Antibacterial activity of Anadendrum microstachyum and Selaginella plana against two pathogenic bacteria (Edwardsiella ictaluri and Streptococcus agalactiae) causing freshwater fish diseases

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Abstract. Aquatic plants are potential to use as antibacterial to control pathogens that cause disease in fish. The antibacterial activity of extracts of Anadendrum microstachyum and Selaginella plana were tested against Edwardsiella ictaluri and Streptococcus agalactiae using disc diffusion Kirby-Bauer method. Plant extracts of A.microstachyum and S. plana at concentration 0.2 g could inhibit E. ictaluri and S. agalactiae; therefore A.microstachyum and S. plana are potential be developed as antibacterial compounds to substitute the use of antibiotic in fish farming and as candidates new drug to prevent and control of diseases in aquaculture.

Keywords: Aquatic plants, Anadendrum microstachyum, Selaginella plana, Edwardsiella ictaluri, Streptococcus agalactiae, Antibacterial.

1. Introduction
Anadendrum is an aquatic plant of the Araceae family and native to China and Southeast Asia. Anadendrum has root fibers and clear stem, green leaves with flat leaf edges (integer), tapered leaf tips (acuminatus), spiky leaf base (acutus), and pinnate leaf reinforcement (penninervis). The Araceae family are one group of plants that are found in Indonesia with 31 genera spread across several regions, including of Sumatra, Java, Kalimantan, Sulawesi and Irian Jaya [1].

Selaginella is estimated to have grown more than 320 million years ago and most species have become extinct, remaining the herbaceous [2]. The blue rane fern (Selaginella plana) has thin, fine and hard stringy roots. The stem is woody green and has two branches in each of the branches. Small leaves are pointed at the tips of the leaves and the edges of the leaves are jagged with a texture in the form of membranes or strands [2]. The plant has a ligula at the bottom of the leaf that functions as a
water absorber. Selaginella plana grows on rocks or river [3]. Rhizome spreads on the rock surface and its roots into the rock crevices.

Selaginella and Anadendrum have been used as medicine because of their antimicrobial effect which contain tannin, saponin, and flavonoid compounds [4, 5]. Flavonoids are active phenol compounds which can denature proteins therefore damaging the structure and changing the permeability mechanism of microsomes, lysosomes, and cell walls [6]. While saponins are phytosterol compounds derived from natural plants and occur in small quantity containing glycoside of triterpenes in which most of the anticancer agents are belong to these compounds [6]. Bioactive compounds extracted from A. microstachyum and S. plana are expected to substitute the antibiotics from chemicals as treatment of fish diseases. The purpose of this study was to determine the antibacterial potentiality of A. microstachyum and S. plana extracts against Edwardsiella ictaluri and Streptococcus agalactiae. These bacteria are pathogenic bacteria which has been reported to cause fatal mortality and morbidity of tilapia, infect catfish culture worldwide, and cause severe economic losses.

2. Materials and Methods
2.1. Aquatic plants extract preparation
Anadendrum microstachyum and S. plana aquatic plants were obtained from Bantimurung, South Sulawesi. The leaf and root of the plants were extracted using ethanol by maceration methods. Ten grams of mixed between the leaves and roots part were macerated using solvents in 10 L 96% ethanol for 48 hours. The macerated extracts were filtered and evaporated using a vacuum rotary evaporator at 50°C. Second evaporation was conducted using water bath at 50°C until thick extract was obtained. The phytochemical assay was done in the Pharmacy laboratory, University of Indonesia.

2.2. Bacterial strains
Two pathogenic bacteria, Streptococcus agalactiae and E. ictaluri, were used to determine the antibacterial effect of the plants extract. The bacterial strains were obtained from the collection of the Research Station for Fish Disease Control, Depok-BRPBATPP (West Java). Each of the bacteria was cultured overnight at 37°C on the Mueller-Hilton medium. The bacteria were then harvested and diluted using 5 mL of sterile saline buffer, with a cell density of 10⁷ CFU/mL (Mac Farland standard).

2.3. Antibacterial activity of plant extracts
The disc diffusion Kirby-Bauer technique [7] was used to evaluate the antimicrobial activity of each plant extract. Plant extracts concentrations of 0.1 g and 0.2 g were dissolved in 1 ml of DMSO, then 50 μL of extracts were dropped on sterile blank disc (6 mm in diameter). Twenty mL Mueller-Hilton agar medium was poured into a sterile petri dish followed by adding 0.1 ml of cultured bacterial suspension. Sterile blank discs loaded with each of the plants extract were placed on top of Mueller-Hilton agar medium. The DMSO was used as the negative control and chloramphenicol (30 mg) was used as positive control. The plates were incubated at 37°C for 24 hours. The presence of the inhibition zone around the discs was measured in mm using a Vernier calipers. The extract is said to have a strong inhibitory category if the diameter of the resulting inhibition ranges from 10 mm to 20 mm. The extract is said to have a medium diameter inhibition diameter if the inhibition range is between 5 mm - 10 mm and the diameter of the inhibition of an extract is said to be weak if the diameter of the inhibition zone produced is less than 5 mm [8]. This test was replicated in duplo.

