

TIK-14_Metabolomic Profiling of Jeruju (*Acanthus ilicifolius*) Leaves Extract with Antioxidant and Antibacterial Activity on *Aeromonas hydrophila* growth *by - Turnitin*

Submission date: 17-Jun-2024 10:16PM (UTC+0700)

Submission ID: 2403160744

File name: TIK-14.pdf (2.04M)

Word count: 9924

Character count: 55491

Metabolomic profiling of Jeruju (*Acanthus ilicifolius*) leaf extract with antioxidant and antibacterial activity on *Aeromonas hydrophila* growth

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ARTICLE INFO

Received on: 07/12/2021

Accepted on: 10/05/2022

Available Online: 04/08/2022

Key words:

Acanthus ilicifolius, Banjar, Jeruju, Mangrove, South Kalimantan.

ABSTRACT

Jeruju (*Acanthus ilicifolius*) is a Kalimantan tidal swamp plant that is potent to treat *Aeromonas hydrophila* infection. This study aimed to determine the potential of Jeruju leaf extract as antioxidant and antibacterial agents to inhibit *A. hydrophila* growth. The research method included the sampling of Jeruju leaves in South Kalimantan, Indonesia. Then, the extracted sample was macerated with ethanol. The sample extracts were screened for phytochemical (Harborne) and metabolomic profiles (liquid chromatography and high-resolution mass spectrometry). The profile of compounds in the extract predicted biological activity using PASS server. Furthermore, the extracts were assayed for antibacterial activity (well diffusion and broth dilution), antioxidant activity 2,2-diphenyl-1-picrylhydrazyl (DPPH), and total phenol content (Folin-Ciocalteu). The phytochemical screening showed that the Jeruju leaves' ethanol extract contained alkaloids, flavonoids, tannins, phenolics, terpenoids, and steroids. The metabolomic profiling was dominated by betaine (41.61%) and choline (40.27%). The prediction of biological activity showed that the Jeruju leaf extract acted as a peptidoglycan glycosyltransferase enzyme inhibitor, DNA synthesis inhibitor, and free radical scavenger. The Jeruju leaf extract can inhibit *A. hydrophila* growth on glutamate starch phenol agar (9.09%). The ethanol extract of Jeruju leaves showed very strong antioxidant potential ($IC_{50} = 49.73 \pm 1.14 \mu\text{g/ml}$ and 70.31% DPPH scavenging effect at 96 $\mu\text{g/ml}$), with a total phenol content of $32,667 \pm 1,778.58 \text{ mg Gallic acid equivalent (GAE)/100 g dry extract}$. These research findings provide potential antioxidant and antibacterial activities for Jeruju (*A. ilicifolius*) leaves' ethanol extract for inhibiting *A. hydrophila* growth.

INTRODUCTION

Aeromonas hydrophila is a Gram-negative and opportunistic bacterial pathogen that infects freshwater fish. *Aeromonas hydrophila* causes motile aeromonad septicemia (MAS) disease and causes losses in freshwater fish farming (Olga *et al.*, 2020). Fish farmers in Kalimantan, Indonesia, usually

use antibiotics (oxytetracycline, chloramphenicol, erythromycin, streptomycin, pefuran, enrofloxacin, and neomycin) to overcome MAS disease (Aisiah *et al.*, 2011). However, antibiotics in conquering illnesses brought about by bacterial contaminations can have an impact on the environment and well-being. Antibiotics not only kill the disease microorganisms but also kill microalgae that could become regular nourishment for refined fish (Agostini *et al.*, 2019) and could be dispensed with nontarget microscopic organisms, for example, probiotic microbes that help refined fish's development (Verschuere *et al.*, 2000). Moreover, the utilization of anti-infection agents in fish cultivation could leave buildups on fish meat (Okocha *et al.*, 2018) and dirty the oceanic climate (Monteiro *et al.*, 2018).

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One of the safe and environment-friendly efforts to overcome *A. hydrophila* infection is by utilizing natural products widely grown around the community. Kalimantan forests contain many types of plants that can be used as natural medicine (Aisiah *et al.*, 2019; Negara *et al.*, 2017). One of the Kalimantan tidal swamp plants that could potentially be used to treat *A. hydrophila* infection is *Acanthus ilicifolius*, locally named Jeruju. *A. ilicifolius* plant belongs to the Acanthaceae family and is included as a mangrove plant.

Jeruju plants (*A. ilicifolius*) are found in wetland regions at stream estuaries as mangrove vegetation. Jeruju was delegated a rising sea-going plant and occupies estuary waters, with a low saltiness level (Irawanto *et al.*, 2015). The Jeruju plant's qualities show a stem encircled by smooth and sharp spines. The natural surroundings of Jeruju are related with wild plants and are infrequently found ashore. Jeruju has enormous serrated leaves, with tightened tips and sharp spines (Noor *et al.*, 2006). In Indonesia, the Jeruju (*A. ilicifolius*) plant is spread across West Sumatra, Bekasi, Central Java, East Java, Bali, East Nusa Tenggara, West Kalimantan, East Kalimantan, South Kalimantan, North Maluku, Maluku, West Papua, and Papua (Fig. 1).

Jeruju is commonly found in the coastal areas of Kalimantan, forming shrubs in areas where salinity is relatively low (Saptiani *et al.*, 2013). Ethnobotanical studies have reported that Jeruju has been used to restore energy after childbirth, medication for stomach pain, rheumatism, hypertension, flatulence, and worm medicine by the Malay community in Sungai Tekong, West Kalimantan, Indonesia (Ernianingsih *et al.*, 2014; Ratnasari *et al.*, 2017). The Jeruju leaves were used as a fever-reducing medicine (antipyretic) in Teluk Selong, South Kalimantan, Indonesia (Forestryana *et al.*, 2018).

