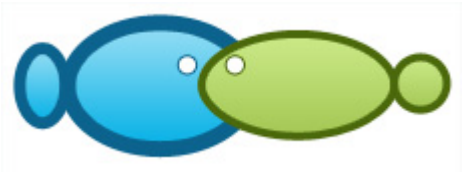

Immunomodulatory and antibacterial effects of *Boesenbergia pandurata*, *Solanum ferox,* and *Zingiber zerumbet* on tilapia, *Oreochromis niloticus*

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Abstract. The aims of the study were to evaluate the potency of antibacterial and immunostimulant effects of some herbs extract, that is, *Boesenbergia pandurata*, *Solanum ferox*, and *Zingiber zerumbet* from East Kalimantan on tilapia *Oreochromis niloticus* infected by *Aeromonas hydrophila* and *Pseudomonas* sp. The focus of this study was to determine the most effective dosage and method of administration to prevent the bacterial infection by in vivo test. Thirty tilapias with the average weight of 15 g were used in this experiment. The tested dosages extracts were 600 ppm *B. pandurata*, 900 ppm *S. ferox*, and 200 ppm *Z. zerumbet*. Meanwhile, three administration methods were applied, that is, injection method, feeding and bath immersion. The results showed that the extract of *B. pandurata*, *S. ferox*, and *Z. zerumbet* can be used as an antibacterial and immunostimulant compound in tilapia. The *B. pandurata* and *Z. zerumbet* extracts effectively were used to prevent *A. hydrophila* infection using the feed method while *S. ferox* extract effectively prevented *Pseudomonas* sp. infection by bath immersion.
Key Words: antibacterial, immunostimulant, *Oreochromis niloticus*, herb plant.

Introduction. The organic compound from plants extract has been commonly used as an antibacterial and immunostimulant to control fish diseases (Cuesta et al 2004; Findlay & Munday 2000; Ratnawati et al 2013; Karina et al 2015; Hardi et al 2016a; Karina et al 2016). In the recent decade, there has been increasing interest in the modulation of the nonspecific immune system of fish using plant extracts or organic matters (Alambra et al 2012; Elkamel & Mosaad 2012; Harikrishnan et al 2011; Menanteau-Ledouble et al 2015; Misra et al 2006; Zokaeifar et al 2012; Satyantini et al 2014). Several studies showed that *Boesenbergia pandurata, Zingiber zerumbet* and *Solanum ferox* have the ability to suppress the *Aeromonas hydrophila* and *Pseudomonas* sp. bacteria growth (Hardi et al 2016a; Hardi et al 2016b) and methanol extract.

According to Siwichi et al (1994) some materials such as β-glucan, lipopolysaccharide, levamisole, chitin, fungi, yeast, mannose, peptidoglycan, microsporidian, and seaweed can be used as immunostimulant in fish culture. Furthermore, Galindo-Villegas & Hosokawa (2004) have evaluated several immunostimulant compounds from animals and plant extract for fish, for example, Tunicate, Abalone, *Quillaja saponaria*, glycyrrhizin, *Laminaria* (seaweed), and bacteria substance; there are peptidoglycan, β-glucan, lipopolysaccharide (LPS), *Clostridium butyricum* cells, *Achromobacter stenohalis* cells, and *Vibrio anguillarum* cells. Based on Hardi et al (2016c) and Hardi & Saptiani (2015) protein fractions of the extracellular product of *Pseudomonas* sp. bacteria were found to be the most effective to inhibit *Aeromonas hydrophila* growth.

Immunostimulant and antibacterial administration in fish can be applied through three methods, namely, injection, feeding and immersion. Galindo-Villegas & Hosokawa (2004) stated that the immersion method has an advantage as the most cost effective method for small fish with no stressful effect on fish. While the oral method has a non-stressful effect on cultured fish, immunostimulation mass allows any size of fish. In this paper we evaluate the efficacy of the *Boesenbergia pandurata, Solanum ferox* and *Zingiber zerumbet* extracts to improve the immune system with several delivery methods (injection, feed, and immersion) for prevention the bacterial infection in tilapia.

Material and Method. This research was done from April to October 2016 at the Laboratory of Environmental Microbiology, Faculty of Fisheries and Marine Science, and Forest Products Chemistry, Faculty of Forestry, Mulawarman University, East Kalimantan, Indonesia.

