Inhibition of fish bacteria pathogen in tilapia using a concoction three of Borneo plant extracts

EH Hardi[1], G Saptiani[1], IW Kusuma[2], W Suwinarti[2], A Sudaryono[3]

[1] Department of Aquaculture, Faculty of Fisheries and Marine Science, Mulawarman University. Jl. GunungTabur, GunungKelua, Samarinda Ulu, Samarinda-75123, East Kalimantan, Indonesia. Tel./Fax.: +62-541-749159.
[2] Faculty of Forestry, Mulawarman University, Samarinda-75123, East Kalimantan, Indonesia.
[3] Department of Aquaculture, Faculty of Fisheries and Marine Science, Diponegoro University

*Corresponding author: estieriyadi2011@gmail.com

Abstract. This study was conducted to evaluate the antibacterial activity of concoction *Solanum ferox*, *Boesenbergia pandurata* and *Zingimber zerumbet* extract (SF, BP, and ZZ) to inhibit pathogenic bacteria in tilapia with the each concentrations 600 ppm BP, 900 ppm SF and 200 ppm ZZ. Antibacterial activity was measured by testing the concoction of three plants extract against single isolate *Aeromonas hydrophila* and *Pseudomonas* sp. and combined both bacteria (10^5 colony-forming units per milliliter). In this research, oxytetracycline was used as a control. Clear zone inhibition was observed at 6, 12, 18 and 24 hours after incubation at 30 ^{o}C. The results showed that the different concoction of BP: SF: ZZ have inhibitory zones against both single and joint isolate bacteria. The ratio of3:3:4 and 1:8:1 had higher antibacterial activity towards *Pseudomonas s*p. and 1:1:3 ratios both inhibit joint bacteria. The ZI% higher of concoction extracts against *A.hydrophila* is 1:1:8; 1:3:1; 3:4:3. The ZI% concoction extracts against *Pseudomonas* sp. ware 3:3:4 and 1:8:1 ratio. While the two bacteria combined, just 1:1:3 ratio had higher Z%. The conclusion is that a concoction of SF:BP:ZZ is effective to inhibit the growth of *A. hydrophila* and *Pseudomonas* sp., even its antibacterial ability is similar to the effectiveness of antibiotic oxytetracycline.

1. Introduction

Aeromonas hydrophila and *Pseudomonas* sp. are two common gram-negative bacteria found to infect freshwater fish [1,2,3,4,5]. Fish infected with *A. hydrophila* bacteria will appear to have ulcers in the external organs while the infected fish *Pseudomonas* sp. will experience a watery internal organ and a ruptured gall bladder [6, 7]. It reinforced by the statement of [8], *A. hydrophila* is a primary pathogen, it produces an ulcerative disease under stress conditions or an association with other pathogens, such as *Aphanomyces* spp. [9].

Research on the use of plant extracts to control the disease has increased showed by many articles that explain the benefits of plant extracts as a natural antibacterial on fish farming. The sole extract from *B. pandurata*, *S. ferox*, and *Z. zerumbet* which contain levamisole, flavonoid, steroid, carbohydrate, can inhibit the growth of pathogenic bacteria such as *Aeromonas hydrophila* and *Pseudomonas* sp. that infecting tilapia both in vitro and in vivo [10, 11, 12, 13], plant extract from

Content from this work may be used under the terms of the Creative Commons Attribution 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.
Published under licence by IOP Publishing Ltd

Ocimum sanctum [14, 15], *Azadirachtin* [16], *Viscum albums*, *Urticadioica* and *Zingiberofficinale* [17], *Radix astragalin* seu Hedysari and *Radix angelicaesinensis* [18, 19], *Astragalus radix* and *Scutellariae radix* [20]. Seven Chinese plant extracts of *Aloe vera* Mill. (Aloaceae), *Angelica s*pecies (Umbelliferae), *Astragalus membranaceous* (Leguminosae), *Ganoderma lucidum* (Fr.) Karst. (Ganodermataceae), *Panax ginseng* C.A Mey. (Araliaceae), *Scutellaria species* (Lamiaceae) and *Zingiber officinale*Rosc. (Zingiberaceae) has an antimicrobial ability [21].