3. Results and Discussion
3.1. Plant extraction
The content of bioactive compounds from both plants qualitatively can be seen in table 1. Anadendrum microstachyum contains flavonoid and glycosides while S. plana contains flavonoid, phenols, saponins, and glycosides. Anadendrum Microstachyum and S. plana produce crude extracts consisting of several secondary metabolites which have synergy antibacterial properties.
Table 1. Bioactive composition of Anadendrum microstachyum and Selaginella plana

| Bioactive composition | A. microstachyum | S. plana |
|-----------------------|------------------|---------|
| Flavonoid             | +                | +       |
| Phenol                | -                | +       |
| Saponin               | -                | +       |
| Glycosides            | +                | +       |

3.2. Antibacterial activity

Anadendrum microstachyum and S. plana were evaluated for their antibacterial activity against two strains of pathogenic bacteria, S. agalactiae and E. ictaluri, causing fish disease in aquaculture. Evaluation of the antibacterial activity can be seen in table 2 and figure 1. Anadendrum microstachyum and S. plana extracts showed higher activity in suppressing the growth of pathogenic bacteria at a concentration of 0.2 g. The extract of A. microstachyum had strong activity with diameter of inhibition zone of 10.67 mm for E. ictaluri and 11.33 mm for S. agalactiae.

Table 2. Average diameter of antibacterial inhibition zones of A. Microstachylum and S. plana plant extracts on pathogenic bacteria, at concentrations of 0.1 g and 0.2 g

| Bacteria     | A. microstachylum | S. plana | Antibiotic chloramphenicol |
|--------------|-------------------|----------|-----------------------------|
|              | Inhibition Zone Concentration (mm) |   | 0.1 g | 0.2 g | 0.1 g | 0.2 g | 30 mg |
| E. ictaluri  |                   |         | 1.33 | 10.67 | -     | 12.33 | 20    |
| S. agalactiae|                   |         | 5.67 | 11.33 | 4     | 9.33  | 23    |

S. plana extract showed potent activity against E. ictaluri (12.33 mm) and moderate activity against S. agalactiae (9.33 mm) (table 2 and figure 1). There was no inhibition activity in the negative controls (DMSO). The efficacy of herbal extracts used in the treatment is caused by the synergy between the active compounds contained in the extract. Synergy gives better antibacterial activity, reduce the potential toxicity of several single compounds, and can prevent drug resistance. Synergy from various secondary metabolites are also claimed to reduce undesirable side effects [9].
The antibacterial test showed that *A. microstachylum* and *S. plana* extracts were able to inhibit the growth of *E. ictaluri* and *S. Agalactiae* with the formation of clear zones around the paper disc containing plant extracts. The antibacterial strength of an extract is determined by the amount of inhibition zone formed. Antibacterial strengths are classified into four categories, namely very strong, strong, moderate, and weak. The extract is said to have a very strong inhibitory category if the diameter of the inhibitory yield is more than 20 mm [8]. Medicinal plants synthesize antimicrobial compounds as part of their defence against invasion by microbial pathogens. It is estimated that almost 50% of synthetic medicines are derived from or patterned after phytochemicals [10]. In the medicinal plant family, secondary metabolites such as alkaloids, phenolics, and other compounds have contributed the largest number of antimicrobial drugs in the pharmacological industry. The safer, biodegradable plant-derived compounds offer a promising solution to the problem of resistant microbes [11].

The efficiency of plant extract compounds and their effectiveness as antimicrobial agents to control the growth of pathogens indicate that the antimicrobial components of plant extracts such as terpenoid, alkaloid, and phenolic compounds interact with enzymes and membrane of microbial cell proteins causing interference with proton flux against cell exterior that induces cell death or inhibits cell death or inhibits enzymes needed for amino acid biosynthesis [12,13]. Eight flavonoid compounds from the leaves of *S. Crispus* showed positive results of the analyses for flavonoids, tannins, alkaloids and saponinic heterosides [14] and have antimicrobial effects. In addition, the inhibitory effect of plant extracts is related to the hydrophobic character of plants which allows them to react with microbial cell membrane proteins and disrupt the structure of mitochondria and change the permeability of cells.

Plant extracts that have proven to be potentially effective can be used as an alternative to controlling fish diseases instead of using antibiotics that will cause mutations in fish pathogens. The detrimental effects of antibiotics on fish health increase the demand for safe and naturally effective antimicrobials.
4. Conclusion
Aquatic plants extracts of S.plana and A. Microstachylum showed higher activity in suppressing the growth of pathogenic bacteria at a concentration of 0.2 g and have proven to be potentially effective to use as an alternative natural prevention for controlling fish diseases and inhibits the growth of E. ictaluri and S. Agalactiae. Further experiments on the two plant extracts are needed to determine the effectiveness, lethal doses 50, formulation in the feed, and the impact to the fish and the environment.

5. References
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