Fish sample. Samples of tilapias, *Oreochromis niloticus,* with average weight ±15 g were purchased from Kutai Kartanegara Fresh Water Hatchery. The fishes were quarantined and isolated to ensure that the experimental fishes are *A. hydrophila* and *Pseudomonas* sp. which are bacteria free.

Plants material. *Boesenbergia pandurata, Zingiber zerumbet,* and *Solanum ferox* plant materials were collected from a traditional market in Samarinda City, Indonesia. The ethanol extracts of the rhizome of plants were modified from proposed method by Limsuwan & Voravuthikunchai (2008).

Bacteria test. *Aeromonas hydrophila* (EA-01) and *Pseudomonas* sp. (EP-01) were cultured in the Laboratory of Environmental Microbiology, Faculty of Fisheries and Marine Science, Mulawarman University. The culture of bacteria was 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}.

Prevention test using injection, feed and bath immersion methods. The concentrations of each extract in preventive bacteria infection that were tested involved 600 ppm of *B. pandurata*, 900 ppm of *S. ferox* and 200 ppm of *Z. zerumbet*. These are the best extract concentrations recorded from our previous studies (Hardi et al 2016a; Hardi et al 2016b).

A total of six experimental groups (30 fishes for each group with average weight of 15 g) were compared: (a) fish injection with *B. pandurata* extract; (b) fish injection with *Z. zerumbet* extract, and (c) fish injection with *S. ferox* extract. The extract was injected via intraperitoneal (0.1 mL fish^{-1}) and cultured for 7 days; then at the day-8 the fish was injected with 0.1 mL fish^{-1} bacteria (10^{10} CFU mL^{-1}) via intramuscular injection and observed until day-14. The fishes that were given *B. pandurata* and *Z. zerumbet* were challenged with *A. hydrophila* and fishes administered with *S. ferox* were injected with *Pseudomonas* sp.; (d) fish injected with Phospate Buffered Saline (PBS) (0.4 mg L^{-1}) and no challenge; (e) fish injected with *A. hydrophila* bacteria without extracts, and (f) fish injected with *Pseudomonas* sp. bacteria without extracts.

In the feeding method, another six experimental groups were performed. In the first step, a total of 500 mL herb extracts were mixed with 2% egg yolk and then sprayed into 500 g commercial fish feed prior to use for the experiment. The experimental fishes were fed two times a day (7 am and 5 pm) for 7 days and in the day-8 the fishes were challenged with pathogenic bacteria via intramuscular injection and observed until day-14. In this trial, six experiments were compared, namely, (a) fish immunized with *B. pandurata* containing 600 mg L^{-1} of extract, (b) fish fed with *S. ferox* containing 900 mg mg L^{-1}, and (c) fish fed with *Z. zerumbet* containing 200 mg L^{-1}, (d) controlled, unimmunized and with no challenge, (e) controlled, unimmunized and injected with *A. hydrophila* bacteria, and (f) controlled, unimmunized and injected with *Pseudomonas* sp. bacteria.

Preventive test method through immersion was done by bathing the experimental fish in respective extract solution dosage (600 mg L^{-1} *B. pandurata*, 900 mg mg L^{-1} *S. ferox*, and 200 mg L^{-1} *Z. zerumbet*) for 30 minutes and then injected intramuscularly with bacteria pathogen. In this trial six experimental fish were compared, (a) fish soaked in a 600 mg L^{-1} *B. pandurata* extract; (b) fish soaked in 200 mg L^{-1} *Z. zerumbet* extract, and (c) fish soaked in a 900 mg L^{-1} *S. ferox* extract, (d) controlled, unimmunized and with no challenge, (e) controlled, unimmunized and injected with *A. hydrophila* bacteria, and (f) controlled, unimmunized and injected with *Pseudomonas* sp. bacteria.

Research parameters. The observed parameters were abnormality of swimming response, feed response, anatomy pathology, total bacteria count in the body of fish, and mortality. Besides that, the total of leukocyte was also examined. The data were presented in the figures and tables and then analyzed descriptively.

