Effectiveness phytochemistry test of aquatic plant *Murdannia* sp. in invito for fish pathogen disease

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**Abstract:** Fish disease is a major factor causing yield losses in fish farming production. The use of antibiotics that treat against diseases can pose the highest risk for the ecosystem and its overuse could cause the side effect, such as antibiotics-resistant diseases. Environmentally friendly production method of fish is thus needed. The objective of this study was to determine the active pharmacological substances and anti-bacterial activities extracted from *Murdannia* sp. as a potential herbal agent as an anti-bacterial treat fish diseases. The study was conducted from October 2017 to May 2018. *Murdannia* sp. aquatic plants were collected from the Bantimurung river waters Maros district, South Sulawesi. Phytochemical analysis was carried out at Laboratory of Pharmacology, University of Indonesia.—Anti-bacterial analysis was carried out using the disc diffusion method in the KKP Fisheries Development Research and Management (IP4I), Depok. The results showed that an application of *Murdannia* sp. crude extract treated at dose of 0.1 g did not inhibit the growth of the bacterial pathogen of *Aeromonas hydrophilla*, *Edwardsiella ictaluri*, *Streptococcus agalactiae*, *Flavobacterium columnare*. However, at 0.2 g of *Murdannia* sp. extract level, the growth of *Edwardsiella ictaluri* was inhibited in intermediate inhibiton category which inhibition zone of 11 mm. Based on the pharmacological test, it was known that *Murdannia* sp. aquatic plants consisted of three majority compound such as 0.084/10 g of flavonoid, 0.0558/10 g of Phenol, and 2/10 g of antioxidant 3036. These findings could be a basic research information in attempting to use aquatic plants as an alternative way for treating anti-bacterial fish disease in future.

1. **Introduction**

Aquaculture is a fishery sector industry that has highly expectation to increase human protein consumes. In intensive aquaculture, the problem of fish disease is undeniably the main problem. Disease is the main cause and economic loss in the aquaculture industry. During this time for
prevention of fish diseases is using antibiotics [1]. Pathogenic bacteria that attack in aquaculture hatcheries are Edwardsiella ictaluri, Flavobacterium columnare and Aeromonas hydrophila which three deadly dangerous pathogens in the aquaculture industry. E. ictaluri, the main cause of enteric septicemia in catfish channels (Ictalurus punctatus Rafinesque), which causes up to 67% catfish industry deaths in one year [2]. Flavobacterium columnare, the cause of death in the freshwater fish aquaculture industry by up to 50% per year [3] and has a wide range of hosts, with known infections in over 36 fish species worldwide [4].

A. hydrophila infects many species of fish and other terrestrial animals [5]. Diseases brought by this fish become residues and infect in the human digestive tract which results in an infection to immune disorders [6]. Streptococcus sp. is a bacterium that attacks tilapia (Oreochromis niloticus) in Indonesia which causes Motile Aeromonas Septicemia (MAS) and Streptococcosis. This infection is characterized by septicemia, ascites, ulcers, bone defects, exophthlimia, and muscle necrosis.

The use of antibiotics as ingredients to overcome fish diseases has long-term effects on the environment, pathogen resistance, and humans who will consume these aquaculture products that will be exposed to fish antibiotic residues [7]. The excessive use of antibiotics will have an impact on the environment and human health which also threatens the sustainability of the fisheries and agriculture industries through irrigation waterways. Sanitary hazards associated with antibiotic residues in fish meat and the spread of antibiotic resistance to pathogenic bacteria in aquaculture [8].

Some researches about herbal antibacterial compounds derived from aquatic flora can be a solution to overcome fish diseases that are environmentally friendly, both for humans and maintain the balance of the aquatic ecosystem [9]. Natural treatment with herbs can increase not only a biological activities such as immunostimulants but also appetite in sick fish, anti-stress, antimicrobial and can be developed as an anti-bacterial in fish diseases in the future. Natural herbal characteristics are safer, cheaper, easy to decompose and there are no environmental hazards [10].

