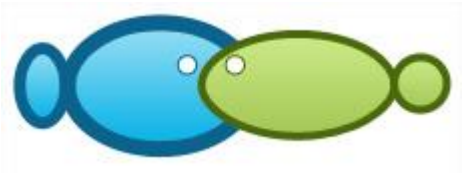

Phytochemical analysis and inhibitory effect of *Bruguiera gymnorrhiza* leaf extract on *Aeromonas salmonicida*, *Aeromonas hydrophila* and *Edwardsiella ictaluri*

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Abstract. Mangroves contain several secondary metabolites which are beneficial for the organisms' defense systems against pests and pathogens. *Aeromonas salmonicida*, *Aeromonas hydrophila* and *Edwardsiella ictaluri* are common fish pathogenic bacteria. The study aimed to determine the chemical composition of the *Bruguiera gymnorrhiza* mangrove tree species' leaf extract and its antibacterial activity on these fish pathogenic bacteria. A complete randomized design method with 6 concentrations was used: 12.5, 25, 50 and 100%, negative control and positive control, with 3 replications. The phytochemical test results showed that the mangrove leaves contained flavonoids, tannins, saponins, alkaloids and steroids. But it did not contain triterpenoids. Mangrove leaf extract of *B. gymnorrhiza* inhibited the growth of *A. salmonicida*, *A. hydrophila* and *E. ictaluri*. However, the inhibition was categorized as of a weak level, since the clear zone was <5 mm. The 100% concentration provided the strongest inhibition.
Key Words: bioactive compounds, mangrove, fish pathogen, secondary metabolites.

Introduction. The aquaculture business is growing significantly in almost all parts of the world, causing, in exchange, a decline in the quality of the aquatic environment, which increases the vulnerability to disease attacks in cultured fish. An integrated approach that considers not only pathogens but also the host and environment will be the most effective (Yoswaty et al 2020).

Mangroves have many benefits directly related to human life on land, ranging from ecological ones to the supply of food and medicines (Effendi et al 2018; Nurjanah et al 2015). Mangroves contain several secondary metabolites which support the organisms' defense against pests and other pathogens, also acting as attractants of animal pollinators and as growth regulating hormones. These compounds are bioactive substances so that humans use them as drugs and fragrances for food and cosmetics industry (Basyuni et al 2009). Phytochemical analysis revealed the presence of saponins, glycosides, tannins, flavonoids, phenol and volatile oils in the secondary metabolite compounds (Basyuni et al 2019).

Some researchers (Haq et al 2011) reported antioxidant and antibacterial activities of the methanolic, ethanolic and chloroform crude extracts of leaves and barks of *Bruguiera gymnorrhiza*. There was no significant difference between the LC 50 value of the ethanol extract of bark and the ascorbic acid on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*. Both ethanol and methanol extracts could inhibit the growth of all pathogenic bacteria while the chloroform extract of leaves showed no activity against any bacteria.

Aeromonas salmonicida is a gram-negative bacterium that is the causative agent of furunculosis, a bacterial septicaemia of salmonid fish, non salmonid fish (like carp), gouramy and tilapia (Kozińska et al 2002). *Aeromonas hydrophila* is associated with disease outbreaks in the aquaculture production in various parts of the world. Data show

that *A. hydrophila* represent approximately 42% of the motile aeromonads isolated from the disease outbreaks in the Zhejiang Province, in China (Nielsen et al 2001). *Edwardsiella ictaluri* is a facultative intracellular bacterium that causes the enteric septicemia of the catfish (ESC) (Attila et al 2006). This study aimed to determine the chemical composition of the *B. gymnorrhiza* mangrove tree's leaf extract and its antibacterial activity on these three fish pathogenic bacteria.

Material and Method

Design, place and object. A complete randomized design (CRD) method with 6 concentrations of mangrove leaf extract i.e. 12.5, 25, 50, and 100%, negative control and positive control, with 3 replications, was used in this experiment. Samples were taken from the mangrove ecosystem of Bandar Bakau, Riau, Dumai Distric, Riau Province Indonesia, during the period from April to August 2019. Fresh samples of *B. gymnorrhiza* leaf were cleaned, washed, chopped into small pieces and dried.

