Phytochemical analysis of *Rhizophora apiculata* leaf extract and its inhibitory action against *Staphylococcus aureus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*

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Abstract. *Rhizophora apiculata* is a species of mangrove widely grown along the coast of tropical area. *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa* are pathogenic bacteria that live in the coastal environment. This study aimed to determine the chemical compounds of the leaf extract of the *R. apiculata* species of mangrove and its inhibitory effect against the 3 above mentioned pathogenic bacteria. This experiment used a completely randomized design (CRD) by preparing a leaf extract at the concentration levels of 12.5% (T1), 25% (T2), 50% (T3), 100% (T4), positive control (T5) and negative control (T0), with 3 repetitions. Phytochemical test showed that *R. apiculata* leaf extract contained: saponins, tannins, flavonoids, steroids, and terpenoids. The extract inhibited the growth of the pathogenic bacteria, with inhibition zones ranging from 5.30 to 3.10 mm for the *S. aureus*, from 6.07 to 3.30 mm for the *A. hydrophila* and from 6.33 to 3.23 mm for the *P. aeruginosa*.
Key Words: mangrove leaf extracts, bioactive compounds, fish pathogenic bacteria, secondary metabolite activity, antibacterial.

Introduction. *Rhizophora apiculata* is a species of mangrove found in several tropical countries, including on the East Coast of Sumatra, Indonesia (Sofian et al 2019). Various species of mangrove contain secondary metabolites, such as alkaloids, flavonoids, steroids, terpenoids, saponins and others. The secondary metabolite compounds are bioactive substances which make plants appropriate for medicinal use (Cheng et al 2007; Kamalifar et al 2016).

Some studies mentioned that in vitro screening of organic solvent extracts of five mangroves, i.e. *Aegiceras corniculatum*, *Aegialitis rotundifolia*, *Aglaia cucullata*, *Cynometra iripa* and *Xylocarpus granatum* showed specific activity in inhibiting the growth of six virulent strains of bacteria pathogenic to fish, i.e. *Edwardsiella tarda*, *Vibrio alginolyticus*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *A. hydrophila* (Choudhury et al 2005). *R. apiculata* extract inhibited the growth of *Vibrio parahaemolyticus*. Endophytic fungi isolated from the leaves of *R. apiculata* can also be used as an inhibitor to *P. aeruginosa* and *Staphylococcus aureus* (Santoso et al 2015).

S. aureus is a pathogenic bacterium to some fish. Staphylococcal infections on fish have caused economic losses to aquaculture. Some researchers have reported and carried out studies on fish infectious diseases topics, for instance on North African catfish (*Clarias gariepinus)* by Oladele et al (2012), and immune response in gills of zebrafish (*Danio rerio*) by Zhang et al (2019). Wang et al (2009) conducted a research upon *S. aureus* infection on amphioxus *Branchiostoma belcheri*, while Su & Chen (2020) studied grouper antimicrobial that modulates *S. aureus* in macrophage cells. Larger scale of work was carried by Canak & Timur (2017), who studied Staphylococcal infections of some marine fish cultured in Turkey.

A. hydrophila is a common pathogenic bacteria of fish (Wamala et al 2018), harmful mainly to carp, tilapia and catfish in tropical countries, and causing considerable losses to aquaculture. Salosso et al (2020) studied the application of Kefa forest honey as

antibacterial in the treatment of the common carp *Cyprinus carpio* infected with bacteria *A. hydrophila*. Andriawan et al (2019) investigated *Holothuria scabra* effect on the immune activity of *Pangasianodon hypophthalmus* against *A. hydrophila*. Sukenda et al (2018) worked on the efficacy of the whole-cell and lipopolysaccharide vaccine against *A. hydrophila* on juvenile tilapia *Oreochromis niloticus*. Payung et al (2017) studied a dietary ginger (*Zingiber officinale*) effect on enhancing the resistance of Nile tilapia against this pathogen. Hardi et al (2016) reported antibacterial activities of some Borneo plant extracts against *A. hydrophila* pathogenic bacteria. Harikrishnan & Balasundaram (2007) discussed modern trends in *A. hydrophila* disease management in aquaculture. Nielsen et al (2001) studied *A. hydrophila* as the dominant motile *Aeromonas* species that caused disease outbreaks in aquaculture production in the Zhejiang Province of China.