Literature studies have reported that the chemical compounds (like bioactive or secondary metabolite) in Jeruju (*A. ilicifolius*) function as a neuralgic, analgesic, anti-inflammatory, antioxidant, hepatoprotective, antileukemic, anticancer, antimicrobial, antiviral, antifungal, and natural insecticide (Irawanto *et al.*, 2015). Jeruju leaf extract could inhibit pathogenic bacteria growth, such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* (Khajure and Rathod, 2010), *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi* (Chundakkadu *et al.*, 2011), *Staphylococcus epidermis* (Govindasamy and Arulpriya, 2013), *Proteus vulgaris*, *Streptococcus pneumoniae*

(Ravikumar *et al.*, 2012), *Streptococcus viridans* (Pringgenies *et al.*, 2020), *Vibrio cholerae* (Thirunavukkarasu *et al.*, 2011a), and *Vibrio harveyi* (Saptiani *et al.*, 2013; Sreenivasa *et al.*, 2015).

Until now, literature studies have not shown the use of Jeruju leaf extract (*A. ilicifolius*) in overcoming *A. hydrophila* infection that causes MAS disease. Therefore, this study aimed to examine the potential of Jeruju leaf extract (*A. ilicifolius*) as a natural product to inhibit the growth of *A. hydrophila*. In addition, this study also evaluated the phytochemical content, metabolomic profiling, antioxidant activity, and total phenol content of the ethanol extract of Jeruju leaves.

MATERIALS AND METHODS

Materials

The research was conducted from July to December 2020 at the Fish Pests and Diseases Laboratory, Faculty of Fisheries and Marine, Universitas Lambung Mangkurat, Banjarbaru, Indonesia. The Jeruju leaves (*A. ilicifolius*) were collected from the riverbank of Bunipah, Aluh-aluh District, Banjar Regency, South Kalimantan, Indonesia. The isolate of *A. hydrophila* was obtained from the Mandiangin Freshwater Aquaculture Center for Fisheries (BPBAT), Banjar, South Kalimantan, Indonesia. The materials used were tryptic soy agar (TSA, Merck), tryptic soy broth (TSB, Merck), glutamate starch phenol agar (GSP agar, Merck), agar (Merck), distilled water, ethanol (Merck), and 2,2-diphenyl-1-picrylhydrazyl (DPPH, Merck). The tools used were digital scales, Whatman filter paper No. 42, ovens (Tungtec Instruments TH-160F), rotary vacuum evaporators (IKA RV 10), petri dishes, and Becker glass.

Jeruju leaves' extraction

The plant materials (Jeruju leaves) were cleaned, cut into small pieces, and dried in an oven at 40°C–50°C. The dried Jeruju leaves were crushed into a fine powder. A total of 100 g of fine powder of Jeruju leaves was macerated in 400 ml of ethanol for 24 hours. Ethanol is used as a solvent that is safe for the consumption for fish and humans. Ethanol is also a natural solvent for both food and natural medicine (Hikmawanti *et al.*, 2021). Ethanol used in this study is of analytical grade (Merck) and safe for the environment, considering it will apply to a fish culture environment. Ethanol is a solvent used to extract compounds from natural materials with

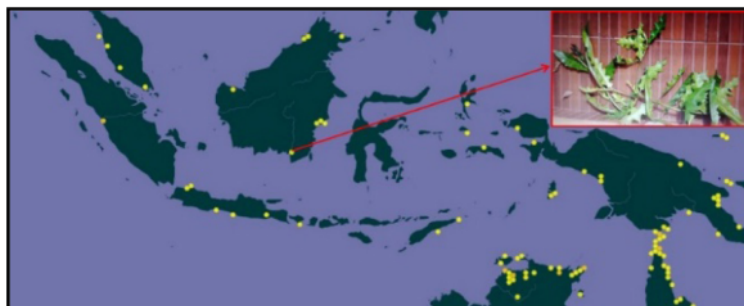


Figure 1. Distribution of Jeruju (*A. ilicifolius*) in Indonesia. Source: Global Biodiversity Information Facility (2021).

good results (Sultana *et al.*, 2009). The extract obtained was then evaporated using a rotary vacuum evaporator. Then, the Jeruju extract was dried in a vacuum desiccator for 4–5 days. The Jeruju leaf extract was stored at 4°C before assaying.

Phytochemical screening

The ethanol extract of Jeruju leaves was subjected to qualitative screening of chemical components to determine the presence of alkaloids, anthraquinones, flavonoids, steroids, terpenoids, saponins, and phenols using conventional standard protocols, as described by Harborne (1998).

Metabolomic profiling

Metabolomic profiling of the Jeruju leaf extract was conducted using liquid chromatography and high-resolution mass spectrometry (LC-HRMS Shimadzu-8040, Japan) with an injection volume of 1 µl. LC-HRMS was equipped with an autosampler, binary pump, column compartment, and a diode array detector for scanning spectroscopy. Chromatographic separation was performed using a C-18 column, Shim Pack FC-ODS (2 mm ϕ \times 150 mm, 3 µm). Two solvents were prepared including solvent A (H₂O: MeOH, 8:2, with 0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile). The two solvents were adjusted to 95:5 ratios, respectively, with an elution gradient of 0/0 at 0 minutes, 15/85 at 5 minutes, 20/80 at 20 minutes, and 90/10 at 24 minutes. Mass spectroscopy (MS) analysis was performed by electrospray ionization (ESI) with positive ions as the source. MS data were obtained through collision energy traps starting at 5.0 V. The ESI source parameters were regulated, including a capillary voltage of 3.0 kV, source temperature of 100°C, desolvation temperature of 350°C, sampling cone of 23 V, and desolvation gas flow of 6 l/hour. The chromatogram data obtained were compared with the data profile from the mzCloud Library system (Riyadi *et al.*, 2020; 2021).