Preparation of extract. Leaf of *B. gymnorrhiza* were dried, finely blended and then macerated in an ethanol solution (70%) for 24 hours. The ratio of *B. gymnorrhiza* leaf to the solvent used was 1:5, i.e. 1 kg of flour was dissolved into 5 L of solvent (Jayaraman et al 2008). The solution was filtered with filter paper and the remainder of the first filter was re-macerated in the same way, to produce a clear color filtrate. The filtrate was collected and evaporated using a rotary vacuum evaporator, at a temperature of 600°C, until the solvent was completely evaporated and the crude extracts of *B. gymnorrhiza* mangrove leaf were obtained.

The crude extract samples were dissolved with 100 mL ethanol solution in a 500 mL beaker glass. The samples were heated and stirred using a stirring rod until boiling. The samples were placed in other 500 mL beaker glasses, and reheated until they became thick. The beaker glass was then filled with ammonia chloroform until there were cracks and added with enough distilled water. The samples were stirred well until 2 layers appeared. The upper layer contained water and extract and the bottom layer contained chloroform extract. The phytochemical analysis was carried out by adding certain color reagents (Mayer, Dragendorff and Wagner (alkaloids), 1% $FeCl_3$ (tannins), $C_4H_6O_3$ (terpenoids and steroids), and concentrated HCl and Mg powder (flavonoids)) to the extract.

Saponin, phenol and tannin analysis. A total of 0.1 g of sample was placed into a beaker glass, then 10 mL of hot water was added and boiled for 5 minutes. The solution was filtered and the filtrate was used as a test solution. The filtrate was placed into a closed test tube, then shaken for approximately 10 seconds and left for 10 minutes and eventually added 1 mL of 2M HCl. The presence of saponins was indicated by the formation of stable froth. For the phenol and tannin test, the extract of *B. gymnorrhiza* mangrove leaf was carried out by adding 3 drops of $FeCl_3$. The presence of phenol and tannin were shown by dark green or blue colors.

Flavonoid, steroid and terpenoid analysis. The mangrove leaf extract was added with 5 drops of Mg and concentrated HCl, and mixed vigorously to form a layer. The reddish yellow to red color indicated that the sample contained a flavonoid compound. Steroid and terpenoid analysis were carried out as follows: First the chloroform was poured into 2 drip plate holes and a xfan to dry. The content of the two holes was added with concentrated anhydrous acetic acid and concentrated H_2SO_4. The presence of steroid compounds was indicated by the formation of a green color, while the presence of a terpenoid was indicated by the formation of a purple color.

Inhibitory test. The concentrate of the mangrove leaf extract was dissolved with distilled water to 12.5, 25, 50 and 100%. The positive control was a 30 µg chloramphenicol-containing paper disc and the negative control was prepared by using distilled water. Pure and young pathogenic cultures (*A. salmonicida*, *A. hydrophila and E.*

ictaluri) were then sub-cultured on nutrient agar. The inhibitory tests of extracts against pathogenic bacteria were carried out by using the disc diffusion method. Bacterial isolates that have been grown were suspended with distilled water, then 0.1 mL of solution were spread using a glass rod on nutrient agar media. Paper discs that have been dropped with the *B. gymnorrhiza* leaf extract (with concentrations of 12.5, 25, 50 and 100%, positive and negative controls) were placed in the inoculation of the test bacteria and incubated for 24 hours. Inhibitory zones are examined by measuring the diameter of the clear zone around the disc paper using calipers.

Results and Discussion

Phytochemical analysis of extracts. From the results of phytochemical analysis it was recorded that *B. gymnorrhiza* leaf extract contained bioactive compounds such as flavonoids, tannins, saponins and steroids, whereas alkaloids and triterpenoids were not found (Table 1).