P. aeruginosa is a causative agent to common disease of tilapia, for example in *Oreochromis mossambicus*. Baldissera et al (2017) carried out an experiment on blood–brain barrier breakdown and myeloperoxidase activity in silver catfish (*Schilbe intermedius*) experimentally infected with *P. aeruginosa*. Other researchers (Matheus et al 2019) worked on the effects of dietary grape pomace flour on the purinergic signaling and inflammatory response of grass carp experimentally infected with *P. aeruginosa*. This study aimed to determine the chemical compounds of mangrove *R. apiculata* leaf extracts and to examine their inhibitory effect on the growth of *S. aureus*, *A. hydrophila* and *P. aeruginosa*.

Material and Method

Sampling and experimental design. *R. apiculata* leaves were collected from the mangrove forest of Bandar Bakau, Dumai, Indonesia. Pure cultures of pathogenic bacteria (*S. aureus*, *A. hydrophila* and *P. aeruginosa*) were obtained from our laboratory in University of Riau. This experiment used a completely randomized design (CRD) using a leaf extract concentration level of 12.5% (T1), 25% (T2), 50% (T3), 100% (T4), positive control (T5) and negative control (T0) with 3 repetitions. Fresh leaves were cleaned using distilled water, air dried and then thinly cut and dried again. The drying process was carried out indoors by aeration.

Preparation of filtrate for the phytochemical test. The dried leaves were finely chopped and ground using a blender machine and then macerated with ethanol solution for 24 hours. The ratio of *R. apiculata* leaves and the solvent was 1:5. 1 kg of mangrove leaf flour was dissolved with 5 L of solvent. The solution was filtered using filter paper. The remainder of the first filtration was re-macerated in the same way, until the filtrate was clear. The filtrate was collected and evaporated using a rotary vacuum evaporator to separate the solvent under a temperature of 60^0C, until the solvent totally evaporated. Thus, a rough extract of *R. apiculata* mangrove leaves was obtained.

The filtrate was moved into a 500 mL beaker glass containing 100 mL ethanol solution. This preparation was heated and stirred using a stirring rod until it was boiled. The filtrate was reheated until it gained consistence in another 500 mL beaker glass. The beaker glass was then filled with ammonia chloroform until cracks appeared, then added with enough distilled water, stirred to form 2 layers. The upper layer was water extract and the lower layer was chloroform extract. The phytochemical test was ended with the addition of certain color reagents to the extract as follows (Gul et al 2017).

Saponin test. This test was carried out by placing 0.1 g of sample into a beaker, then adding 10 mL of hot water and boiling for 5 minutes, and finally filtering before using the filtrate as a test solution. The filtrate was removed into a closed test tube then shaken for ±10 seconds and left for 10 minutes, then added with 1 mL of 2M HCl. The presence of saponins was indicated by the formation of stable froth (Gul et al 2017).

Phenol and tannin test. The test was carried out by adding 3 drops of $FeCl_3$ solution to the filtrate. The presence of phenol compounds was indicated by dark green or blue,

while the presence of tannin compounds was characterized by the formation of bromine-colored compounds.

Flavonoid test. The presence of flavonoids was tested by adding 5 drops of concentrated Mg and HCl to the filtrate, then by shaking vigorously to form a layer. The formation of a reddish yellow to red layer indicated that the sample contains flavonoid compounds.

Steroid and terpenoid test. Two drip plate holes were filled with sample filtrate. Chloroform drops were then poured into the 2 drip plate holes and the mixture was fan-dried. The holes were 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 extract solution was prepared as experimental unit of 12.5% (T1), 25% (T2), 50% (T3), 100% (T4), positive control (T5), and negative control (T0). The positive control solution (chloramphenicol) had a concentration of 30 µg and the negative control used distilled water solution. A pure culture of pathogenic bacteria (*A. hydrophila*, *P. aeruginosa* and *S. aureus*) was grown on nutrient agar.

The inhibitory test on pathogenic bacteria was carried out by using the disc diffusion method. Bacterial isolates that have been rejuvenated were suspended with distilled water, then 0.1 mL of solution was spread using a glass rod on nutrient agar media. Paper disc that had been dropped with *R. apiculata* leaf extract (12.5%, 25%, 50% and 100%), positive and negative controls were placed in the inoculation of test bacteria and incubated for 24 hours. The inhibition zone is examined by measuring the diameter of the clear zone around the disc paper by using a caliper. The data obtained was inserted into tables and analyzed descriptively.

Results and Discussion

Phytochemical tests. Phytochemical tests were carried out to detect secondary metabolites of plants based on their class. It was noted that *R. apiculata* leaf extract contained saponins, tannins, flavonoids, steroids, and terpenoids (Table 1).

