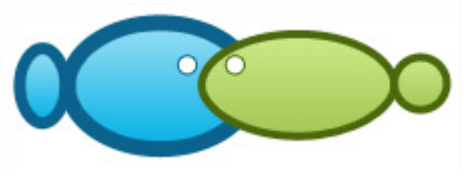

Antibacterial activities of some Borneo plant extracts against pathogenic bacteria of *Aeromonas hydrophila* and *Pseudomonas* sp.

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Abstract. The objective of the present study was to explore the antibacterial activity from some plant extracts against *Aeromonas hydrophilla* and *Pseudomonas* sp. pathogenic bacteria for tilapia (*Oreochromis niloticus*). The plant extracts were obtained by using ethanol and the concentration of the extract was 500-600 mg L^{-1} and the the inhibition zone was measured after 24 h at 30°C. The results showed that 16 extracts have antibacterial activity with more than 10 mm against *A. hydrophila* and eight extracts against *Pseudomonas* sp. The highest activity was shown by the black cumin (*Nigella sativa*) extract against both bacteria. The effective dose of the extract as an antibacterial substance, against both bacteria, were 800 and 900 mg L^{-1} *Boesenbergia pandurata* to *A. hydrophila*, 600 and 900 mg L^{-1} *Solanum ferox* to *Pseudomonas* sp., and concentration of *Zingiber zerumbet* 200 and 2,000 mg L^{-1} effective to inhibit *A. hydrophila* bacteria.
Key Words: antibacterial, inhibition zone, *Oreochromis niloticus*, septicemia, traditional plant.

Introduction. *Aeromonas hydrophila* and *Pseudomonas* sp. infection plays an important role on fish mortality, especially in a culture system. Generally, these bacteria infect simultaneously, but clinical symptoms in infected fish are different. The *Aeromonas* infection causes the fish to bleed on the outside of infected organs such as the surface of the body, skin and operculum, while the clinical signs of *Pseudomonas* sp. are mostly observed by pale and watery internal organs, and the gall bladder ruptures (Hardi & Pebrianto 2012). Both bacterial infections are mostly found in freshwater fish farms.

There have been some ways to inhibit the fish disease caused by bacteria pathogens. Vaccines and antibiotics are the most common method used by fish farmers. However, vaccines are specific for bacteria and must be combined with boosters and repeated administration, while application of antibiotics is very susceptible to pathogen resistance and environmental deterioration (Kesarcodi-Watson et al 2008; Nugroho & Fotedar 2013). According to Findlay & Munday (2000) and Cuesta et al (2004), organic and inorganic materials can be used as an immunostimulant to control fish diseases. In the recent decade, there has been increasing interest in the modulation of the non-specific immune system of fish, as both treatment and prophylactic measures against diseases, using plant extracts (Misra et al 2006; Harikrishnan et al 2011; Menanteau-Ledouble et al 2015).

Prevention of bacterial infections, using plant extracts, is highly desirable due to low cost, environmental friendliness, and effectiveness against certain bacteria, compared to antibiotics which might be harmful to the environment (Cheng et al 2014). Some plants, for example *Cinnamomum verum* (Rattanachaikunsopon & Phumkhachorn 2010), *Olea europaea* (Micol et al 2005), *Solanum trilobatum* (Divyagnaneswari et al

2007), and *Eclipta alba* (Christybapita et al 2007), have shown some effectiveness to control fish diseases caused by various pathogens. Haniffa & Kavitha (2012) reported that 26 species of seaweed have antibacterial activities for five species of fish pathogens i.e. *Aeromonas salmonicida, Aeromonas hydrophila, Pseudomonas anguilliseptica, Vibrio anguillarum,* and *Yersinia ruckeri*. While dichloromethane extract from *Asparagopsis armata, Ceramium rubrum, Drachiella minuta, Falkenbergia rufolanosa, Gracilaria cornea* and *Halopitys incurvus* showed antibacterial activity against V. *anguillarum* and *P. anguilliseptica*. Moreover, Babuselvam et al (2012) reported that pathogenic bacteria in shrimp and fish can be inhibited by extracts of *Rhizophora mucronata* and *Salichornia brachiata*.