Prediction of biological activity

The metabolomic profile detected from the ethanol extract of Jeruju leaves with LC-HRMS was predicted for biological activity using the PASS server (<http://www.pharmaexpert.ru/passonline/index.ph>). The PASS server is a software that is useful for predicting the biological activity of a compound (Rumengan *et al.*, 2021). The predicted biological activity requires a structural formula in the form of canonical SMILE obtained from the National Center for Biotechnology Information (<https://pubchem.ncbi.nlm.nih.gov/>) (Aisiah *et al.*, 2020).

Antibacterial activity assay

The antibacterial activity assay was conducted using the well-diffusion method on a petri dish with modifications (Balouiri *et al.*, 2016; Tanod *et al.*, 2019a). The well-diffusion method used two media layers, namely the base media and the seed media layer. The modification was carried out by adding TSA composition (2 g) with 2 g of bacto agar in 100 ml of distilled water as the base medium. The seedling layer was made from 70% TSA in 100 ml of distilled water, then put into a tube containing 9 ml of seed media and sterilized. Furthermore, 1 ml of *A. hydrophila* isolate was added to warm seedling with a density of 1×10^7 colony/ml (bacterial solution compared to the McFarland, HiMedia

standard). The *A. hydrophila* isolates used pure culture from BPBAT Mandiangin. The seeding medium that was added with *A. hydrophila* was vortexed, and then poured onto the base media layer. After the media hardens slightly, a well hole was made at a certain distance, using a 5 mm diameter glass tube. Each well was filled with 50 µl of Jeruju leaves' ethanol extracts with concentrations of 50, 100, 150, 200, and 300 mg/ml and incubated at 37°C for 24 hours. Cefadroxil and tetracycline (1 mg/ml each) were used as comparison controls. After that, the zone of inhibition was observed and measured. All experimental measurement data were carried out in three replications and expressed as mean \pm standard deviation ($n = 3$).

Antibacterial activity was evaluated using the broth dilution method based on the guidelines (EUCAST, 2000; Wiegand *et al.*, 2008) with modifications. Exactly 10 ml of TSB was inoculated with 100 µl of *A. hydrophila* (density 1×10^7 colony/ml), then incubated at 37°C for 24 hours. After that, 100 µl of the Jeruju leaves' ethanol extract (200 mg/ml) was added. *Aeromonas hydrophila* culture with aquadest was used as the negative control, and cefadroxil and tetracycline (1 mg/ml each) were used as positive controls. The total colony count was carried out on GSP agar, based on the total plate count method, following the Indonesian National Standard No. 01-2332.3 of 2006 with modifications (Indonesian National Standardization Agency—BSN, 2006). Modifications made using TSB on broth media and solid media using GSP selective media. If the GSP is red, it indicates *A. hydrophila* is not growing, whereas if the GSP is yellow, it indicates *A. hydrophila* growth.

Antioxidant activity assay

Antioxidant activity was determined using the DPPH radical scavenging method (Molyneux, 2004; Tanod *et al.*, 2019a). The ethanol extract of Jeruju leaves was added with ethanol so that the concentration was 100 µg/ml; then serial dilutions were made (6, 12, 24, 48, and 96 µg/ml). A 2 ml aliquot of each concentration's extract solution was added to 2 ml of the 50 µM DPPH solution. The mixture was homogenized and left for 30 minutes in a dark room at room temperature. Then, the mixture measured the free radical scavenging at a wavelength of 517 nm with a spectrophotometer (UV-VIS spectrophotometer T90 + PG Instruments Ltd).

The absorbance value of the DPPH solution was also measured and determined by IC₅₀ (half-maximal inhibitory concentration). Ascorbic acid was used as a positive control. IC₅₀ was determined as the concentration of the extract solution required to scavenge 50% DPPH free radicals (Dewanto *et al.*, 2021). The assay was carried out in three repetitions, and the measurement results were expressed with a standard deviation. The DPPH scavenging effects were calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Blank Absorbance} \times \text{Sample Absorbance}}{\text{Blank Absorbance}} \times 100\%$$

Total phenol content assay

The ethanol extract of Jeruju leaves was evaluated for total phenol content according to the Folin–Ciocalteu method (Blainski *et al.*, 2013; Lamuela-Raventós, 2017). Exactly 25

mg of Jeruju leaves' ethanol extract was dissolved in 25 ml of ethanol: aquadest (1:1) solution. Then, 1 ml from the extract solution and 10 ml of distilled water + 1 ml of Folin–Ciocalteu reagent (homogenization) were added. After that, it was let to stand for 8 minutes and 3 ml of 20% Na₂CO₃ was added (which was let to stand for 2 hour at room temperature). Then, the absorption with a UV-Vis spectrophotometer at a wavelength of 750 nm was measured, which gave a blue color. In the same way, a gallic acid solution was prepared, i.e., 25 mg of gallic acid was dissolved in ethanol: water (1:1) to a volume of 25 ml. Then, the gallic acid solution in a series of dilutions of 5, 20, 40, 60, 80, and 100 g/ml was made. The standard curve of gallic acid was prepared with the concentration of gallic acid (µg/ml) against the absorbance value. Total phenolic was determined using the standard curve regression equation for gallic acid. The total phenol content was expressed in mg GAE/100 g dry extract (Muliadin *et al.*, 2021; Riyadi *et al.*, 2021b).

RESULTS AND DISCUSSION

Phytochemicals of Jeruju leaves' ethanol extract

The Jeruju leaves were extracted using ethanol solvent to make the resulting extract more environment-friendly and safe to use for fish and humans. The extraction of 100 g of simplicia Jeruju leaves with 400 ml of ethanol solvent (analytical grade) obtained an extract weight of 21.30 g (extract yield of 21.30%). Phytochemical analysis was carried out to determine the type of natural products in the ethanol extract of Jeruju leaves. The phytochemicals' screening of the ethanol extract of Jeruju leaves (*A. ilicifolius*) is presented in Table 1.