The combined use of multiple extracts for disease control has a higher antibacterial ability compared to the use of a single extract. *Curcuma longa*, *Ocimum sanctum*, and *Azadirachta indica* extract combined with a 1:1:1 had higher antibacterial activity ratio in Goldfish *Carassius auratus* were challenged with *A. hydrophila* infection [24]. Further trials on the treatment using a combination of the three extracts can improve survival and assist the healing process of wounds caused by bacterial infection of *A. hydrophila* in carp (*Carassius auratus*) [22]. In this paper, we will discuss the antibacterial activity of the combined *S. ferox, B. pandurata* and *Z. zerumbet* extracts against single bacteria and a combination of *A. hydrophila* and *Pseudomonas* sp.

2. Method

This research was conducted from January to March 2017 at Aquatic Microbiology Laboratory, Faculty of Fisheries and Marine Sciences and Wood Chemical Laboratory, Faculty of Forestry Mulawarman University, East Kalimantan.

2.1. Bacteria test

The pathogen bacteria used in this study were *A. hydrophila* (EA-01) and *Pseudomonas* sp. (EP-01) from the Laboratory of Aquatic Microbiology Faculty of Fisheries and Marine Sciences Mulawarman University. The bacteria was grown in BHI (Brain Heart Infusion Broth, DIFCO®) and BHIA (Brain Heart Infusion Agar, DIFCO®) solid medium for 24 hours at 30 °C, the density used was each of the bacteria was 10^5 CFU mL-1. The test bacteria used in the antibacterial test was single bacteria *A. hydropila*, *Pseudomonas* sp. and the combined both bacteria with a ratio 1:1.

2.2. Preparation of B. pandurata, Z. zerumbet and S. ferox Extract

Spices obtained from traditional markets in Samarinda, East Kalimantan Indonesia and the extraction process using ethanol solution by following the basic procedures [23, 10]. The extraction process was carried out at the Wood Chemical Laboratory at the Faculty of Forestry, Faculty of Fisheries, and Marine Sciences at the University of Mulawarman. 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 sterileWhatman no. 42 filtration paper and filtered sample was centrifuged for 24 h at 50 rpm to obtain a crude extract.

The extract concoction was prepared by mixing 0.6 g *B. pandurata*, 0.9 g *S. ferox* and 0.2 g *Z. zerumbet* ware weighed, soaked in 1 L of sterile distilled water [23, 25]. In this study will use a combination of *S. ferox* extract, *B. pandurata* and *Z. zerumbet* (SF: BP: ZZ) with a ratio of 1: 1: 8; 8: 1: 1; 1: 8: 1; 1: 1: 3; 3: 1: 1; 1: 3: 1; 3: 3: 4; 4: 3: 3; 3: 4: 3; 2: 2: 1; 1: 2: 2; 2: 1: 2.

Table 1. Combined extract of *S. ferox*, *B. pandurata* and *Z. zerumbet* used in the study

Extract concoction	*S. ferox* (mL)	*B. pandurata* (mL)	*Z. zerumbet* (mL)
1:1:8	10	10	80
1:1:3	20	20	60
3:3:4	30	30	40
2:2:1	40	40	20
8:1:1	80	10	10

Extract concoction	*S. ferox* (mL)	*B. pandurata* (mL)	*Z. zerumbet* (mL)
3:1:1	60	20	20
4:3:3	40	30	30
1:2:2	20	40	40
1:8:1	10	80	10
1:3:1	20	60	20
3:4:3	30	40	30
2:1:2	40	20	40

2.3. Antimicrobial activity of concoction plant extracts

The In vitro antibacterial test was performed by following diameter clear zone method [26]. It began with growing each test bacteria at BHIA at room temperature of 0.5 mL for 30 min. It was followed by dripping 25 µl of each extract on a 6 mm sterile antibacterial paper, and placed on the inoculated BHIA media with each of the test bacteria and incubated for 24 hours at 30 °C [27]. Observation of the inhibitory zone diameter was performed at 6, 12, 18 and 24 incubation periods. Sterile aquadest (distilled water) is used as a negative control and commercial antibiotic Oxytetracycline (OT) is used as a positive control.

The measured result was a clear zone or zona inhibition (ZI); the ZI% of the extract used was compared with the antibiotic OT as the standard. The percentage of ZI was calculated using the [28].

$$ZI\% = 100\%X(\frac{extract\ ZI - ZI\ water}{antibiotic\ ZI - ZI\ water}) \quad (1)$$

2.4. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the extracts against single and combined bacteria was carried out using TSA media following the Mandal et al (2002)method. The test was performed by adding each of the combined extracts of 0.5 mL or 0.5 mL of oxytetracycline antibiotics into TSB medium containing bacterial cultures each (10^3 CFU / mL). The cultures were then incubated for 24 h at 30 °C [30, 31, 25].