Previous studies found that various secondary metabolites, such as alkaloids, flavonoids, glycosides, phenols, saponins, and steroids, can be extracted by phytochemical screening, and these compounds have antimicrobial properties against both gram-positive and gram-negative organisms (Baser 1993; Jouad et al 2001; Anyanwu & Dawet 2005; Koche et al 2010).

The objectives of the present study were to examine the antibacterial activities of several plant extracts against *A. hydrophila* and *Pseudomonas* sp. bacteria that infect tilapia (*Oreochromis niloticus*). The plants are commonly used by Indonesian people as traditional medicine. However, the application of those plant extracts on fish pathogens, especially *A. hydrophila* and *Pseudomonas* sp. bacteria have not been reported.

Material and Method. The study was conducted in January to December 2014 at the Laboratory of Environmental Microbiology, Faculty of Fisheries and Marine Science, and Forest Products Chemistry, Faculty of Forestry, Mulawarman University, East Kalimantan, Indonesia.

Collection and identification of plant samples. Plant samples were collected from traditional markets in Samarinda, Indonesia. Plant species were selected based on the information of ethnobotany by local communities, especially the species that are frequently used as spice and aromatic in cooking, food preservatives and fragrances. Based on these criteria, 32 species of plants have been selected for further examination.

Extraction of plant. The extraction was done in Laboratory of Forest Products Chemistry, Faculty of Forestry, Mulawarman University. The air-dried plant samples were mashed using a blender, then dried at room temperature for 48 hours. A total of 100 g of dried samples were soaked in 100 mL of ethanol in an Erlenmeyer at room temperature. The extract solution was filtered with Whatman no. 42 filtration paper and filtered sample was centrifuged for 24 h at 50 rpm to obtain a crude extract.

Bacterial isolate and culture condition. The *A. hydrophila* (EA-01) and *Pseudomonas* sp. (EP-01) were used as test bacterial strains. The bacteria were isolated from unhealthy tilapia from a pond in Loa Kulu Kutai Kartanegara, East Kalimantan, Indonesia. The culture of bacteria were grown in BHI (Brain Heart Infusion Broth, DIFCO®) and BHIA (Brain Heart Infusion Agar, DIFCO®) media for 24 h at 30^{o}C and the density of bacteria was 10^{10} CFU mL^{-1}.

Antibacterial activity test. The in vitro inhabitation antibacterial test was performed following method Dulger & Gonuz (2004). The extract concentrations ranged from 500 to 600 mg L^{-1}, and the procedure as follows: a total 25 µm of extracts were dropped on Weidman sterile paper, then placed on a media that already contained cultured bacteria on TSA medium (Tryptic Soy Agar), then incubated for 24 h at 30^{o}C. The inhibition zone was observed 24 h after inoculation. The sterile distilled water was used as a negative control and commercial antibiotic (tetracycline) used as a positive control.

Determination test of plant extracts effective dosage. Based on anti-bacterial tests, three plants, i.e. *Boesenbergia pandurata*, *Solanum ferox,* and *Zingiber zerumbet,* were examined for in vitro test to determine the optimum dosage of these plant extracts against *A. hydrophila* and *Pseudomonas* sp. The effective dosage was determined based on the form of inhibition zone of more than 12 mm. A commercial antibiotic

(Tetracycline) and PBS (Phosphate Buffer Saline) were used as control. The diffusion assay was done at the concentration of the extract, which ranged between 100-6,000 mg L^{-1} following Dulger & Gonuz (2004). Twenty five µm of each extract of *B. pandurata* and *Z. zerumbet* were impregnated on 6 mm diameter sterile discs and placed on BHIA medium plates previously swabbed with *A. hydrophila* and 25 µm of *S. ferox* extract to *Pseudomonas* sp. culture. The plates were incubated at 30°C for 18, 24, 36, 48 and 60 h after incubation, respectively.

Phytochemical analysis. The alkaloids, flavonoids, saponins, tannins, triterpenoids, steroids and carbohydrate components from *B. pandurata*, *S. ferox* and *Z. zerumbet* extracts were examined using the methods proposed by Poongothai et al (2011), and Tiwari et al (2011).

