table 2. Relative percentage of the most dominant compound (number of compound) of the best fraction

Compound type	Relative percent (%)
Butylated hydroxyoluene	98
Hexanedioic acid, bis(2-ethylhexyl)ester	95
Diisooctyl adipate	91
Dimethylpyrroline	49
Cyclohexane	47
1-Ethyl-1-methylcyclohexane	47

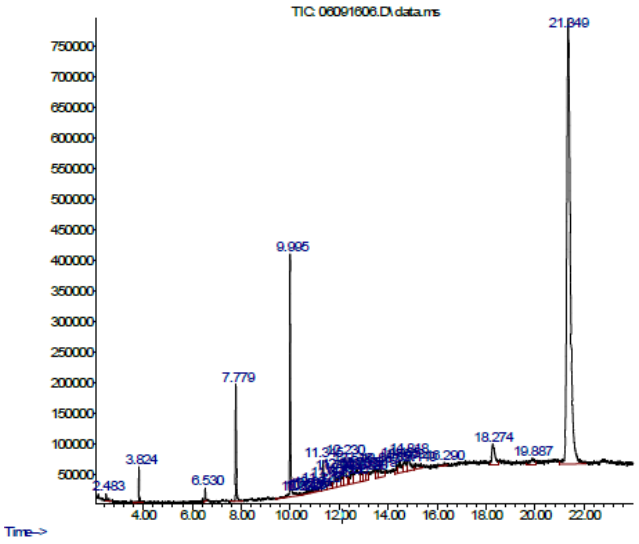

Figure 5. GC-MS analysis results of the best fraction of *Anredera cordifolia* leaf extract

Bioinformatics analysis (in silico)

The analysis results of GC-MS analysis at the peak point of 21.359 m/z identified two main compounds of Hexanedioic acid (Figure 6.A), namely bis (2-ethylhexyl) ester ($C_6H_{10}O_4$) and Diisooctyl adipate ($C_{22}H_{42}O_4$) (Figure 6.B). Bis(2-ethylhexyl) ester (CID 196) belongs to the group of carboxylic acids (RCO_2H) with canonical structure smiles C(CCC(=O)O)CC(=O)O. Diisooctyl adipate (CID 66932) is an ester compound (RCO_2R) with the molecular formula $C_{22}H_{42}O_4$ and canonical smiles CC(C)CCCCCOC(=O)CCCCC(=O)OCCCCCCCC(C)C.

At the peak point of 9.998 m/z, three compounds were found, namely Dimethylpyrroline ($C_6H_{13}N$) (Figure 6.C), Cyclohexane (C_6H_{12}) (Figure 6.D), and 1-Ethyl-1-methylcyclohexane (C_9H_{18}) (Figure 6.E). Dimethylpyrroline (CID 641767) belongs to the amide (N-H) compound group with a molecular weight of 99.177 g/mol. The canonical smile structure is CC1CCC(N1)C, which shows the characteristics of a pyrrole ring with a methyl substituent. Cyclohexane (CID 8078) is a hydrogen-bonded aldehyde (C-H bending) compound with a molecular weight of 82.162 g/mol, with canonical smile structure of C1CCCCC1, showing a cyclic structure typical of cycloalkane compounds. Additionally, 1-Ethyl-1-methylcyclohexane (CID 35411) belongs to the aldehyde group with a structure containing hydrogen bonds and a molecular weight of 126,243 g/mol, with canonical smiles CCC1(CCCCC1)C.

At the peak point of 7.781 m/z, only BHT ($C_{15}H_{24}O$) was identified (Figure 6.F). This compound belongs to the group of carboxylic acids (RCOH) and is a phenol derivative with hydrogen bonds. BHT is an antioxidant, comprising a molecular weight of 220.356 g/mol. The canonical smiles structure is CC1=CC(=C(C(=C1)C(C)(C)O)C(C)(C)C, which describes a phenolic structure with butyl and methyl substituents that contribute to the stability as an antioxidant.

Analysis of antibacterial properties based on GC-MS identification results

After identifying the compounds' group, molecular formula, molecular structure, and canonical smiles from GC-MS identification, the antibacterial properties of the compounds are analyzed using PassOnline software. The results are predictions based on Pa (Probability Activity) and Pi (Probability Inactivity) values (Table 3).