Result and Discussion

Mortality rate. The mortality caused by *Pseudomonas* sp. infection was higher (80%) compared to *A. hydrophila* infection (60%) (Figure 1). This finding is similar to Eissa et al (2010) who reported that infected tilapia by *Pseudomonas anguilliseptica* caused hingher mortality of fish (96.66%). However, the survival rate of infected tilapia by *A. hydrophila* was increased up to 100% when the fishes were injected and fed with *B. pandurata*. The study indicates that the application of *S. ferox* through injection, feed and bath was able to reduce the mortality rate of tilapia after being infected by *Pseudomonas* sp. The oral administration of *Z. zerumbet* was the best method for reducing the mortality rate of fish sample after infection of *A. hydrophila.*

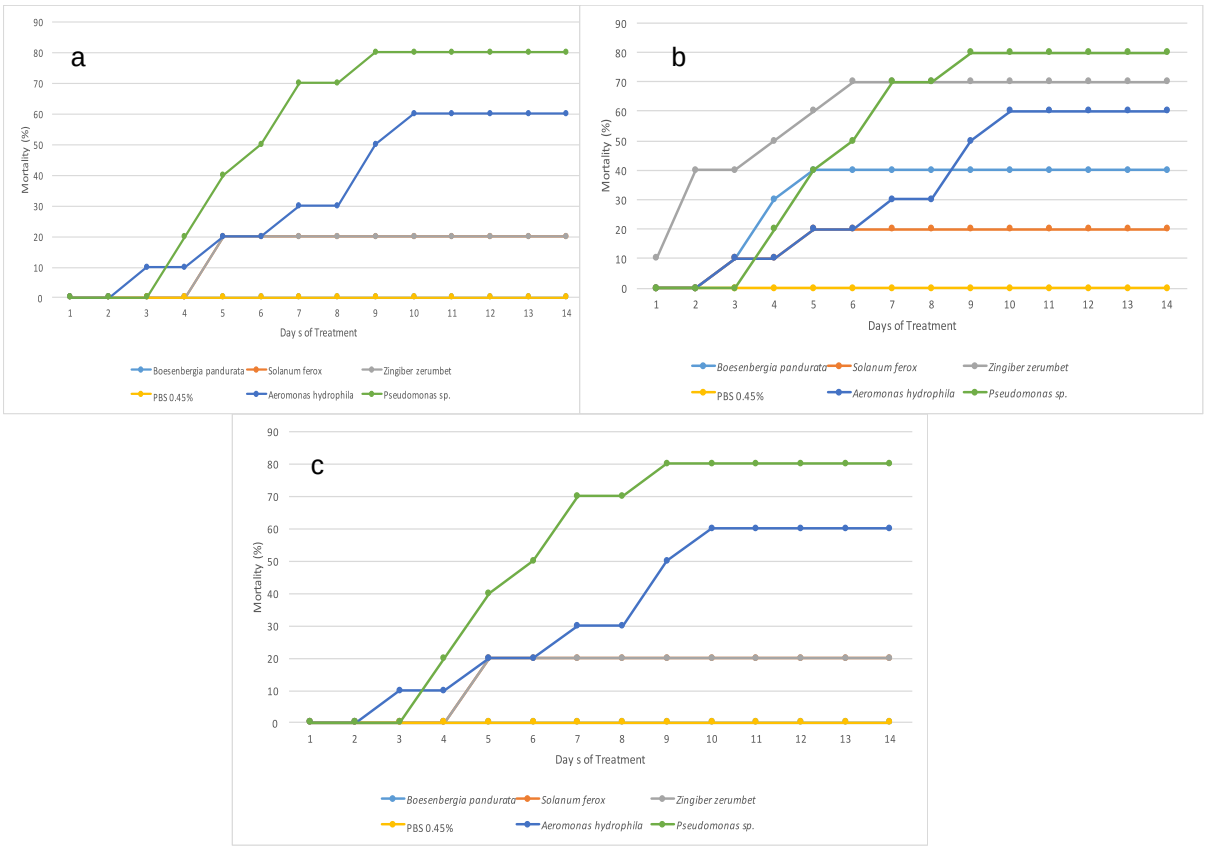

Figure 1. Fish mortality in the prevention using *Boesenbergia pandurata*, *Solanum ferox,* and *Zingiber zerumbet* extract test: (a) injection method, (b) feed method, and (c) bath method.