Result and Discussion. The results showed that 31 traditional plant extracts had an antibacterial activity against *A. hydrophila* and *Pseudomonas* sp. bacteria (Figure 1). Among the 31 extracts, 15 of the herbal plants extracted inhibited the growth of *A. hydrophila* with inhibition zones more than 10 mm; they are *Amomum compactum, Artocarpus camansi, Boesenbergia pandurata, Citrus hystrix, Curcuma aeruginosa, Curcuma domestica, Illicium verum, Nigella sativa, Ocimum basilicum, Piper nigrum* (white pepper), *Solanum ferox, Syzygium aromaticum, Tamarindus indica, Trigonella foenum-graecum, Zingiber zerumbet*. Seven extracts that could suppress the *Pseudomonas* sp. growth were *Citrus hystrix, Curcuma longa, Illicium verum, Myristica fragrans, Nigella sativa, Solanum ferox, Tamarindus indica* (Table 1).

Table 1

Antibacterial activities of 32 plants extract against *Aeromonas hydrophila* and *Pseudomonas* sp.

Plant species	*Antibacterial activity*	
	Pseudomonas sp.	*Aeromonas hydrophila*
Tetracycline (commercial antibiotic)	+	+
Aquadest (negative control)	-	-
Alpinia galanga	-	-
Amomum compactum	-	+
Artocarpus camansi	-	+
Boesenbergia pandurata	-	+
Cinnamomum verum	-	-
Citrus hystrix	+	+
Coriandrum sativum	-	-
Cuminum cyminum	-	-
Curcuma aeruginosa	-	+
Curcuma domestica	-	+
Curcuma heyneana	-	-
Curcuma longa	+	-
Cymbopogon citratus	-	-
Cymbopogon nardus	-	-
Etlingera elatior	-	-
Foeniculum vulgare	-	-
Illicium verum	+	+
Kaempferia galanga	-	-
Myristica fragrans	+	-
Nigella sativa	+	+
Ocimum basilicum	-	+
Ocimum sanctum	-	-
Pandanus amaryllifolius	-	-
Piper nigrum (white pepper)	-	+
Piper nigrum (black pepper)	-	-
Solanum ferox	+	+
Syzygium aromaticum	-	+
Tamarindus indica	+	+
Trigonella foenum-graecum	-	+
Zingiber officinale	-	-
Zingiber zerumbet	-	+

(+) = inhibition zone ≥ 10 mm; (-) = inhibition zone < 10 mm.

The highest antibacterial activity against *A. hydrophila* and *Pseudomonas* sp. was *N. sativa* extract with inhibition zone around 20-23 mm against the bacteria, followed by *B. pandurata* (23 mm) and *Z. zerumbet* (16 mm) against *A. hydrophila* and *S. ferox* (15 mm) against *Pseudomonas* sp.