Indonesia is a mega biodiversity country that has endemic aquatic flora and fauna biota and is of global concern. One of Indonesia’s biodiversity richness which is endemic and untouched and has not yet become a global concern is aquatic plants [11].

Ethnobotany is reliable in sustainable pharmacopoeia in aquaculture field [12]. Murdannia sp. is very famous and much reported from India, Pakistan, and China. In India Murdannia sp. genus to date consists of 29 taxa and 58 species [13,14,15,16,17,18]. The western part of India consists of 22 species. This western region of India is a major centre of Murdannia sp. diversification. Some reports showed that there are 50 species in tropical and subtropical regions and 20 endemic species in China [19]. Also, the general character of Murdannia sp. has been discussed [19]. Murdannia sp. in this study came from Bantimurung, Maros Regency, South Sulawesi. Murdannia sp. aquatic plants, have never been examined the content of active compounds and their effectiveness as antibacterial. Therefore, the purpose of this study is to characterize the content of active substances (allelochemical compounds) Murdannia sp. and their effectiveness as antibacterial compounds in fish diseases in vitro.

2. Methods
2.1. Plant material
Murdannia sp. is an aquatic plant that was used in this study originated from the Bantimurung river flow Maros Regency, South Sulawesi. The observation and sample collection was conducted in October 2017. The aquatic plants were identified based on standard botanical techniques for species identification in the Research centre for Biology of Indonesian Institute of Science (LIPI), Cibinong, West Java. The fresh samples were washed by tap water to remove impurities on the sample surface and then dried in the greenhouse of the Indonesian Centre for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAAD), Bogor. Those dried samples were powdered by using a blender. Phytochemical analysis was tested in Laboratory faculty of Pharmacy University of Indonesia (UI). Antibacterial fish Pathogen Analysis was conducted in Laboratory Installation of Research on The Development and Control of fish Disease BRPBATTP – Ministry Marine and Fisheries.
2.2. Chemical and reagents
The chemicals and reagents used in this study include gallic acid standard, quercetin standard, 1,1-Diphenyl-2-picrylhydrazine (DPPH) from Sigma-Aldrich, Folin Ciocalteu’s phenol, methanol for analysis, ethanol for analysis from Merck Indonesia.

2.3. Extraction procedure
Aquatic plants samples were extracted according to the procedures described by Indonesian Materia Medika and Harborne [20, 21]. The dried powder sample (1 g) was diluted in 10 mL ethanol 70% and extracted by using a sonicator for an hour. Then, the filtrate and the residue were separated by using a funnel size 10µm and 58x58 cm for wide.

2.4. Phytochemistry screening
The aquatic plants extracts were diluted using ethanol. Identification of the presence of total phenolic content, flavonoid content, antioxidant activity, accordance with procedures of Indonesian Materia Medika and Harborne [20, 21].

2.5. Determination of Total Phenolic Content
Total phenolic content in the leaf extracts of Murdannia sp. was determined by the Folin–Ciocalteau colorimetric method. The microplate TPC method was based on the 96-well microplate Folin–Ciocalteau method with some modifications [22]. A total of 25 µL of the sample solution or the standard solution was mixed with 100 µL of 1:4 dilute Folin–Ciocalteau reagent and shaken for 60s in a 96-well microplate and incubated for 4 min. Then the solution added with 75 µL of sodium carbonate solution (1%), and shaken for 60s. The solution was incubated within two hours at room temperature. The absorbance was measured at λ 750 nm using a microplate reader 96-well® (Versa Max ELISA Microplate Reader, USA). The calibration curve of standards (gallic acid) was measured by the absorbance from microplate reader instrument and then calculated. The total phenolic content was derived from the calibration curve. The total phenolic contents were expressed as mg gallic acid equivalents (GAE)/g extract.