As shown in Table 3, BHT is the active compound in the best fraction of *A. cordifolia* leaf extract with the highest predicted antibacterial properties. The analysis of BHT obtained the highest Pa of 0.789 and the lowest Pi value of 0.004 as an antiseptic. This suggests that the compound is 78% likely to have antibacterial activity and 0.4% likely to lack antibacterial activity. Based on previous studies, BHT has effective antibacterial activity against various pathogenic bacteria, including those commonly found in aquaculture ecosystems. This compound can inhibit the growth of *Staphylococcus aureus* (Sipriyadi et al. 2022). BHT was also successfully isolated from the microalga *Scenedesmus obliquus* (Marrez et al. 2019). Antibacterial activity is not only limited to Gram-positive bacteria but also includes Gram-negative bacteria, often associated with severe diseases in fish (Lalnunfela et al. 2020).

Antibacterial mechanism of Butylated Hydroxytoluene (BHT) compound against *Aeromonas hydrophila*

Antibacterial mechanism of BHT against *A. hydrophila* can be observed using Stitch 5.0 software and Uniprot software to determine the bacteria proteins bound or damaged by the compound (Figure 7).

Based on Figure 7, the antibacterial mechanism of BHT compound above can be explained that BHT compound can damage *A. hydrophila* bacterial cells simulated using *A. hydrophila* subsp *hydrophila* A449 bacteria by inhibiting and damaging DXS (1-deoxy-D-xylulose-5-phosphate synthase) proteins and CAH (carbonic anhydrase) proteins indicated by red lines in the figure.

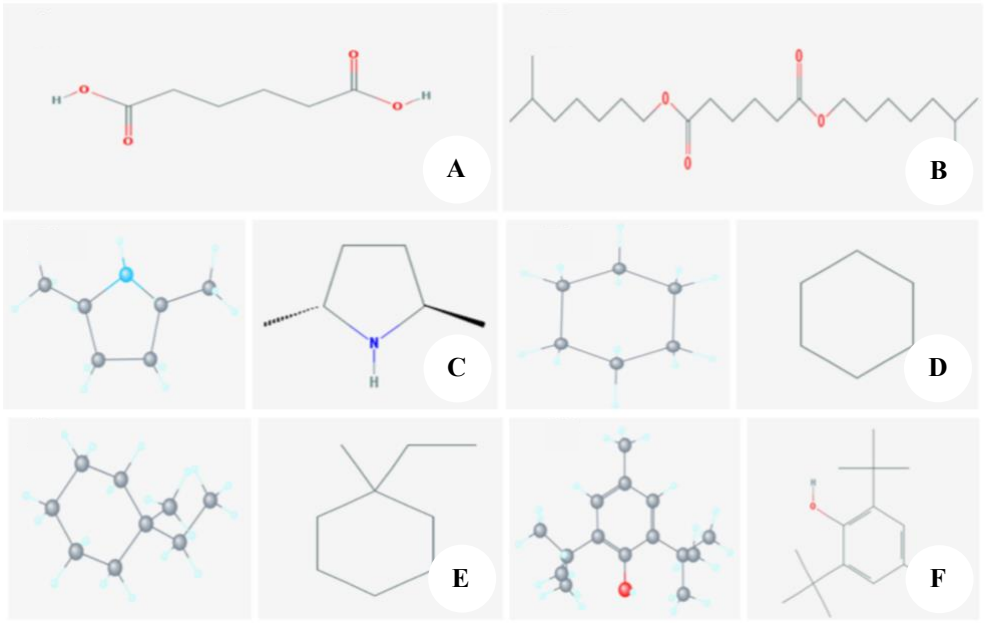

Figure 6. Molecular structure: A. Hexanedioic acid ($C_6H_{10}O_4$), B. Diisooctyl adipate ($C_{22}H_{42}O$), C. Dimethylpyrroline ($C_6H_{13}N$), D. Cyclohexane (C_6H_{12}), E. 1-Ethyl-1-methylcyclohexane (C_9H_{18}), F. BHT ($C_{15}H_{24}O$)

Table 3. Antibacterial properties analysis table of Hexanedioic acid, Diisooctyl adipate, Dimethylpyrroline, Cyclohexane, 1-Ethyl-1-methylcyclohexane, and BHT