Table 1 shows the phytochemical screening of the ethanol extract of Jeruju leaves, indicating the presence of flavonoids, alkaloids, tannins, phenolics, steroids, and terpenoids. Jeruju was also reported to contain lignans (Kanchanapoom *et al.*, 2001). *Acanthus ilicifolius* was also reported to contain alcohol, alkanes, fatty acids, lignans, steroids, and terpenoids (Wöstmann and Liebezeit, 2008). *Acanthus ilicifolius* collected from Kollam, Kerala, India, detected phytochemical components of saponins, tannins, terpenoids, flavonoids, alkaloids, and anthraquinones (Chundakkadu *et al.*, 2011). The leaf extract of *A. ilicifolius* isolated 4-coumaric acid compounds, including coumarin compounds, lignans, flavonoids, and phenylethanoid (Ravikumar *et al.*, 2012). Leaves of Jeruju collected

from Sungai Tekong, Kubu Raya, West Kalimantan, Indonesia, detected phytochemical components of alkaloids, saponins, flavonoids, terpenoids, and phenol (Ernianingsih *et al.*, 2014). Jeruju leaves collected from Kaligawe, Semarang, and the coastal area of Teluk Awur, Jepara, Central Java, Indonesia, were reported to contain saponins, quinone, and tannins compounds (Ardiantami *et al.*, 2015). In *A. ilicifolius*, there were also found terpenes and flavonoids (Sreenivasa *et al.*, 2015). Phytochemical studies of *A. ilicifolius* reported chemical constituents of triterpenoids, alkaloids, saponins glycosides, flavonoids, steroids, phenols, and coumarins (Bora *et al.*, 2017). The methanol extract of *A. ilicifolius* leaves contained flavonoids, alkaloids, and phenols (Handayani *et al.*, 2018). Jeruju also produces compounds of steroids, flavonoids, and tannins (Pringgienis *et al.*, 2020).

The use of methanol, ethanol, and water solvents in plant extraction did not significantly affect the results of qualitative screening of phytochemical components. The statement is according to the results of Godwill *et al.* (2013), Chigayo *et al.* (2016), and Nurdyansyah and Widyastuti (2020). However, if quantitative screening of phytochemical components is carried out, the use of methanol showed higher amounts (especially for phenols, flavonoids, alkaloids, and terpenoids) than ethanol and water (Truong *et al.*, 2019). So, it is indicated that methanol can extract better than ethanol and water.

Therefore, it strongly suspected that the difference in the phytochemical components in *A. ilicifolius* was due to environmental influences. The factors that influence differences in the production of chemical components are environmental conditions (Dewanto *et al.*, 2019). Chemical components produced by organisms play a role in the defense system in maintaining life (Gallo *et al.*, 2004), then organisms will produce more phytochemical components if they live in extreme environments. Liu *et al.* (2016) reported the difference of total tannin, flavonoid, rutin, and phenol in the same sample extract from different locations. Differences in phenol content were also reported in Mykhailenko *et al.* (2020); environmental factors affect the accumulation of phenolic compounds and their derivatives such as flavonoids, isoflavonoids, and xanthenes in plants.

The phytochemical components in Jeruju leaves, like flavonoids, alkaloids, tannins, phenols, steroids, and terpenoids, reported having antibacterial and antioxidant properties. The

Table 1. Phytochemical screening of Jeruju leaves' ethanol extract (*A. ilicifolius*).

Phytochemicals	Methods		Indicators
Flavonoids	Alkaline reagent	+	The greenish-yellow color (sample + NaOH) faded after dilute acid was added
	Lead acetate	+	Yellowish-brown precipitate formed
Alkaloids	Dragendorff's	+	Red precipitate formed
	Mayer's	+	Yellow precipitate formed
Tannins	Gelatin	+	A thick green precipitate formed
Phenolics	Iron (III) Chloride	+	The blackish green precipitate formed
Saponins	Foam	–	No stable foam formed for 10 minutes
Anthraquinones	Anthraquinone	–	No red color produced
Steroids	Liebermann Burchard's	+	A brown ring formed
Terpenoids	Salkowski's	+	Formed golden yellow color

flavonoid action mechanism as an antibacterial damaged bacterial cell walls and membranes, binding to cell adhesions and deactivating enzymes (Cowan, 1999). Flavonoid compounds act as scavengers for free radicals that arise due to bacterial infection to protect cells from negative enzymatic reactions (Xie *et al.*, 2015). Alkaloids are chemical components that act as scaffolds for antibacterial drugs (Cushnie *et al.*, 2014). The alkaloid action mechanism as an antibacterial is by inhibiting reductase dihydrofolate and topoisomerase type I enzymes, which play a role in DNA synthesis (Kittakoop *et al.*, 2014; Samoilenko *et al.*, 2009). Alkaloids have also been reported as inhibiting bacterial virulence without affecting growth or survival (LaSarre and Federle, 2013). An N group in the alkaloid structure can be an antioxidant because it acts as a free radical scavenger (Neganova *et al.*, 2012). Alkaloid components were reported to increase phenolic compounds' performance to provide a more potent antioxidant effect in plant extracts (Gan *et al.*, 2017).

Tannin antibacterial mechanism was conducted by inactivating microbial adhesion cells and inhibiting iron availability for microorganisms. The antibacterial mechanism of tannins showed phenolic damage the bacterial cell wall of polypeptides (Akiyama *et al.*, 2001; Ogbuagu, 2008). Tannins are known as antioxidants because they have the -OH(hydroxyl) group attached to aromatic rings. Tannins effectively scavenged free radicals as an electron donor and a source of hydrogen atoms, active in metal chelation because of the -OH groups and conjugated double bonds that allow the formation of electron delocalization (Dewanto *et al.*, 2018). The phenol antibacterial mechanism deactivated proteins (enzymes) in the bacterial cell membrane. Phenol binds to proteins through hydrogen bonds resulting in a damaged protein structure where most of the cell wall and cytoplasmic membrane structures of bacteria contain protein and fat (Susanti *et al.*, 2008). Phenolic components have the potential as antioxidants because they have -OH(hydroxyl) group on the aromatic ring (Agati *et al.*, 2009). The hydroxyl group can donate H atoms to free radical compounds to reduce free radicals. Phenol hydroquinone and its derivatives act as oxidative inhibitors that bind to free radicals and react with reactive oxygen species (ROS) molecules to form more stable compounds (Harborne, 1998).