Furthermore, 1 mL of the suspense was diluted with 9 ml sterile aquadest and the dilution was inoculated into TSA medium and culture for 24 hours at 30 °C, then the total number of growing bacteria was calculated based on [30, 31, 29, 10]. The percentage of MIC is calculated using the following formula.

$$MIC\% = 100\%X(\frac{extract\ CFU - aquadest\ CFU}{antibiotic\ CFU - aquadest\ CFU}) \quad (2)$$

3. Result and Discussion

3.1. Inhibition zone

Figure 1 shows the combined effectiveness of the three extracts of *A. hydrophila* bacteria. The inhibition zone formed between 8 - 20.33 mm, in general the 6th hour after the incubation period of the inhibition zone begins to form and continues to increase until 24 hours. There are 7 compounds extracts that have a sensitive inhibitory zone (> 14 mm) against *A. hydrophila* bacteria, which is a combination of extract with a ratio of 1: 1: 8; 2: 2: 1; 3: 1: 1; 4: 3: 3; 1: 2: 2; 1: 8: 1; and 1: 3: 1. Combined extract with a ratio of 1: 2: 2 is the best ratio with 20.33 mm inhibition zone at 24 hours incubation compared to other ratios.

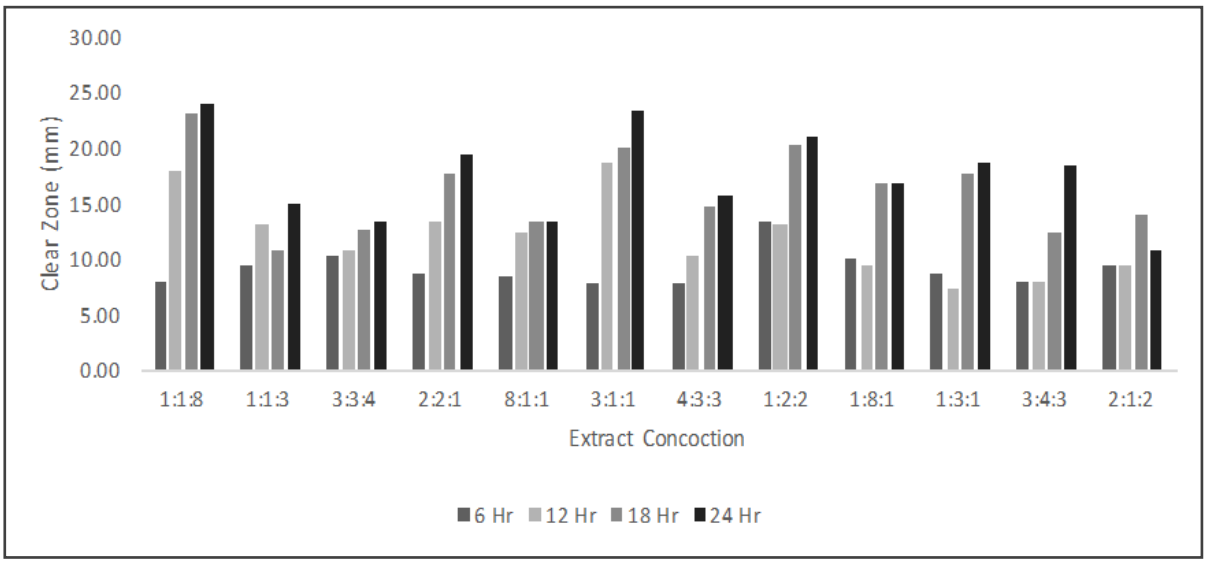


Figure 1. Antibacterial activity of concoction of *S. ferox*, *B. pandurata* and *Z. zerumbet* against *Aeromonas hydrophila*