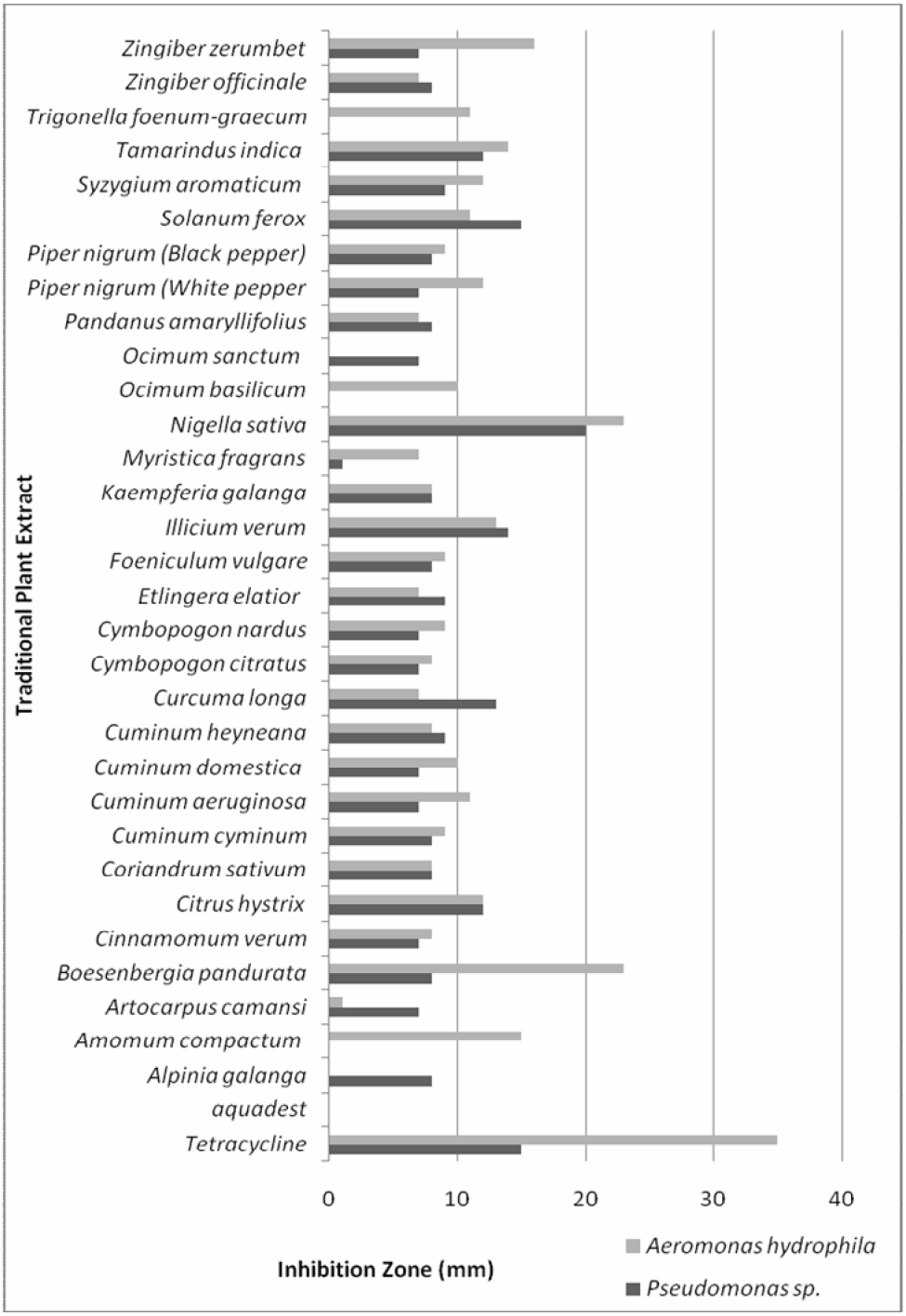

Figure 1. Sensitivity of several herbs against *Aeromonas hydrophila* and *Pseudomonas* sp.

The results revealed that concentrations of all extracts from the tested plants have different sensitivity inhibitions; for example, concentrations of 800 and 900 mg L^{-1} of *B. pandurata* resulted in the widest inhibitory zone that ranged between 12 to 23 mm (Table 2), and they were classified as an intermediate group to inhibit the *A. hydrophila* growth. These concentration levels were also likely to be used as antibacterial for fish. Meanwhile, 20 concentrations of *Z. zerumbet* have the inhibition sensitivity to *A. hydrophila*, where concentrations of 200 and 2,000 mg L^{-1} of *Z. zerumbet* were found as the best

concentration that can be used for antibacterial material to suppress the *A. hydrophila* infection on fish (Table 3). Similarly, *S. ferox* was sensitive to *Pseudomonas* sp. and the concentrations of 600 and 900 mg L^{-1} of *S. ferox* were indicated as the best concentration to inhibit the growth of *Pseudomonas* sp. (Table 4).