Steroids have been reported to fight Gram-positive and Gram-negative bacteria (Polat *et al.*, 2011). Steroid components can disrupt the cell membrane of bacteria such as *S. aureus*, *E. coli*, and *K. pneumoniae* via the quaternary amine groups carried by the conjugate (Figuroa-Valverde *et al.*, 2009). Steroids also exert cytotoxic effects on bacterial cells (Dogan *et al.*, 2012). Steroid components were reported to play a role in enhancing endogenous antioxidants (Mooradian, 1993). The antibacterial mechanism of terpenoids is by damaging the bacterial cell membrane and dissolving the constituent membrane lipids (Cowan, 1999). The low concentrations of the terpenoid components only affect the enzymes involved in energy production, whereas the high concentrations of the terpenoids can lyse the membrane (Jasmine *et al.*, 2011). Terpenoids have a relatively complex cyclic structure (consisting of alcohol, aldehyde, or carboxylic acid), so they have a hydroxyl group that could act as an antioxidant (Dewanto *et al.*, 2019).

Metabolomic profiling of Jeruju leaves' ethanol extract

Metabolomic profiles screening of the Jeruju leaves' ethanol extract with LC-HRMS detected 95 peaks. The results

of the mass spectrum of each peak, compared with the mass spectra in the mzCloud library database, showed 67 compound profiles. However, based on the mzCloud score, only 35 peaks (24 compounds) were confirmed with an accuracy level above 85. Screening of the metabolomic profiles of the Jeruju leaves' ethanol extract with LCHRMS is presented in Table 2.

Table 2 shows that the list of metabolomic profiles was dominated by betaine (41.61%) and choline (40.27%). Betaine is an antioxidant substance that has been used in agriculture and health industry. Betaine is a precursor to *S*-adenosylmethionine, contributing to glutathione synthesis (endogenous antioxidant) (Jung *et al.*, 2013). The mechanism of betaine as an antioxidant is by scavenging ROS in cells by regulating the endogenous nonenzymatic antioxidant defenses. In addition, betaine inhibits ROS formation by isolating cells from oxidative stress inducers (Zhang *et al.*, 2016).

Choline was reported to reduce oxidant damage and regulate the antioxidant system in the immune system of Jian carp (*Cyprinus carpio* var. Jian), which was subjected to a challenge with *A. hydrophila* (Wu *et al.*, 2014). Choline also reported increasing the antibacterial properties of gills and the relative level of gene expression for tight-jointed proteins, decreasing the inflammatory status, and regulating the mRNA level of the associated signaling molecule in grass carp gills (*Ctenopharyngodon idella*) (Zhao *et al.*, 2016). Choline deficiency could cause oxidative damage due to changes in the transcription of antioxidant enzymes and signaling molecules Nrf-2/Keap-1 in the hepatopancreas and intestine (Wu *et al.*, 2017).

Biological activity prediction with PASS server

Furthermore, the list of compounds in Table 2 predicted their potential biological activity using the PASS server. The predicted value of the compound's biological activity in the ethanolic extract of Jeruju leaves was expressed as a probability to be active (Pa) (Fig. 2). The prediction was carried out as an inhibitor of cell wall biosynthesis, peptidoglycan membrane inhibitor, protein synthesis inhibitor, nucleic acid synthesis inhibitor, and free radical scavenger (Madigan *et al.*, 2019).

Figure 2 shows the potential of Jeruju leaves' ethanol extract as an antibacterial against the growth of *A. hydrophila*, closely related to its mechanism, which is thought to be a peptidoglycan glycosyltransferase enzyme inhibitor, DNA synthesis inhibitor, and free radical scavenger. Peptidoglycan glycosyltransferase enzyme plays a role in peptidoglycan biosynthesis in bacterial cell wall formation (Derouaux *et al.*, 2013). By inhibiting the peptidoglycan glycosyltransferase enzyme action, bacteria cannot synthesize peptidoglycan; so, bacteria cannot maintain their shape and protect themselves from osmotic pressure.

Biological activity prediction of the Jeruju leaves' ethanol extract as an inhibitor of the peptidoglycan glycosyltransferase enzyme, DNA synthesis inhibitor, and free radical scavenger was supported by the results phytochemical screening and metabolomic profiling with LC-HRMS. The flavonoid and phenol compounds in the extract are thought to deactivate and inhibit the peptidoglycan glycosyltransferase enzyme's performance. In addition, alkaloid components could inhibit DNA synthesis by inhibiting the enzyme's dihydrofolate reductase and topoisomerase type I (Kittakoop *et al.*, 2014). Metabolomic profiles shown in

Table 2. Metabolomic profiles of the Jeruju leaves' ethanol extract with LC-HRMS.