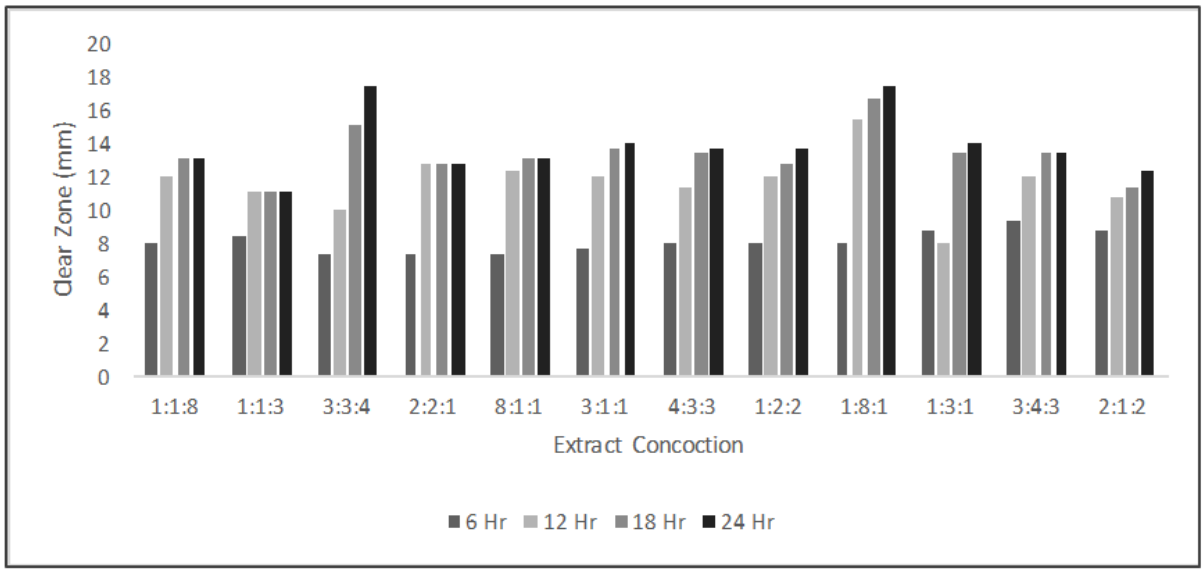


Figure 2. Antibacterial activity of *S. ferox, B. pandurata* and *Z. zerumbet* extracts against *Pseudomonas* sp.

Pseudomonas sp. is a pathogenic bacterium that is also inhibitory assay using a combination of all three extracts. Figure 2 shows the inhibitory zone from the overall combination of extracts to the bacteria *Pseudomonas* sp. The clearly zone is generally smaller than the zone of inhibition against *A. hydrophila* bacteria that is ranged from 8 - 17.33 mm. There are only 3 combined extracts that have a sensitive inhibitory zone (> 14 mm) against *Pseudomonas* sp. namely the combination of extract with a ratio of 3: 3: 4; 3: 1: 1; and 1: 8: 1. Combined extract with 3: 3: 4 and 1: 8: 1 ratio is the best ratio with 17.33 mm inhibition zone at 24 h incubation time compared to other ratio

Both bacteria *A. hydrophila* and bacteria *Pseudomonas* sp. always found to infect tilapia simultaneously and the pathogenicity rate of bacterial compounds is generally higher than that of single infections [35]. The trials of the combined trials of the extracts were carried out on a combination of both in vitro bacteria. The results show the average inhibition zone formed from the

overall combination of extracts to the two bacteria can be seen in Figure 3. What formed is ranged from 8 - 18.67 mm. There are 8 combinations of extracts that have a sensitive inhibitory zone (> 14 mm) against both bacteria (*A. hydrophila* and *Pseudomonas* sp.), Which are combined with a ratio of 1: 1: 3; 3: 3: 4; 2: 2: 1; 8: 1: 1; 4: 3: 3; 1: 3: 1; 2: 1: 1; and 3: 4: 3. Combined extract with a ratio of 1: 1: 3 is the best ratio with 20.67 mm inhibition zone at 24 h incubation compared with other ratios.