Pa	Pi	Activity
Hexanedioic		
0.478	0.014	Antiseptic
0.292	0.064	Antibacterial, ophthalmic
0.251	0.005	Antibacterial
0.212	0.028	Antibiotic Glycopeptide-like
0.165	0.041	Antibiotic
Dimethylpyrrolin		
0.462	0.098	Antibiotic Glycopeptide-like
0.351	0.055	Antimycobacterial
0.318	0.054	Antibacterial
0.282	0.004	Antibacterial, ophthalmic
1-Ethyl-1-methylcyclohexane		
0.278	0.068	Antibacterial
0.212	0.011	Antibacterial, ophthalmic
0.165	0.041	Antibiotic
0.137	0.099	Antiseptic
Diisooctyl		
0.394	0.022	Antiseptic
0.241	0.088	Antibacterial
0.140	0.140	Antibacterial, ophthalmic
0.114	0.069	Antibiotic
Cyclohexan		
0.333	0.062	Antimycobacterial
0.313	0.004	Antibacterial, ophthalmic
0.280	0.040	Antiseptic
0.267	0.074	Antibacterial
0.157	0.045	Antibiotic
Butylated Hydroxytoluene		
0.789	0.004	Antiseptic
0.274	0.098	Antimycobacterial
0.230	0.095	Antibacterial
0.156	0.048	Antibacterial, ophthalmic
0.108	0.074	Antibiotic

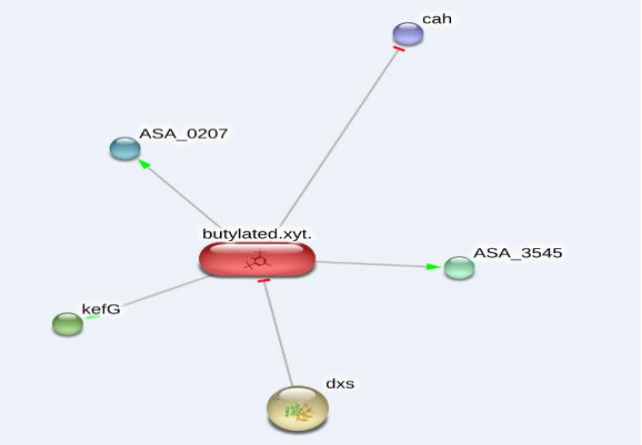

Figure 7. Antibacterial mechanism of BHT compound against *Aeromonas hydrophila*

Discussion

Phytochemical discussion

The results showed phenol and its derivative were the most dominant active compounds in the best fraction of *A. cordifolia* leaf extract (Figure 2). As a group of secondary metabolites known for antimicrobial properties, Phenol compounds inhibit pathogenic bacteria through interactions with proteins that cause inactivation and structural changes (Lima et al. 2019; Faheem et al. 2022). Antibacterial mechanism includes several aspects, such as protein inactivation through oxidative stress that generates Reactive Oxygen Species (ROS) (Shalaby and Horwitz 2015), alters protein structure (Vazquez-Armenta et al. 2024), and interferes with enzymatic activities important for bacterial metabolism (Nandi et al. 2020). Additionally, phenolic

compounds interact with bacterial proteins through hydrogen bonding and hydrophobic interactions (Bhat et al. 2021). This interaction changes protein conformation, weakens membrane integrity, and increases cell permeability, leading to leakage of intracellular components. Phenolic compounds also inhibit bacterial virulence factors, such as adhesion proteins, toxins, and quorum-sensing regulators, thereby reducing colonization and pathogenicity without inducing resistance (Vazquez-Armenta et al. 2024). Disruption of membrane proteins and lipid bilayers also causes changes in membrane fluidity, inhibits nutrient uptake, and leads to cell lysis, further strengthening the bactericidal effects (Naiel et al. 2023). GC-MS analysis showed that the active compounds contained in the best fraction of *A. cordifolia* leaf extract are Hexanedioic acid ($C_6H_{10}O_4$), Diisooctyl adipate ($C_{22}H_{42}O_4$), Dimethylpyrroline ($C_6H_{13}N$), Cyclohexane (C_6H_{12}), 1-Ethyl-1-methylcyclohexane (C_9H_{18}), BHT ($C_{15}H_{24}O$) (Figure 6).