RT (min)	Metabolomic profiles	Area (%)	Mz-Cloud score	Bioactivity potential	References
1.029	α -Amino-caproic acid	0.45	86.4	Antibacterial	Midura-Nowaczek <i>et al.</i> (2013)
1.033			89.1		
1.155	Choline	40.27	96.1	Antibacterial and regulates the inflammatory response	Zhao <i>et al.</i> (2016)
1.367			94.0		
14.360			93.2		
1.104	Adenosine	0.66	91.8	Anticancer	Prestegard <i>et al.</i> (2009)
1.180	Betaine	41.61	95.6	Antimicrobial, cytotoxic and	Radošević <i>et al.</i> (2018);
				Antioxidant	Angelini <i>et al.</i> (2019)
1.224	Trigonelline	0.84	93.7	Antibacterial and	Almeida <i>et al.</i> (2006); Zhou <i>et al.</i> (2012)
1.402			93.2	Antidiabetes	
1.440			86.1	Hepatoprotective, antihyperlipidemic, antinociceptive, antiarrheal, antioxidant, anticancer, antimicrobial, an inhibitor of nephrolithiasis and carcinogenesis	
1.757	Isoleucine	1.42	88.1		Mujahid <i>et al.</i> (2016)
1.870	L-Phenylalanine	0.04	85.8	Antioxidant, analgesic, antipyretic, anti-inflammatory,	Fikri <i>et al.</i> (2020)
				Antimicrobial, and cytotoxic	
6.573	Schaftoside	0.02	89.9	Antioxidant	Thiem <i>et al.</i> (2001)
7.501	2-(3,4-Dihydroxyphenyl) ethyl 3-O-(6-deoxy- β -L-mannopyranosyl)-6-O-[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl]- β -D-glucopyranoside	0.08	90.2	Anti-inflammatory, cytotoxic, and antineoplastic	Pettit <i>et al.</i> (1990); Simamora <i>et al.</i> (2020)
8.015	4-Indolecarbaldehyde	0.24	88.1	Anti-inflammatory	Carpes <i>et al.</i> (2020)
11.095	9S,13R-12-Oxophytodienoic acid	0.55	90.7	Antimicrobial and Antiproliferative	Fudyma <i>et al.</i> , (2019); Laila <i>et al.</i> (2020)
17.106			88.7		
11.749			91.2		
17.817	9-Oxo-ODE	0.30	92.5	–	–
17.155	2-Amino-octadec-4-ene-1,3-diol	0.12	91.1	–	–
17.623	9-Oxo-10(E),12(E)-octadecadienoic acid	1.17	93.3	–	–
18.019			93.8		
18.731	(±)-12(13)-DiHOME	0.09	87.5	Antibacterial	Son <i>et al.</i> (2018)
19.787			90.0	Antimicrobial, Antioxidant,	
19.968	1-Linoleoyl glycerol	0.87	90.7	Anti-inflammatory, anti-arthritis,	Parthipan <i>et al.</i> (2015)
20.232			92.6	Antiasthma and diuretic	
20.451	α -Linolenic acid	0.20	92.4	Antibacterial and antimalarial	Das (2018)
21.357	Monoolein	0.17	88.3	Antioxidant and antiatherosclerotic	Fadzir <i>et al.</i> (2018)
21.531			88.7		
21.406	Bis(2-ethylhexyl) phthalate	0.20	89.4	Antimicrobial, antifouling, and cytotoxic	Habib and Karim, (2009); Osuntokun and Cristina (2019); Roy (2020)
22.208	Oleamide	1.16	95.9	Antioxidant and antibacterial	Elmi <i>et al.</i> (2020)
22.982	Hexadecanamide	0.15	96.1	Antibacterial, anticancer, anti-inflammatory, analgesic, antipyretic, and antioxidant	Al-Snafi (2019); Aldakheel <i>et al.</i> (2020)
25.026	Stearamide	0.15	89.7	Antimicrobial, antioxidant, antiseptic, and anti-inflammatory	Zayed <i>et al.</i> (2014); Mary and Giri (2016)
26.214	Erucamide	0.07	94.9	Antibacterial, antitumor, and cytotoxic	Saha <i>et al.</i> (2020)
26.411	Triethanolamine	0.35	87.8	Antimicrobial	Petrović <i>et al.</i> (2012)

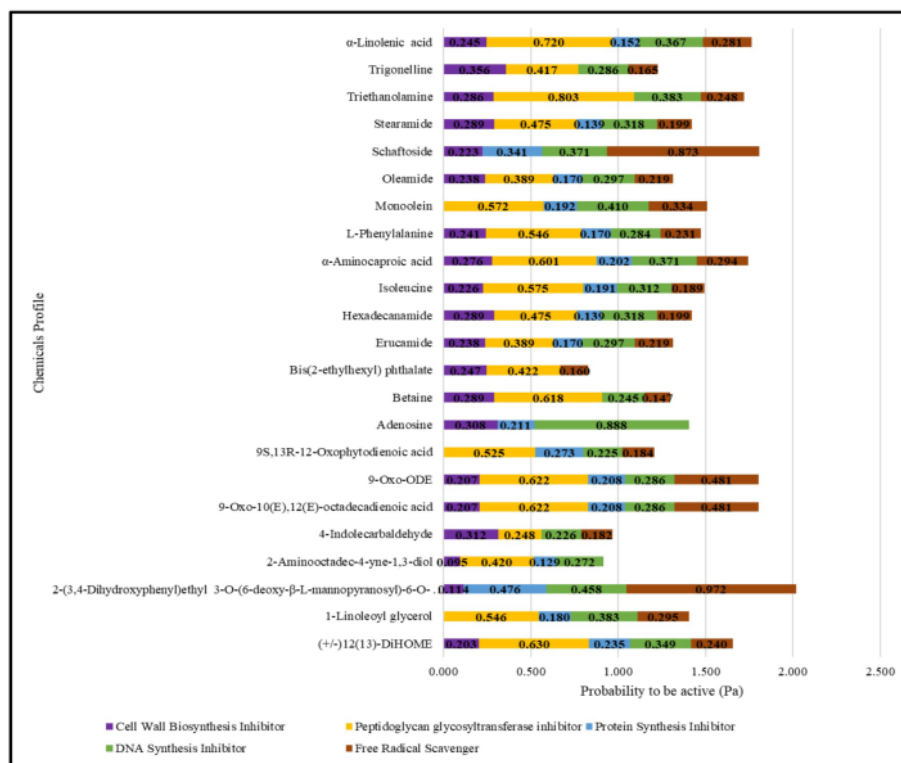


Figure 2. Prediction of the biological activity of Jeruju leaves' ethanol extract with PASS server.