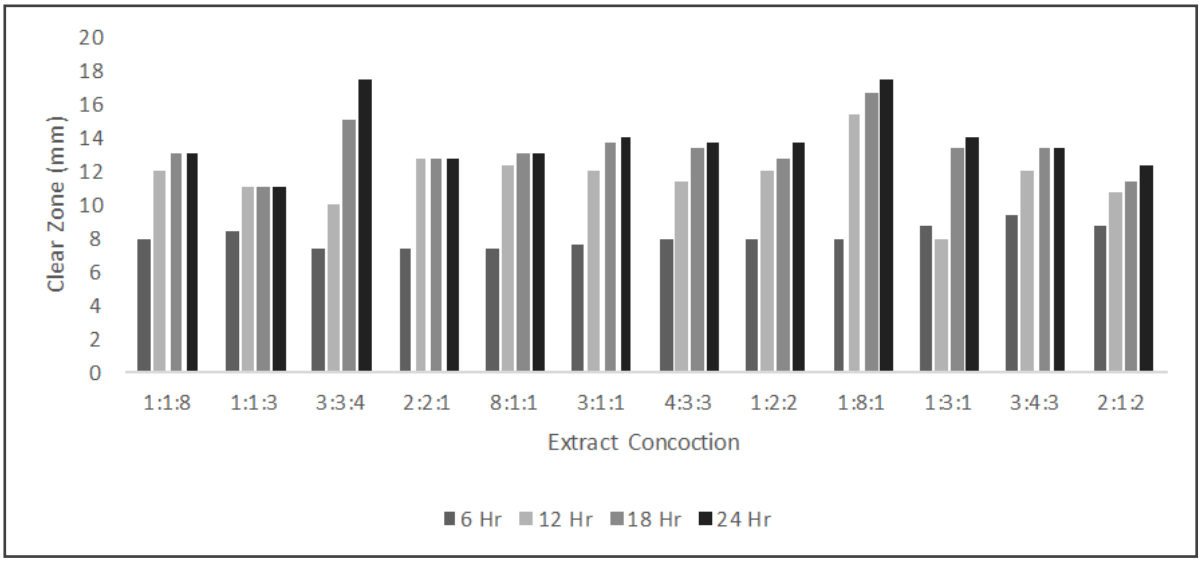


Figure 3. Antibacterial activity of concoction *S. ferox* extract, *B. pandurata* and *Z. zerumbet* against *Aeromonas hydrophila* and *Pseudomonas* sp. combination.

The ethanol extracts of the various comparison ratios have different inhibitory zones when compared with the antibiotic oxytetracycline, only a few ratios that have a ZI% value of percentage. Generally, a ZI% value is formed after 12 h of incubation time and begins to increase with increasing length of incubation. Zone inhibition (ZI%) against *A. hydrophila* bacteria ranged from 5.6 to 26.32%; 19.5% against *Pseudomonas* sp. and 5.7-42.5% against both bacteria. The overall average ZI% formed can be seen in Table 2.

Table 2. Percentage of Zone Inhibition (ZI%) concoction extract of *S. ferox*, *B. pandurata* and *Z. zerumbet* against *Aeromonas hydrophila*, *Pseudomonas* sp. and combination of both bacteria based on time (hours/h) of incubation

Concoction Extract (SF:BP: ZZ)	Bacterial test											
	A. hydrophila				*Pseudomonas* sp.				Combined Bacteria			
	6 hr	12 hr	18 hr	24 hr	6 hr	12 hr	18 hr	24 hr	6 hr	12 hr	18 hr	24 hr
1:1:8	0	1	21.05	26.32	0	0	0	0	0	0	0	0
1:1:3	0	1	21.05	26.32	0	0	0	0	0	0	26.4	42.5
3:3:4	0	1	21.05	26.32	0	0	3.45	19.5	0	0	0	5.7
2:2:1	0	0	0	0	0	0	0	0	0	0	0	28.7
8:1:1	0	0	0	0	0	0	0	0	0	17.2	28.7	0
3:1:1	0	0	0	0	0	0	0	0	0	0	0	0
4:3:3	0	0	0	0	0	0	0	0	0	0	26.4	35.6
1:2:2	0	0	0	0	0	0	0	0	0	0	0	0
1:8:1	0	0	0	0	0	5.7	14.9	19.5	0	0	0	0

1:3:1	0	0	1	5.26	0	0	0	0	0	0	0	0
3:4:3	0	0	0	1	0	0	0	0	0	0	5.7	12.6
2:1:2	0	0	0	1	0	0	0	0	0	0	0	26.4

The ratio of SF: BP: ZZ of 3: 3: 4 is a ratio that has a good ZI% against single bacteria *A. hydrophila, Pseudomonas* sp. as well as the combination of both bacteria, compared with other ratios. Successive amount of the ratio of the three combined extracts to *A. hydrophila* bacteria, *Pseudomonas* sp. and both bacteria are as follows 4, 2, 7 ratio. The largest ZI% score was 35.6% in a 4: 3: 3 ratio against both bacteria.The value of combined MICs SF: BP: The highest ZZ is a 1: 1: 8 ratio to *A. hydrophila*; 1: 8: 1 against *Pseudomonas* sp. and 1: 1: 3 against the combined bacteria.