2.6. Determination of Total Flavonoid Content
The total flavonoid content in aquatic plants was determined using the aluminium chloride colorimetric assay. 50 µl of extracts or standard solution of quercitin (6.25, 12.5, 25, 50, 100 µg/mL) in 70% ethanol was added to 10 µl of 10% aluminium chloride solution, followed by 150 µl of 95% ethanol. 80% ethanol was used as reagent blank. 10 µl of 1 M sodium acetate was added to the mixture in a 96-well plate. All reagents were mixed and incubated for 40 minutes at room temperature, protected from light. The absorbance was measured at 415 nm using a Microplate Reader (VersaMax™ ELISA Microplate reader, USA) [19].

2.7. Qualitative antioxidant activity test with DPPH solution
A qualitative test was carried out by spraying 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in the sample solution which was bottled on the TLC plate and incubated for 30 minutes. The TLC profile in UV rays was observed with a wavelength of 254 nm.

2.8. Quantitative antioxidant activity test with the DPPH assay for Antioxidant activity
This assay was carried out using the method of [22], with minor modifications. The antioxidant activity test was performed on extracts from maceration and UAE (dry matter adjusted to a working solution of 1000 µg/mL in methanol) and standards (100 µg/mL quercetin stock solution in methanol). The DPPH solution absorption was measured using a UV-Vis spectrophotometer first.

A total of 20 µL of quercetin solution, negative control or sample was added 180 µL of DPPH 150 mmol/L solution. The mixture was shaken for 60 seconds in the well then, the solution was incubated at room temperature for 40 minutes in dark conditions [22]. Absorbance of the test solution was...
measured at the wavelength obtained in the DPPH maximum wavelength test. The percentage of inhibition of extracts against DPPH was calculated, calculated by the following formula:

\[
\text{Percentage inhibition} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100\%
\]

After the inhibition percentage of each concentration was obtained, linear regression was then made so that the equation \( y = a + bx \) could be obtained, where \( x \) is the concentration (μg/mL) and \( y \) is the percentage of inhibition (%). Antioxidant activity is expressed by 50% Inhibition Concentration or IC50, which is the concentration of the sample which can reduce DPPH radicals by 50% from the initial concentration.

2.9. Quantitative antioxidant activity test with the FRAP assay

This test was based on the microplate reader method described by [23], with minor modifications, using ammonium ferrous sulfate (AFS) as the standard. Antioxidant activity in the Ferric Reducing Antioxidant Power (FRAP) method was calculated as ferrous equivalent antioxidant capacity (FeEAC) in μmol/g, using the equation:

\[
\text{FeEAC (μmol/gr)} = \frac{\Delta A}{\text{GRAD}} \times \frac{\text{Av}}{\text{Spv}} \times D \times \frac{1}{C} \times 10^5
\]

Where \( \Delta A \) = pathlength correction value, \( \text{GRAD} \) is the gradient of the AFS calibration curve, \( \text{Av} = \) aliquot volume (300 μL), \( \text{Spv} = \) test sample volume (20 μL), \( C = \) sample concentration, and \( D = 1 \).

2.10. Antibacterial test with paper disc diffusion method

*Aeromonas hydrophila, Edwardsiella ictaluri, Flavobacterium columnare* were cultured on Trypton Soy Broth (TSB) media. Incubated for 24 hours at 28°C, except *Edwardsiella ictaluri* was incubated 48 hours. *Streptococcus agalactiae*, cultured on Brain Heart Infusion (BHI) media, was incubated for 48 hours at 28°C (the growth period for these two bacteria is 48 hours). Antibacterial susceptibility tests use Clinical and Laboratory Standards Institute (CLSI 2005) procedures [24, 25]. Calculation of the number of colonies using the CLSI Standard [26]. Determination of the diameter of the antibacterial inhibitory zone by following the Bauer method [25], with inhibition zone classification based on Lorian method [27].