Hexanedioic acid, including bis(2-ethylhexyl)ester, has been identified as a significant component in various biological studies, with antioxidant and antibacterial activity (Eyasu et al. 2024). The presence of this compound in bacteria membranes can disrupt the lipid layer's structure, thereby affecting its fluidity level. Membrane fluidity is crucial in determining permeability, as higher fluidity allows more passage of various solutes through the membrane. This relationship is reinforced by findings showing that compounds such as squalane can liquefy the lipid layer, increasing the membrane's permeability to certain substances (Salvador-Castell et al. 2021). The presence of bis(2-ethylhexyl)ester is also associated with antifungal activity. This suggests that integrating bis(2-ethylhexyl)ester into the membrane affects fluidity and contributes to some bacterial strains' antimicrobial properties (Soliman et al. 2022). Changes in membrane properties due to incorporation can make bacteria cells more susceptible to environmental stresses, including the influence of antimicrobial agents. Diisooctyl also shows similar effects on membrane characteristics. Changes in membrane lipid composition, particularly in the presence of branched-chain fatty acids, can increase its fluidity and permeability (Naradasu et al. 2021).

Dimethylpyrroline is a compound that can potentially inhibit DNA replication in bacteria. This compound functions by inhibiting the activity of essential enzymes in DNA replication, such as topoisomerase and DNA gyrase. Both enzymes break and reconnect DNA strands, where disruption in their activity can cause bacteria to lose the potential to replicate DNA, thereby causing cell death (Isnaini et al. 2021). Compounds that interfere with peptidoglycan synthesis in bacteria cell walls also contribute to their antibacterial effects. Dimethylpyrroline and its derivatives have been recognized as important compounds in inhibiting the quorum-sensing mechanism. Quorum Sensing (QS) is a complex communication system that allows bacteria to adjust their behavior based on population density. Dimethylpyrroline compounds have the potential to act as Quorum Sensing Inhibitors (QSI) by competitively interacting with receptors such as LuxR and LasR, which inhibit communication between bacteria. This inhibition process can reduce the pathogenicity of *A. hydrophila* by decreasing the ability to form biofilms and produce toxins, increasing susceptibility to the immune system and antibacterial agents. Welsh et al. (2018) showed that structural analogs of natural Acyl-Homoserine Lactones (AHLs) could effectively compete with endogenous ligands in binding to the receptor, leading to changes in virulence phenotype in *Pseudomonas aeruginosa*. Other studies have also demonstrated the competitive inhibition mechanism, indicating compounds such as ortho-vanillins act as competitive antagonists to LasR, which bind at the exact location of the native ligand 3OC12-HSL (Woods et al. 2024).

Cyclohexane and 1-ethyl-1-methylcyclohexane can destabilize bacterial membranes through hydrophobic interactions, thereby increasing susceptibility to external stressors (Zhang et al. 2019b). The high hydrophobic properties allow these compounds to partition into the lipid layer, disrupt lipid packing, increase membrane fluidity, and enhance permeability (Vierros and Sammalkorpi 2015). The lipid disruption effects weaken membrane integrity, leading to leakage of intracellular contents and high susceptibility to osmotic stress, antimicrobial agents, and oxidative damage. Although cyclohexane integrates into the membrane, 1-ethyl-1-methylcyclohexane can cause greater disruption (Koyanagi et al. 2016). Gram-negative bacteria experience increased permeability of the outer membrane, while Gram-positive bacteria are susceptible to depolarization and loss of function, compromising bacterial survival.

BHT is a known antioxidant with paradoxical pro-oxidant effects on bacterial cells. In high concentrations, this compound triggers oxidative stress by generating ROS (Ousji and Sleno 2020), which causes oxidative damage to bacteria lipids, proteins, and nucleic acids. Excessive accumulation of ROS can disrupt essential cellular functions, triggering apoptosis-like cell death in *A. hydrophila*. BHT also inhibits the enzymatic defense mechanisms of bacteria against oxidative stress, including the enzymes catalase and superoxide dismutase, which increases antibacterial effectiveness. Previous studies reported that BHT could inhibit the growth of some beneficial bacteria, potentially impacting the overall health and productivity of aquaculture organisms (Shallangwa et al. 2022).

Aeromonas hydrophila is one of the Gram-negative bacteria with a distinctive and highly organized cell envelope structure, serving as a strong barrier against various external compounds, including antibiotics, toxins, and environmental stress factors (Rasmussen-Ivey et al. 2016; Mulia et al. 2023b). This cell envelope comprises three main layers: the inner cytoplasmic membrane, a thin peptidoglycan layer, and the outer membrane (Liu 2014). Lipopolysaccharide (LPS) in *A. hydrophila* plays a crucial role in limiting the penetration of hydrophobic compounds, contributing to the bacteria's resistance to various antimicrobial agents. Based on previous studies, LPS functions as a selective permeability barrier, which is more difficult for hydrophobic agents to penetrate than the more accessible membranes of Gram-positive bacteria (Lim et al. 2022; Hudecová et al. 2024). Additionally, LPS molecules

can trigger an immune response in the host organism, potentially complicating therapeutic strategies. The expression pattern of genes regulating LPS synthesis varies among *A. hydrophila* strains, affecting their sensitivity to antimicrobial agents (Erickson et al. 2023; Kurniawan et al. 2024), between the presence of LPS and certain antibacterial compounds' reduced effectiveness due to difficulty penetrating the protective outer membrane (Patel et al. 2017).