Table 3. MIC% concoction of *S. ferox*, *B. pandurata* and *Z. zerumbet* against *A. hydrophila*, *Pseudomonas* sp. and a combination of both bacteria at the 24 h incubation period.

Concoction extract (SF:BP:ZZ)	Bacteria Test		
	A.hydrophila	*Pseudomonas* sp.	Combined Bacteria
1:1:8	94%	57%	56%
1:1:3	70%	45%	85%
3:3:4	63%	64%	67%
2:2:1	80%	52%	67%
8:1:1	63%	57%	45%
3:1:1	92%	59%	54%
4:3:3	70%	55%	67%
1:2:2	80%	55%	45%
1:8:1	77%	67%	45%
1:3:1	80%	60%	65%
3:4:3	79%	55%	64%
2:1:2	55%	45%	70%

The three-plant extracts namely *S. ferox*, *B. pandura* and *Z. zerumbet* have antibacterial ability against bacteria *A. hydrohila* and *Pseudomonas* sp. If the extract of *B. pandurata* and *Z. zerumbet* effectively inhibited the growth of *A. hydrophila* bacteria both in vitro and in vivo, *S. ferox* extract was more effective in inhibiting *Pseudomonas* sp. also in vitro [10, 11] and in vivo [12, 13]. The concoction of the three extracts effectively inhibited both bacteria in vitro, and the results showed higher compared with a single extract. The concoction of the extract to inhibit pathogenic bacteria proved to be better than that of a single extract such as the results of [25] study that combined the *O. support*, *T. avicennoides*, and balsam apple (*Momordica balsamina*) (290.6-750.0 lg / mL) against some bacteria: *A. hydrophila*, S. *dysenteriae*, *Shigella flexneri, Shigella sonnei, Shigella bodyii,* and *E. coli.* Similarly, the results of the [24] study combined three extract *Curcuma longa, Ocimum sanctum*, and *Azadirachta indica* with a 1: 1: 1 ratio effectively suppressing *A. hydrophila* bacteria compared to a single extract.

Phytochemical results show that the extract of *S. ferox* contains alkaloids and carbohydrates; *B. pandurata* contains alkaloids, flavonoids, and carbohydrates while *Z. zerumbet* contains alkaloid flavonoids, steroids and carbohydrates [10]. Crude extracts are potential to inhibit bacterial growth than fractionation of components that would normally affect the loss of antibacterial cytokines [34], so the use of crude extracts for field applications would be more effective.

Differences of the material contained resulted in differences in antibacterial activity against bacteria, when the three extracts were combined, showed an increase in antibacterial ability due to synergism between the three extracts. The components contained in the extracts increase each other's ingredients. However, further research is needed for in vivo testing whether the incorporation of the three extracts can be useful in enhancing fish immunity or not. The control of *A. hydrophila* bacteria, *Pseudomonas* sp. both single and combined in fish in vivo can be performed.

4. Conclusion

In this study showed that the combined extract of *S. ferox, B. pandurata*, and *Z. zerumbet* with the ratio 1: 1: 8; 1: 8: 1; and 1: 1: 3has the potential to inhibit the growth of *A. hydrophila* bacteria, *Pseudomonas* sp. or both.

Acknowledgments

Authors are grateful to the RISTEK DIKTI with the project in 2016 and 2017, Mulawarman University, which made this work possible. The authors are grateful to the Department of Fisheries and Marine Science for the facilities made available.