Table 1

Phytochemical test results of *Rhizophora apiculata* leaves extract

Active compound	*Color indicator*	*Result*
Saponin	Yellow with foam	+
Tanin	Dark green or blue	+
Flavonoid	Yellowish red or red	+
Steroid	Green	+
Terpenoid	Purple	+

Phytochemical test is an approach method used in determining the presence of secondary metabolite compounds in plants. Classes of secondary metabolites are determined qualitatively using several phytochemical test reagents. The secondary metabolites contained in the extract were detected by the change of color and by the deposition or formation of foam in accordance with the reagents used (Campagna et al 2012; Lima et al 2011).

The results of phytochemical tests showed that the extracts of *R. apiculata* leaves contain secondary metabolites: saponins, indicated by the presence of foam; tannins, indicated by the change color to dark green or blue; flavonoids, indicated by the change color to reddish yellow; steroids indicated by the green color of the test and terpenoids by the purple color of the test (Malik et al 2017; Asha et al 2012).

Inhibitory test. *R. apiculata* mangrove leaf extracts showed an inhibitory effect against pathogenic bacteria *S. aureus*, *A. hydrophila*, and *P. aeruginosa*. This can be noted from the inhibitory zone that formed around the paper discs placed on the surface of nutrient agar media (Figure 1). Growth inhibition zones ranged between 5.30-3.10 mm (*S. aureus*), 6.07-3.30 mm (*A. hydrophila*) and 6.33-3.23 mm (*P. aeruginosa*). More detailed data is presented in Table 2. It was also noted that the higher the concentration of the extract used, the higher the inhibitory effect generated on all types of test pathogens. More detailed data is presented in Table 2.

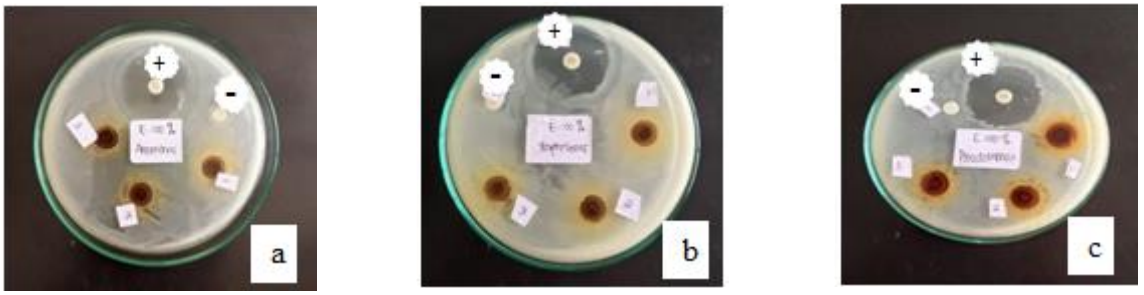

Figure 1. Inhibition zones of *Rhizophora apiculata* leaf extract: (a) *Aeromonas hydrophila,* (b) *Staphylococcus aureus*, and (c) *Pseudomonas aeruginosa*. (+ positive control; - negative control).

Table 2

Inhibition of *Rhizophora apiculata* mangrove leaf extracts against pathogenic bacteria

Pathogenic bacteria	*Experimental units*	*Clear zone (mm) ± standard deviation*
Staphylococcus aureus	T1	3.10±0.721
	T2	4.38±0.057
	T3	4.57±0.152
	T4	5.30±0.100
	T5	24.8±1.778
	T0	0±0.000
Aeromonas hydrophila	T1	3.30±0.100
	T2	4.40±0.200
	T3	5.03±0.776
	T4	6.07±0.692
	T5	23.32±1.350
	T0	0±0.000
Pseudomonas aeruginosa	T1	3.23±0.288
	T2	3.77±0.550
	T3	4.63±0.763
	T4	6.33±0.115
	T5	13.85±1.461
	T0	0±0.000

Saponin compounds are found in a large number of plants and some marine animals such as the sea cucumbers (Lacaille-Dubois & Wagner 2000) and the *Ophiuroidea* (brittle star) (Vincken et al 2007). In plants, saponins are spread evenly in parts such as roots, stems, tubers, leaves, seeds and fruit. The highest concentration of saponin in plant tissue is found in plants that are susceptible to attack by insects, fungi or bacteria, thus showing that this compound can act as a defense mechanism for plant bodies (Wina et al 2005).