Table 2

Antibacterial activity of *Boesenbergia pandurata* against *Aeromonas hydrophila*

Treatment (ppm)	*Mean diameter of inhibitory zones (mm)*				
	18 h	*24 h*	*36 h*	*48 h*	*60 h*
Tetracycline	11.3	12	12.3	12.3	12.3
Aquades	0	0	0	0	0
100	8	8	8	8	8.3
200	8.6	8.6	8.6	8.6	8.6
300	8.6	9	9	9	9.3
400	9	9	9	9	9
500	7.6	8	8	8	8.6
600	10	10	10	10	10.5
700	9	9.3	9.3	9.3	9.3
800	12	12	12	12	12.3
900	11.6	11.6	13	13	13
1000	8.3	10	10.3	10.3	10.3
1500	6	6.3	7	7	7
2000	7.3	7.6	9	9	9
2500	8	8.6	8.6	8.6	8.6
3000	7.3	9.3	9.6	9.6	9.6
3500	7	8	8.3	8.3	8.3
4000	7	7.6	7.6	7.6	7.6
4500	9.6	9.6	10	10	10
5000	7.6	9	10	10	10
5500	8.5	9	10	10	10
6000	9.5	10	10	10	10

Table 3

Antibacterial activity of *Zingiber zerumbet* against *Aeromonas hydrophila*

Treatment (ppm)	*Mean diameter of inhibitory zones (mm)*				
	18 h	*24 h*	*36 h*	*48 h*	*60 h*
Tetracycline	11.3	12	12.3	12.3	12.3
Aquadest	0	0	0	0	0
100	8	9	9	8	9
200	9	10	10	10	10
300	6	6	6	6	6
400	9	9	9	9	9
500	6	7	7	7	7
600	8	8	7	6	7
700	7	7	7	7	7
800	7	9	9	9	9
900	9	9	9	10	9
1000	9	9	9	8	9
1500	9	9	9	9	9
2000	10	10	10	10	10
2500	8	9	9	9	9
3000	10	10	10	10	10
3500	8	8	8	7	8
4000	8	9	9	9	9
4500	8	8	8	8	8
5000	9	9	9	9	9
5500	9	9	9	8	9
6000	9	9	9	9	9

Table 4

Antibacterial activity of *Solanum ferox* against *Pseudomonas* sp.

Treatment (ppm)	*Mean diameter of inhibitory zones (mm)*				
	18 h	*24 h*	*36 h*	*48 h*	*60 h*
Tetracycline	11.3	12	12.3	12.3	12.3
Aquades	0	0	0	0	0
100	8	8	8	8	8.3
200	8.6	8.6	8.6	8.6	8.6
300	8.6	9	9	9	9.3
400	9	9	9	9	9
500	7.6	8	8	8	8.6
600	10	10	10	10	10.5
700	9	9.3	9.3	9.3	9.3
800	12	12	12	12	12.3
900	11.6	11.6	13	13	13
1000	8.3	10	10.3	10.3	10.3
1500	6	6.3	7	7	7
2000	7.3	7.6	9	9	9
2500	8	8.6	8.6	8.6	8.6
3000	7.3	9.3	9.6	9.6	9.6
3500	7	8	8.3	8.3	8.3
4000	7	7.6	7.6	7.6	7.6
4500	9.6	9.6	10	10	10
5000	7.6	9	10	10	10
5500	8.5	9	10	10	10
6000	9.5	10	10	10	10

A variety of plant species are capable of synthesizing many substances with antibacterial activity. Several plant extracts have various activities like antistress, growth promotion, appetite stimulation, enhancement of tonicity and immune-stimulant, maturation of culture species, aphrodisiac and anti-pathogen properties in fish cultures due to active principles, such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids or essential oils (Citarasu 2010; Chakraborty & Hancz 2011; Reverter et al 2014). According to current results, *B. pandurata*, *Z. zerumbet*, *N. sativa*, and *S. ferox* plants extract showed the diverse activity caused by the ingredients contained in the extracts. Phytochemical analysis results showed that *B. pandurata* contains alkaloids, flavonoids and carbohydrates and *Z. zerumbet* contains alkaloids, flavonoids, steroids and carbohydrates, which are able to suppress the bacteria growth. In addition, the bacteria growth can be also inhibited by sterols, hydroxyl chavicol, eugenol and phenolic (Wink 2010). Other chemicals, such as fatty acids (stearic acid and palmitic acid) and hydroxyl fatty acids esters (hydroxyl esters, myristic and palmitic acids), also have the ability to inhibit the growth of bacteria (Bhattacharya et al 2007). The material of fatty acids could damage the wall surface of the bacteria that grow, particularly at low temperatures. Fatty acids are believed to damage the structure and function of the bacterial cell wall and membrane (Hayes & Berkovitz 1979).