The three autoinducer systems in *A. hydrophila*, namely AHL, LuxS, and QseBC type systems, play an essential role in controlling the expression of virulence factors and supporting communication between bacterial populations (Jin et al. 2020). The autoinducer systems enable diffusion in and out of cells, allowing *A. hydrophila* to adjust behavior based on population density. This process contributes to pathogenicity in various hosts, including fish (Rasmussen-Ivey et al. 2016). Interfering with the synthesis, transport, or identification of autoinducers produced by *A. hydrophila* is a strategy to prevent QS. *A. hydrophila* uses N-Acyl Homoserine Lactones (AHLs) as autoinducers to communicate in the bacterial community, enabling coordination of various behaviors, including biofilm formation and virulence factor expression (Papenfort and Bassler 2016; Srinivasan et al. 2020; Arumugam et al. 2023). Previous reports showed that quorum-sensing inhibitors, such as naringin and tannic acid, had been shown to effectively suppress biofilm formation and reduce virulence factor expression in *A. hydrophila* (Patel et al. 2017). Additionally, recombinant enzymes, such as AHL-lactonase, significantly reduces effectively degrade AHL, and considerably decreases virulence levels and biofilm formation in aquaculture environments (Zhang et al. 2019a).

In silico discussion

As shown in Table 5, BHT is the best fraction compound of *A. cordifolia* leaf extract, which interferes with cellular protein function, including enzymes such as DXS and CAH. These compounds have hydroxyl (-OH) groups interacting with polar amino acid residues in DXS and CAH proteins. The interactions lead to the formation of hydrogen bonds, disrupting the natural hydrogen bonding network necessary to maintain stable protein structures. Therefore, these changes can affect the enzyme's active site, inhibiting substrate binding and reducing its catalytic activity. The structural instability that occurs due to hydrogen bond disruption can also exposes the protein's hydrophobic areas, making it more susceptible to denaturation processes.

DXS protein in *A. hydrophila* has a crucial role in 1-deoxy-D-xylulose-5-phosphate (DXP) biosynthesis pathway, which is a fundamental part of synthesizing essential biomolecules (Zhou et al. 2017). In the path, DXS acts as a catalyst in the acyloin condensation reaction, which includes pyruvate as a three-carbon molecule and glyceraldehyde 3-phosphate (G3P) to form DXP. This reaction becomes an essential early stage in DXP pathway, producing terpenoids, isoprenoids, and various secondary metabolites required for cellular functions. Additionally, CAH acts as the primary carbon source in the metabolic process of *A. hydrophila,* directly contributing to its survival and growth (Supuran 2016; Occhipinti and Boron 2019). Due to the significant role of DXS and CAH in cellular metabolism, disruption to their function can seriously impact bacterial survival. One compound that has the potential to disrupt these proteins is BHT. Specifically, the interaction of BHT with DXS and CAH is capable of causing structural changes or impaired protein function, which has implications for the smooth metabolic processes of *A. hydrophila*. These disruptions inhibit the DXP biosynthesis pathway through mechanisms including oxidative stress or direct interaction with protein active sites, causing changes in enzymatic activity. Therefore, the production of DXP can be halted, leading to the failure of important metabolite synthesis, impaired growth, reduced adaptability, and cell death in *A. hydrophila*.

In conclusion, UV-Vis spectrophotometer, FT-IR, and GC-MS analysis showed that BHT was the main active compound of *A. cordifolia*, with significant antibacterial activity. In silico studies showed that BHT could inhibit essential enzymes of *A. hydrophila*, such as DXS and CAH, playing a role in the metabolic process of bacteria. BHT also showed the potential to interfere with quorum sensing mechanisms, inhibit biofilm formation, and increase oxidative stress, leading to bacterial cell death. Based on the results, *A. cordifolia* leaf extract could be used as a natural solution in controlling *A. hydrophila* disease in the aquaculture sector. However, further studies through in vivo tests were recommended to confirm its effectiveness and support its application in fishing industry.

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