Tannin compounds are classified as polyphenolic compounds (Syawal et al 2019), having the effect of growth inhibiting and killing the fungus *Candida albicans* and also of inhibiting the bacterial growth. Therefore, tannin has anti-fungal and anti-bacterial properties Syahidah & Subekti (2019). Tannin compounds found in the leaves extract of *Rhizophora* sp. can shrink the cell wall or cell membrane, thereby disrupting the cell's permeability itself. As a result, cells cannot carry out living activities, experiencing a

stunted growth or even death. The antibacterial effects of tannins, through reactions with cell membranes, enzyme inactivation and destruction or inactivation of the genetic material functions, were also reported by Thirunavukkarasu et al (2013).

Flavonoids are typically contained in the green plants and can be found in plant parts including leaves, roots, wood, bark, pollen, flowers, fruit and seeds. Flavonoids readily dissolve in water and have biological activity, being cytotoxic to cancer cells and inhibiting the release of histamine, anti-inflammatory, anti-fungal and anti-bacterial (Li et al 2006). Malik et al 2017 mentioned that the effects of flavonoids can also prevent the division of bacteria and form complex compounds against extracellular proteins, disrupting the cell membranes integrity.

Steroid compounds, as secondary metabolites, are known to have bio-insecticidal, antibacterial, antifungal, and anti-diabetes activities. Steroids an inhibitory mechanism damages the bacterial cell membranes by increasing cell permeability, resulting in cell leakage, followed by the release of the intracellular material (Liu et al 2018).

Terpenoid, as antibacterial compounds, are effective in inhibiting the growth of bacteria, fungi, viruses and protozoa. The most common mechanism of action of the terpenoids consists of inhibiting bacterial growth by irritating the cell wall, by coagulating bacterial protein and by causing hydrolysis and diffusion of the cell fluid due to differences in osmotic pressure (Basyuni et al 2009).

As reported in several studies, mangrove species, such as *Avicennia marina*, have antibacterial properties against *S. aureus*, containing alkaloid compounds, terpenoids and flavonoids (Amirkaveei & Behbahani 2011). Jairaman et al (2019) reported another generic antibacterial plant (*A. marina*) leaf extract from the backwaters of Muthukadu Lake, Tamil Nadu, due to their phenolic compounds, such as alkaloids and flavonoids. In a previous study (Pimpliskar et al 2011), there was reported that the leaves of *R. apiculata*, extracted using hot water, ethanol, ethyl acetate and n-hexane, and were able to inhibit the growth of *A. hydrophila*. The antimicrobial substances, such as alkaloids and flavonoids against *A. hydrophila*, can be expected to inhibit bacterial enzymes, resulting in a disruption of the metabolism or even into death of the bacterial cells and inhibiting the formation of the virulence factors in bacteria, consisting of extra cellular toxins (Dhayanithi et al 2012).

The inhibition found in this study is not surprising, considering that several researchers have reported similar results. Saptiani et al (2018) reported that *A. marina* and *Acanthus ilicifolius* extracts inhibited *S. aureus* with an inhibition zone of 13.33±0.58 mm. All the extracts can inhibit *A. hydrophila* (13.00±1.00 mm) and *E. coli* (12.67±0.58 mm). Ethanol extract of *Sonneratia alba* can inhibit *Vibrio harveyi* (12.67±0.58 mm) and *A. ilicifolius* (12.33±0.58 mm) against *Saprolegnia* sp. Another study mentioned that mangrove leaf extract had antibacterial properties against *E. coli* and antifungal against *Penicillium digitatum* (Amirkaveei & Behbahani 2011).

P. aeruginosa and *A. hydrophila* are Gram negative bacteria, while *S. aureus* is Gram positive bacteria. In each bacterium the highest inhibitory zone was found at a concentration of 100% where the highest inhibitory zone was found in the *P. aeruginosa*, followed by the *S. aureus* and *A. hydrophila*. Gram-negative bacteria have a thinner layer of peptidoglycan than Gram-positive bacteria. This makes Gram negative bacteria more sensitive to anti-bacterial substances. Based on the results of the study, it was observed that the higher the concentration, the greater the inhibition zone produced, as confirmed by previous studies reporting that the inhibitory effect increases with the inhibitor concentration (Brown et al 2014).

Conclusions. The current study revealed that *R. apiculata* mangrove leaf extract contains secondary metabolites of saponins, tannins, flavonoids, steroids and terpenoids, having the potential to inhibit the growth of pathogenic bacteria *S. aureus*, *A. hydrophila,* and *P. aeruginosa*, presenting inhibition zone diameters in the medium category.

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