Table 2, which have a hydroxyl group ($-\text{OH}$), an amine group ($-\text{NH}_2$), and a cyclic nitrogen structure, are thought to act as free radical scavengers.

Antibacterial activity of the Jeruju leaf extract

Antibacterial activity evaluation of the Jeruju leaves' ethanol extract was carried out by observing the inhibition zone formed from *A. hydrophila* isolates. The measurement of the inhibition zone diameter of the Jeruju leaves' ethanol extract (*A. ilicifolius*) compared to the control is shown in Table 3.

Table 3 shows the antibacterial activity, which increases depending on the extract concentration against the growth of *A. hydrophila*. The Jeruju leaves' ethanol extract showed weak antibacterial activity against *A. hydrophila* with agar diffusion method ($p < 0.05$). According to the inhibition zone category by Paudel *et al.* (2014), there are four categories of antibacterial activity: very strong (inhibition zone $\phi > 20$ mm), strong (inhibition zone, $\phi = 15-20$ mm), moderate (inhibition zone $\phi = 10-15$ mm), and weak (inhibition zone $\phi < 10$ mm). As dominant profile components in Jeruju leaves, betaine and choline have mechanisms to increase endogen antioxidants. In addition, flavonoids, phenols, and alkaloids in Jeruju leaves have hydroxyl and amine groups, which are hydrophilic. *Aeromonas hydrophila* has a hydrophilic side, namely carboxyl, amino acid, and hydroxyl (Madigan *et al.*, 2019). The hydrophilic side is a factor that

determines the penetration, binding, and activity of antibacterial compounds (Sefa *et al.*, 2020).

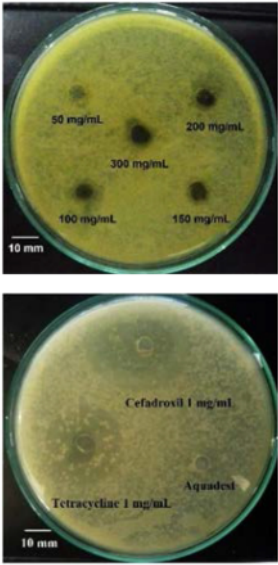
This study also observed the antibacterial power of the ethanol extract of Jeruju leaves in inhibiting the growth of *A. hydrophila*. Observations were made using the broth dilution method to count the quantitative number of *A. hydrophila* that could be inhibited. The results showed that Jeruju leaves' ethanol extract (200 mg/ml) could suppress the number of *A. hydrophila* that grew on glutamate starch phenol agar (GSP agar) ($p < 0.05$) (Table 4). According to the media guidelines for *Aeromonas* Jeppesen (1995), *A. hydrophila* will degrade the red color of the GSP agar medium to yellow. The color change is because *A. hydrophila* degrades the starch in GSP agar by producing acid, causing the phenol red to turn yellow (Naviner *et al.*, 2006).





Antioxidant activity and total phenol content of the Jeruju leaf extract

This study also evaluated the antioxidant activity of the Jeruju leaf extract using the DPPH radical scavenging method. DPPH was a stable free radical and can accept electrons or hydrogen radicals to form a stable diamagnetic molecule (Tanod *et al.*, 2019b). Antioxidant activity indicates chemical components' ability to inhibit oxidation reactions, expressed as the percentage of DPPH radical scavenging. The percentage of DPPH radical scavenging for Jeruju leaf extract and ascorbic acid as a control is shown in Figure 3.

Table 3. Diameter of the inhibition zone from Jeruju leaves' ethanol extracts against *A. hydrophila*.

Sample	Diameter (mm)
Jeruju leaf extract	
300 mg/ml	9.78 ± 0.19 ^d
200 mg/ml	8.89 ± 0.38 ^c
150 mg/ml	8.22 ± 0.51 ^c
100 mg/ml	4.89 ± 0.69 ^b
50 mg/ml	1.22 ± 0.51 ^a
Cefadroxil (1 mg/ml)	28.78 ± 0.77 ^f
Tetracycline (1 mg/ml)	19.33 ± 0.77 ^e
Aquadest	0


Table 4. Antibacterial activity of Jeruju leaves' ethanol extract against *A. hydrophila* after 24 hours.

Sample	Antibacterial Activity	
	Broth dilution method (Colony/ml)	Selective media (GSP agar)
Jeruju leaves' extract 200 mg/ml	$(3.15 \times 10^7 \pm 1.71 \times 10^6)^c$	
Cefadroxil 1 mg/ml	$(1.61 \times 10^3 \pm 4.30 \times 10^3)^a$	
Tetracycline 1 mg/ml	$(2.57 \times 10^4 \pm 1.76 \times 10^3)^c$	
Aquadest	$(3.47 \times 10^{10} \pm 4.48 \times 10^8)^d$	

GSP is red = *A. hydrophila* = no growth; GSP is yellow = *A. hydrophila* growth.

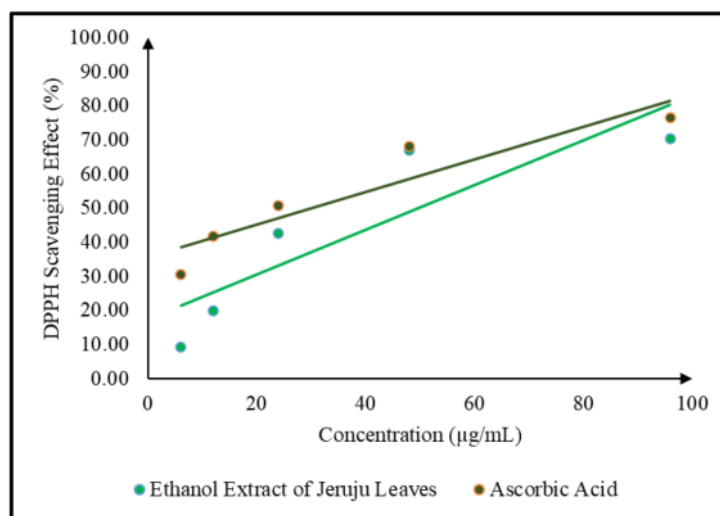


Figure 3. DPPH scavenging effect of Jeruju leaves' ethanol extract compared with ascorbic acid.