According to current results, *N. sativa* extract was recorded as the highest antibacterial activity against *A. hydrophila* and *Pseudomonas* sp.; it is having inhibition zones between 20 and 23 mm against both bacteria. Similarly, the previous finding by Bakathir & Abbas (2011) showed that concentrations of 300 mg mL^{-1} extract of *N. sativa* could inhibit *Staphylococcus aureus* bacterium, resulting in 10-20 mm inhibition zones. Some studies have been reported *N. sativa* seed has immuno-modulatory, immunosuppressive and anticancer properties (Islam et al 2004; Hassieb 2006; Aljabre et al 2015) and antimicrobial activity (Bakathir & Abbas 2011). The mechanism of the antimicrobial effect of these seeds could be attributed to the active ingredients, especially thymoquinone and melanin (Hassieb 2006; Roy et al 2006). The thymoquinone caused cells oxidative activity that inhibited gram positive cocci (*S. aureus* and *Staphylococcus epidermidis*) (Halawani 2009; Chaieb et al 2011) Though, the *N. sativa* has many benefits as antibacterial, anti-cancer, anti-inflammation, antioxidant, and imunomodulator (Ishtiaq et al 2013; Haseena et al 2015), the price of this seed is very

expensive compared to the other herbs. Thus, this plant might not be suitable to be implemented in small scale aquaculture.

Besides, the present study found that the extracts of *B. pandurate* and *Z. zerumbet* have a wide inhibition zone against *A. hydrophila,* while *S. ferox* was sensitive to *Pseudomonas* sp., and therefore, this is the best plant extract to inhibit the growth of *Pseudomonas* sp. This finding is in agreement to Ali et al (1990) and Hardi et al (2016), who reported that *S. ferox* gave a good immune response to *Pseudomonas* sp. This is because *S. ferox* has higher levels of alkaloids that play an important role as antibacterial properties (Huang et al 2008). In addition, alkaloids from solanum have antimicrobial activity to inhibit growth of *Escherichia coli* and *Staphylococcus aureus* (Kumar et al 2009; Amanpour et al 2015). Although the mechanism for alkaloids to inhibit growth of bacteria is unclear, probably the solanin might induce reactive oxygen species (Meng et al 2016) that could inhibit the growth of bacteria.

Conclusions. *S. ferox* and *N. sativa* plant extracts showed to be the best extracts to inhibit *Pseudomonas* sp., while *B. pandurata*, *Z. zerumbet* and *N. sativa* were shown as good antibacterial against *A. hydrophila*. The highest antibacterial activity against both bacteria was shown on *N. sativa*. The in vitro test showed that the effective doses were 800 and 900 mg L^{-1} *B. pandurata* to *A. hydrophila*, 600 and 900 mg L^{-1} of *S. ferox extract* to *Pseudomonas* sp., and 200 mg L^{-1} and 2,000 mg L^{-1} of *Z. zerumbet, which* were effective to inhibit *A. hydrophila* bacteria.

Acknowledgements. This research was supported by Directorate General of Higher Education of the Republic of Indonesia (DIKTI) with National Strategic Research (Grant no.248/UN 17.16/PG/2015). We thank the Faculty of Fisheries and Marine Sciences, Mulawarman University and Office of Marine and Fisheries Kutai Kartanegara, East Kalimantan for their assistance during in the field experiment.

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Received: 16 April 2016. Accepted: 15 June 2016. Published online: 18 June 2016.
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How to cite this article:
Hardi E. H., Kusuma I. W., Suwinarti W., Agustina, Abbas I., Nugroho R. A., 2016 Antibacterial activities of some Borneo plant extracts against pathogenic bacteria of *Aeromonas hydrophila* and *Pseudomonas* sp. AACL Bioflux 9(3):638-646.