References

[1] Newman S G 1983 *Aeromonas hydrophila*: a review with emphasis on its role in fish disease. *Collection Foundation Marcel Merieux* (France: Lyon).
[2] Cipriano R C, Bullock G L, Pyle S W 1984 *Fish Disease Leaflet* **68**:87-97.
[3] Choudhury D, Pal A K, Sahu N P, Kumar S, Das S S, Mukherjee S C 2005 *Fish and Shellfish Immunology* **19**:281–291.
[4] Kumari J, Sahoo P K 2005 *Journal of Fish Diseases* **29**:95–101.
[5] Yavuzcan H 1998 *The Israeli journal of aquaculture* **50**(2):82-85.
[6] Hardi E H, Pebrianto C A, Hidayanti T, Handayani R T 2014 *Jurnal Kedokteran Hewan* **8**(2):130- 134. [Indonesia]
[7] Hardi EH, Pebrianto C A 2012 *Jurnal Ilmu Perikanan Tropis* **16**(2):35-39. [Indonesia]
[8] Ansary A, Haneef R M, Torres J L, Yadav M 1992 *Journal of Fish Diseases* **15**:191–196.
[9] Lilley J H, Hart D, Richards R H, Roberts R J, Cerenius L, Soderhall K 1997 *Veterinarian Record* **140**:653–654.
[10] Hardi E H, Kusuma I W, Suwinarti W, Agustina, Abbas I, Nugroho R A 2016 *AACL Bioflux* **9**(3):638-646.
[11] Hardi E H, Kusuma I W,Suwinarti W, Agustina, Nugroho RA 2016 *Nusantara Bioscience* **8**(1):18- 21.
[12] Hardi E H, Saptiani G, Kusuma I W,Suwinarti W, Nugroho R A 2017*AACL Bioflux* **10**(2):182:190.
[13] Hardi E H, Kusuma I W, Suwinarti W, Saptiani G, Sumoharjo, Lusiastuti A M 2017*Nusantara Bioscience* **9**(2):220- 228.
[14] Logambal SM, Venkatalakshmi S, Michael R D 2000 *Hydrobiologia* **430**:113–20.
[15] Venkatalakshmi S, Michael R D 2001 *Journal Aquatic Tropics* **16**:1–10.
[16] Logambal SM, Michael R D 2001 *Journal Aquatice Tropics* **16**:339–47.
[17] Dugenci S K, Arda N, Candan A 2003*Journal Ethnopharmacol***88**:99-106
[18] Jian J, Wu Z 2003 *Aquaculture* **218**:1–9.
[19] Jian J, Wu Z 2004 *Fish Shellfish Immunol* **16**:185–91.
[20] Yin G, Jeney G, Racz T, Xu P, Jun X, Jeney Z 2006 *Aquaculture* **253**:39–47.
[21] Tan B K, Vanitha J 2004 *Current Medicinal Chemistry* **11**:1423-1430.
[22] Harikrishnan R, Balasundaram C, Heo M S 2010 *Fish and Shellfish Immunology* **28** :354-361.
[23] Limsuwan S, Voravuthikunchai S P 2008 *FEMS Immunology Med Microbiol* **53**: 429–436.
[24] Harikrishnan R, Balasundaram C 2008 *Journal of Aquatic Animal Health* **20**:165–176.
[25] Iwalokun BA, Gbenle GO, Adewole TA, Akinsinde K A 2001 *Journal of Health, Population,*

and Nutrition **19**:331–335.
[26] Dülger B, Gonuz A 2004 *Pharma Biology* **42**: 301-304.
[27] Bradshaw L J 1992 Laboratory of microbiology, 4th edition. Saunders College, Philadelphia.
[28] Singh R, Chandra R, Bose M,Luthra P M 2002 *Current Science* **83**:737–740.
[29] Mandal S, Mandal M, Pal NK, Halder P K 2002 *Indian Journal of Experimental Biology* **40**:614–616.
[30] Ilori MO, Sheteolu AO, Omonigbehin EA, Adeneye A A 1996 *Journal of Diarrhoeal Diseases Research* **14**:283–285.
[31] Nagamura CV, Nakamura TU, Bando E, Fernandes A, Melo N, Cortez DAG, Filho B P 1999 *Memorias do Instituto Oswaldo Cruz* **94**:675– 678.
[32] Mandal S, Mandal M, Pal NK, Halder PK 2002 *Indian Journal of Experimental Biology* **40**:614–616.
[33] Harikrishnan R, Balasundaram C 2008 *Journal of Aquatic Animal Health* **20**:165–176.
[34] Babu B, Jisha VK, Salitha CV, Mohan S, Valsa A K 2002 *Indian Journal of Microbiology* **42**:361–363.
[35] Rauhun 2014 *SKRIPSI* Fakultas Perikanan dan Ilmu Kelautan Universitas Mulawarman Samarinda [Indonesia].