Table 5. IC₅₀ and total phenol content of Jeruju leaves' ethanol extract (*A. ilicifolius*).

Evaluation	Value	Regression
IC ₅₀ of Jeruju leaves	49.73 ± 1.14 µg/ml	$y = 0.6553x + 17.375$ $R^2 = 0.7691$
IC ₅₀ of ascorbic acid	29.84 ± 0.34 µg/ml	$y = 0.4771x + 35.762$ $R^2 = 0.8636$
Total phenol content	32,667 ± 1778.58 mg GAE/ 100 g dry extract	$y = 0.01x - 0.032$ $R^2 = 0.9987$

Figure 3 shows an increase in the effect of DPPH radical scavenging, along with the increase in the concentration of Jeruju leaf extract. The antioxidant activity indicates the ability of an extract to scavenge free radicals (Tanod *et al.*, 2019a). The ethanol extract of Jeruju leaf is thought to donate H atoms/electrons to interact with DPPH radicals. This study evaluated the IC₅₀ value and total phenol content of the Jeruju leaves' ethanol extract (*A. ilicifolius*) using the Folin–Ciocalteu method (Table 5). The Jeruju leaves' ethanol extract showed very strong potential as an antioxidant. Antioxidant activity was evaluated according to Blois (1958) and Riyadi *et al.*'s (2019) studies, namely very strong (IC₅₀ < 50 µg/ml), strong (IC₅₀ between 50 and 100 µg/ml), moderate (IC₅₀ between 100 and 150 µg/ml) and weak (IC₅₀ between 150 and 200 µg/ml).

Previous research has also evaluated the percentage of DPPH radical scavenging from Jeruju leaf extract (*A. ilicifolius*). The percentage of DPPH radical scavenging from the ethanol extract of *A. ilicifolius*, collected from Poondiyankuppam, northeast coast of India, ranged from 50.55 ± 2.88 to 86.87 ± 5.04%, which was compared with DPPH radical scavenging of ascorbic acid ranging from 65.12 ± 5.40 to 90.32 ± 5.12% (concentration = 0.1–2 mg/ml, with a DPPH concentration of 0.1 mM). In addition, it was also reported that the total phenol

content of the ethanolic extract of *A. ilicifolius* was 257 mg GAE/g (Thirunavukkarasu *et al.*, 2011b).

The ethanol extract of *A. ilicifolius* leaves collected from Alapakkam, Tamil Nadu, India, detected flavonoid and phenol components, with DPPH scavenging activity ranging from 20.59 to 76.79%, which was compared with DPPH scavenging activity of ascorbic acid ranging from 91.18 to 96.43% (concentration = 200–1000 µg, with a DPPH concentration of 0.1 mM). This study also reported that the ethanol extract of *A. ilicifolius* leaves' total phenol content was 17.22 mg/10 ml extract (Vani and Manikandan, 2018).

Previous studies also reported IC₅₀ of *A. ilicifolius* leaves' ethanol extract of 78.90 ± 1.87 µg/ml and ascorbic acid of 10.08 ± 1.79 µg/ml (DPPH concentration = 0.2 mM), and total phenol content of 128.86 ± 0.01 mg GAE/g dry weight (Biswas *et al.*, 2019). The methanol extract of *A. ilicifolius* leaves collected from Wonorejo, East Java, Indonesia, reported alkaloid components, flavonoids, glycosides, polyphenols, steroids, and tannins. In addition, it also reported an IC₅₀ value of 17.51 µg/ml of the methanol extract of *A. ilicifolius* leaves, with a DPPH concentration of 0.06 mM (Andriani *et al.*, 2020).

CONCLUSION

These research findings provide a potential activity for Jeruju (*A. ilicifolius*) leaves' ethanol extract as antioxidant and

antibacterial for inhibiting *A. hydrophila* growth. The antibacterial action mechanism of Jeruju leaf extract is thought to be closely related to its antioxidant properties. Metabolomic profile structure indicates the alkaloid and flavonoid components that play a role in the antioxidant and antibacterial activity of the Jeruju extract. Further studies on an ethanol extract of Jeruju leaves *in vivo* on fish infected with *A. hydrophila* need to be carried out to observe the Jeruju leaf extract's toxicity and stability.

ACKNOWLEDGMENTS

The authors acknowledge the Rector, Dean of Faculty of Fisheries and Marine, Head of Institute for Research and Community Service, Universitas Lambung Mangkurat, and Head of Mandiangin Freshwater Aquaculture Center for Fisheries (BPBAT) for providing research facilities.

AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

FUNDING

PNBP funds funded this research through the Compulsory Research Lecturer Program with DIPA funding of 2020 (No.023.17.2.6777518/2020), Universitas Lambung Mangkurat, Indonesia.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

PUBLISHER'S NOTE

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How to cite this article:

Aisiah S, Rini RK, Tanod WA, Fatmawati F, Fauzana NA, Olga O, Riyadi PH. Metabolomic profiling of Jeruju (*Acanthus ilicifolius*) leaf extract with antioxidant and antibacterial activity on *Aeromonas hydrophila* growth. J Appl Pharm Sci, 2022; 12(08):057–069.

TIK-14_Metabolomic Profiling of Jeruju (*Acanthus ilicifolius*) Leaves Extract with Antioxidant and Antibacterial Activity on *Aeromonas hydrophila* growth

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