Table 1

Phytochemical analysis of *Bruguiera gymnorrhiza* leaf extract

No.	*Parameters*	*Result*	*Mark*
1.	Flavonoid	+	Positive
2.	Tanin	+	Positive
3.	Saponin	+	Positive
4.	Alkaloid	-	Negative
5.	Steroid	+	Positive
6.	Triterpenoid	-	Negative

The presence of these chemicals in *B. gymnorrhiza* leaf extracts is not surprising. Mangrove plants contain compounds that can be used as antimicrobials, anti-cancer and anti-leukemia (Nurjanah et al 2015; Basyuni et al 2019). Flavonoids are good reducing agents, which inhibit many oxidation reactions, both enzymes and non-enzymes. Flavonoids will be dissolved in polar solvents, such as ethanol, methanol, butanol, acetone, dimethylsulfoxide and water. The mechanism of action of flavonoid compounds damage the cell membrane without being irreparable (Panche et al 2016).

Tannins are complex phenolic compounds that can inhibit the bacterial activity so plants that contain tannins are often used in the pharmaceutical field because tannins contain tanic acids that have been used as antiseptics. Tannins have spasmolytic properties, which can shrink the cell membrane so that it interferes with the cell's permeability, preventing it to carry out biological processes which cause its growth to be stunted or even its death (Vincken et al 2007; Amini et al 2014).

Saponins are secondary metabolites with high molecular weight. These compounds are found in a large number of plants and in some marine animals such as sea cucumbers (Woo et al 2017), *Ophiuroidea* or brittle star (Amini et al 2014). In plants, saponins are spread evenly in parts such as roots, stems, tubers, leaf, seeds and fruit (Rahmania 2018; Wina et al 2005). The highest concentration of saponin in plant tissue was found in plants that are susceptible to be attacked by insects, fungi or bacteria, thus showing that this compound can act as a defense mechanism for plant bodies. Other authors (Florian & Wink 2011) stated that saponin compounds are substances that, when interacting with bacterial cell wall, can cause bacterial cell lysis or rupture.

Some authors have also reported that mangroves contain alkaloids and saponins, benzoquinone derivatives, naphthoquinone, naphthofurans, flavonoids, polyphenols, rotenones, flavoglicans, sesquiterpenes, di-triterpenes, limonoids, essential oils, sterols, carbohydrates, o-methyl-inocytes, o-methyl inocytes, iridoids glycosides, alkaloids and free amino acids, pheromones, gibberellin, forbol esters, sulfur compounds, fats and hydrocarbons, long chain aliphatic alcohols and saturated fats, and free fatty acids, including polyunsaturated fatty acids (Dia et al 2015; Syawal et al 2020; Poompozhil & Kumarasamy 2014; Basyuni et al 2019).

Saponins are a class of glycosides and sterols which, when fully hydrolyzed, will produce a sugar and a non-sugar fraction called sapogenin or genin. Saponin is a surface active compound, like a soap, and can be detected based on its ability to form foam and produce the hemolysis of the blood (Oleszek et al 2002; Tamura et al 2012). Saponins have received considerable attention because of their various biological activities including hepatoprotective, anti-ulcer, anti-tumor, antimicrobial, adjuvant and anti-inflammatory activities. They consist of aglycone (a lipid-soluble compound) which commonly contains either water soluble sugar residues and a sterol or a triterpenoid. Saponins are highly surface-active compounds, due to their amphiphilic nature, and their biological activities are rather due to their chemical structure (Moghimipour & Handali 2015).

Steroids are compounds that exert a wide range of biological activities. They are essential for plant growth, reproduction and responses to various abiotic and biotic stresses (Cécile et al 2012). Steroids inhibit the bacterial growth by a mechanism of protein synthesis inhibition and causes changes in the components of the bacterial cells and lysis (Poompozhil & Kumarasamy 2014). Steroids can increase the permeability of cell membranes so that the cell leakage will occur, followed by the release of intracellular material (Oleszek 2002).