*Murdannia* sp. leaf extract as much as 0.2 g, from maceration dissolved in a 1 mL tube with 5% Dimethyl sulfoxide (DMSO) solvent. The Mueller Hinton agar (MHA) media which had contained the inoculum in the test tube was poured into a Petri dish containing 10 mL of MHA media, then homogenized by shaking and waited until the media solidified. After sterile paper discs were prepared, the extract was dropped onto the paper discs. Negative control using extract sample solvent, DMSO, was dropped on paper discs by 20μL. The discs was then incubated for 24 hours at 30°C. The antimicrobial agent diffuses into the agar and inhibits growth of the test microorganism and then the diameters of inhibition growth zones were measured with units of millimeters (mm).

3. Results

3.1. Phytochemical tests: flavonoids, phenols and antioxidants.

Phytochemical screening test results *Murdannia* sp. showed that aquatic plants, *Murdannia* sp. only contain flavonoids, phenols, glycosides and antioxidants (table 1). This aquatic plant does not have Alkaloids, terpenoids, anthraquinone and saponins.
Table 1. Results of screening phytochemical tests of aquatic plants Murdannia sp.

| Phytochemical test Result | Alkaloids | Flavonoids | Terpenoids | Phenols | Anthraquinone | Saponins | Glycosides | Antioxidants |
|--------------------------|-----------|------------|------------|---------|---------------|----------|------------|-------------|
|                          | -         | 0.84mg/QE/g | -          | -       | -             | +        | 458.325 GAE/g IC<sub>50</sub>(µg/mL) |

Glycoside test qualitatively using Anhydrous acetic acid + Sulfuric acid, the indicator shows there is a purple ring (figure 1.a) and Flavonoid Test using Shinoda reagents and AlCl<sub>3</sub> spray and UV light to see the scattering zones formed (figure 1.b).

![Figure 1](image1.png)

(a) (b)

Figure 1. Qualitative result screening Phytochemical test (a) glycosides test and (b) Flavonoid test.

3.2. Antibacterial Activity Test of Murdannia sp.

The inhibition zone test results of the plant extract at 0.1 and 0.2 g/mL extract (paste) and form a zone around the disc paper. The average area of antibacterial inhibition zones in A. hydrophila, E. ictaluri, S. agalactiae, F. columnare, and C. violaceum bacteria at extract concentrations of 0.1 g/mL between 2-7 mm and the inhibition zone diameter of extract concentrations 0.2 g/mL range from 7-11 mm (table 2).

Table 2. Average diameter of antibacterial inhibition zones of Murdannia sp. in the bacteria A. hydrophila, E. ictaluri, S. agalactiae, F. columnare, and C. violaceum (concentrations of 0.1 g/mL and 0.2 g/mL).

| Fish Pathogen | 0.1 g/mL concentration | 0.2 g/mL concentration |
|---------------|-------------------------|-------------------------|
|               | Average inhibition zone diameter (mm) and standard deviation | Inhibited zone response | Average inhibition zone diameter (mm) and standard deviation | Inhibited zone response |
| A. hydrophila | 3 ± std 0                | Resisten                | 8.67 ± std 2.08166 | Resisten                |
| E. ictaluri   | 2 ± std 0                | Resisten                | 11 ± std 5.291502 | Intermediet            |
| S. agalactiae | 3.67 ± std 0.57735       | Resisten                | 9.33 ± std 4.04145 | Resisten                |
| F. columnare  | 7 ± std 4.58257          | Resisten                | 7.33 ± std 2.51661 | Resisten                |
| C. violaceum  | 2 ± std 1.52752          | Resisten                | 1 ± std 0          | Resisten                |
| Control (-)   | Bacteria grow in infinite amounts | No inhibition zone | Bacteria grow in infinite amounts | No inhibition zone |

Control (-)
4. Discussion

S. agalactiae is a gram-positive bacterium and the others are gram-negative bacteria. Both types of bacteria have different protective membranes. Gram-positive bacteria are more resistant to physical treatment, with low lipid content and sensitive to penicillin. Gram-negative bacteria cannot stand physical treatment, high lipid content and are more resistant to penicillin. The focus of this research is on the E. ictaluri bacteria, which are gram-negative bacteria. According to the literature, E. ictaluri is resistant to Cationic Antimicrobial Peptides (CAMPs) caused by genes that regulate O-polysaccharide synthesis (OPS) [28]. This result showed that the content of Murdannia sp. extract compound with high concentration can inhibit the work of E. ictaluri bacteria.

The same thing is also happened to A. hydrophila, and S. agalactiae, as the concentration of Murdannia sp. extract increased, the inhibition of bacteria also increased, although the inhibition was not as good as the bacteria E. ictaluri. In F. columnare bacteria the inhibitory power of bacteria from Murdannia sp. extract is very stable even though the extract concentration is increased. Significantly, the difference from C. violeceum which is a quorum sensing bacteria, where the higher concentration of extract makes C. violeceum more resistant.

Murdannia sp. response, resistant to all bacteria tested both at a concentration of 0.1 g/mL and 0.2 g/mL due to failure of the compound contained in Murdannia sp. inhibits pathogenic bacteria, except E. ictaluri bacteria at a concentration of 0.2 g/mL responding to intermediates with an area of inhibition zone of 11 mm. The intermediate response to E. ictaluri [0.2 g/mL] shows the effectiveness of compounds contained in E. ictaluri at these concentrations giving a vague effect (whether sensitive or resistant). This response occurs because the compound allelochemical Murdannia sp. has not been able to inhibit the growth of pathogenic bacteria.

Pelczar and Chan [29] said that the mechanism of inhibition of bacterial growth by antibacterial compounds can be in the form of cell wall damage by inhibiting its formation or changing it after it is formed, changes in cytoplasmic membrane permeability so that it causes the release of food material from the cell, changes in protein molecules and nucleic acids, inhibits the action of enzymes, and inhibits the synthesis of nucleic acids and proteins.

The antioxidant activity of Murdannia sp. as well as the total phenolic content and total flavonoids per mg of dry weight extract in this study, as expected, there were inhibitory zones in each of the test bacteria, i.e. in the amount of extract the more the inhibitory zones will get bigger. De Freitas et al [30] reported that there was a correlation between the antioxidant content of Leiothrix spiralis on antimicrobial, hemolytic and antiproliferative plants.

Cowan [31] said that the inhibitory effect on microbes by Flavonoid compounds is due to the interaction between these compounds and cell membranes of target microorganisms, as well as their ability to bind extracellular proteins with cell walls. In this research, Murdannia sp. showed the differences in antimicrobial activity against gram-positive and gram-negative organisms. These differences can be related to the fact that cell walls in gram-positive bacteria are one layer, whereas gram-negative cell walls are multi-layered structures. This tendency can be explained by the fact that gram-negative bacteria have an outer membrane that surrounds the cell wall, which limits the diffusion of bioactive compounds due to the presence of lipopolysaccharides [32] and protects biological membranes from free radicals [33, 34].

5. Conclusions

Aquatic plant Murdannia sp. extracts contain phytochemical secondary metabolites such as flavonoids, phenols, glycosides and antioxidants. Murdannia sp. can be an intermediate antibacterial in the pathogen E. ictaluri at a concentration of 0.2 g/mL. The small inhibitory zone in other test bacteria does not mean that the compound has no antibacterial activity, the inhibitory activity of Murdannia sp. extract increases with the concentration of the extract given dose. The benefit of this research for fish farmers is that the farmers can use the aquatic plants as antibacterial, especially for fish diseases treatment. Outlook for the future of aquaculture system can protect aquatic plant, to balance the aquatic ecosystem.
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**Conflicts of interest**

The authors declare that there are no conflicts of interest in this study.