Inhibitory test. Inhibitory test of *B. gymnorrhiza* mangrove leaf extract against fish bacteria *A. hydrophila*, *A. salmonicida* and *E. ictaluri* can be noted from the clear zone (free of pathogenic bacterial growth) formed around the diffusion paper disc, on the nutrient agar (Figure 1, 2 and 3).

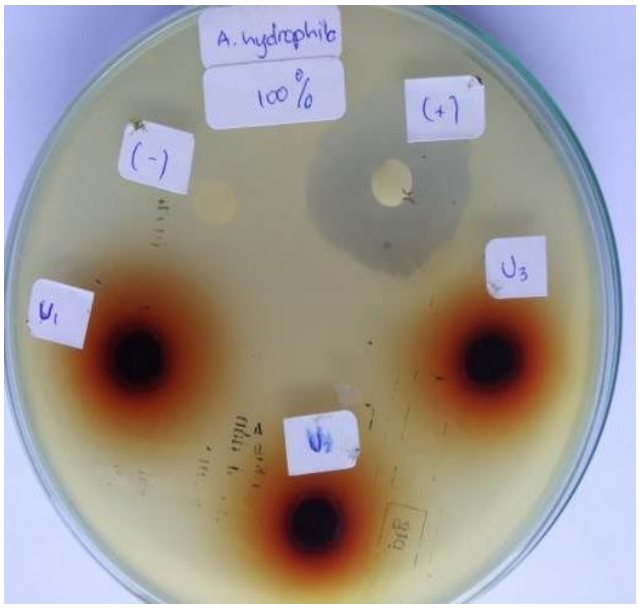

Figure 1. Clear zone around the paper disc containing *Bruguiera gymnorrhiza* mangrove leaf extract tested to *Aeromonas hydrophila*.

Inhibitory levels were examined by measuring the diameter of the clear zone. The score indicated that the inhibiting zones (of the substances contained in the mangrove extracts against the three pathogenic bacteria) were not clearly visible. The clearest zone was recorded at a concentration of 100% mangrove leaf extract, against *A. salmonicida*. The average inhibition of *B. gymnorrhiza* leaf extracts against *A. hydrophila* ranged from 0.48 to 3.58 mm. A similar result was also scored for *A. salmonicida* where the clear zone ranged from 0.45 to 3.93 mm. Likewise, with the pathogen of *E. ictaluri*, the inhibition ranged from 0.65 to 3.60 mm (Table 2). A clear zone area <5 mm is classified as weak inhibition.

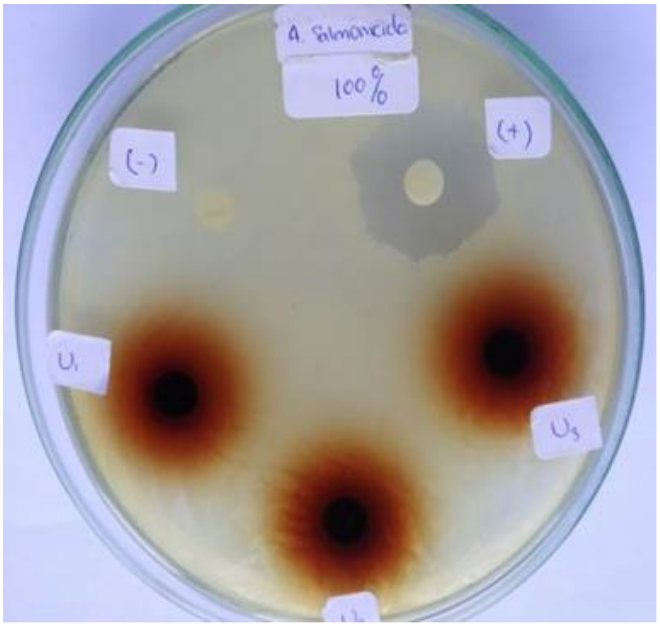

Figure 2. Clear zone around the paper disc containing *Bruguiera gymnorrhiza* mangrove leaf extract tested to *Aeromonas salmonicida*.

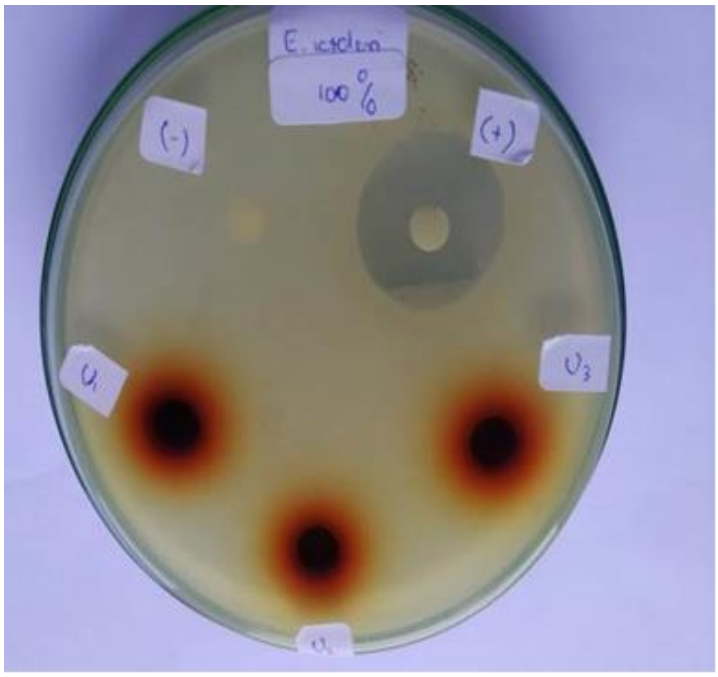

Figure 3. Clear zone around the paper disc containing *Bruguiera gymnorrhiza* mangrove leaf extract tested to *Edwardsiella ictaluri*.

Some researchers reported that increasing concentrations of medicinal plant extracts would increase the bacterial growth inhibition level (Nurjanah et al 2015). This is due to the presence of higher levels of bioactive compounds in extracts with high concentrations.

A. hydrophila, *A. salmonicida* and *E. ictaluri* are gram negative bacteria. Gram-negative bacteria contain more lipids, less peptidoglycan and the outer membrane is in the form of a bilayer. The outer membrane consists of phospholipids (inner layer), and lipopolysaccharides (outer layer) composed of lipid A, which is nonpolar. This makes it harder for the antibacterial compounds to enter the cell so that the antibacterial activity is weak.

The mechanism of action of the antibacterial compounds include inhibiting cell wall synthesis, inhibiting the integrity of microbial cell membranes, inhibiting microbial cell protein synthesis, interfering with microbial cell metabolism and inhibiting the synthesis of nucleic acids and proteins (Simões et al 2009; Wikaningtyas & Sukandar 2016).

Table 2

Inhibition of *Bruguiera gymnorrhiza* mangrove leaf extracts against pathogenic bacteria

Pathogenic bacteria	*Experimental treatments*	*Clear zone (mm) ± standard deviation*
Aeromonas hydrophila	12.5	0.48±0.07
	25	1.35±0.21
	50	2.16±0.20
	100	3.58±0.92
	Positive control	18.43
	Negative control	0
Aeromonas salmonicida	12.5	0.45±0.05
	25	1.30±0.34
	50	2.21±0.24
	100	3.93±0.85
	Positive control	17.57
	Negative control	0
Edwardsiella ictaluri	12.5	0.65±0.21
	25	1.10±0.26
	50	1.91±0.37
	100	3.60±0.36
	Positive control	18.36
	Negative control	0

Conclusions. The mangrove *B. gymnorrhiza* leaves contained flavonoids, tannins, saponins, alkaloids and steroids and did not contain triterpenoids. The leaf extract inhibited the tested fish pathogenic bacteria: *A. salmonicida*, *A. hydrophila* and *E. ictaluri*. However, the inhibition level was in the weak category (clear zone <5 mm). The higher the leaf extract concentration, the stronger the inhibition. It is recommended to carry out further research by testing other fish pathogenic microorganisms.

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Conflict of interest. The authors declare no conflict of interest.

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