The study revealed that *Z. zerumbet, B. pandurata*, and *S. ferox* had immunostimulatory and antibacterial activities. Besides, several herb plants were reported to have immunostimulatory and antibacterial substance against the bacteria pathogen with the

different methods; for example, the extracts of *Curcuma longa*, *Ocimum sanctum,* and *Azadirachta indica* (Harikrishnan & Balasundaram 2008; Harikrishnan et al 2009; Harikrishnan et al 2010b) were able to suppress the *A. hydrophila* infection in goldfish.

The *S. ferox* and *B. pandurata* extracts had the active compounds such as alkaloids, flavonoids, and carbohydrates (Hardi et al 2016a). Flavonoid is a dominant substance as antibacterial, while *Z. zerumbet* extract does not have this substance.
Based on prevention method application showed that injection and oral methods are the most effective method for reducing the mortality of infected tilapia compared to bath immersion method. A similar finding was also reported in Atlantic salmon (*Salmo salar*) by Bridle et al (2011). In addition, Wahjuningrum et al (2012) also reported that the application of *Phyllanthus niruri* and *Allium sativum* powder through feeding method during 21 days was effectively preventing *A. hydrophila* infection in catfish (*Clarias gariepinus*). Moreover, Alambra et al (2012) recorded that the total haemocytes were increased when the shrimp *Macrobrachium rosenbergii* was fed turmeric (*Curcuma longa*) extract in the diet for seven days, and it was effectively against *Vibrio alginolyticus* infection.

Abnormality behavior and fish pathology. The direct observation on the fish after being infected by *A. hydrophila* and *Pseudomonas* sp. showed that the appetite of the tilapia fish was decreased significantly and the fishes suffered weakness and anorexia and were swimming into surface of the water (gasping), and unstably swimming on the bottom of the aquarium (Table 1). However, after treatment the fish showed normal behavior. It indicates that extract of the *B. pandurata*, *S. ferox,* and *Z. zerumbet* applied through injection, oral administration and immersion can reduce the infection effects of the *A. hydrophila* and *Pseudomonas* sp.

Table 1

Distribution of fish abnormal swimming and pathology anatomy

Group	*Injection*			*Oral*			*Immersion*		
	Gasping	*Weakness*	*Aggressive*	*Gasping*	*Weakness*	*Aggressive*	*Gasping*	*Weakness*	*Aggressive*
Control	-	-	-	-	-	-	-	-	-
Boesenbergia pandurata	+	+	+	+	++	+	+	+	+
Solanum ferox	-	+	+	-	+	+	-	+	+
Zingiber zerumbet	-	-	+	+	+	+	+	+	+
Aeromonas hydrophila	++	+++	++	++	+++	++	++	+++	++
Pseudomonas sp.	+	++	+	+	++	+	+	++	+

Note : (-) none, (+) mild, (++) moderate, and (+++) severe

The study revealed that, after injection with the pathogenic bacteria, the experimental fish shows darkening in skin, fin rot, and moderate hyperemia of the eye (exophthalmia) (Table 2 and Figure 2). Several researchers reported that in some cases of *Pseudomonas* sp. infection the infected fish has the symptoms such as hemorrhage, darkness in skin, scales, abdominal ascetics, and exophthalmia (Altinok et al 2006; Austin & Austin 2007; Hardi & Pebrianto 2012; Toranzo et al 2005; Yardimci & Aydin 2011).

The exophthalmia was not found in fish administration with *Z. zerumbet* extract via injection and *S. ferox* through injection, feed, and immersion method. These are because the group of *Zingiber* plant contains antibacterial and immunostimulant compounds (Tan & Vanitha 2004). The plant extracts have a component bacterial as flavonoids, alkaloids, and carbohydrates that can suppress bacteria growth and increase the leukocyte production (Cowan 1999). The compound of phenolics, flavonoids, and terpenoids can be used as antibacterial agents (Cowan 1999; Shan et al 2007). This

compound caused membrane bacteria disruption, that related to a phospholipid bilayers (cell death) rupture. Phenolics and flavonoids from *Rabdosia rubescens* acetone extract caused *Bacillus subtilis* bacteria disrupting cell wall, increasing cell membrane permeability and leading to leaking of cell constituents (Cheng & Hu 2015; Hammuel et al 2014).

Table 2

Distribution of fish organ pathology

Group	*Injection*			*Oral*			*Immersion*		
	Fin rot	*Darkness*	*Exophthalmia*	*Fin rot*	*Darkness*	*Exophthalmia*	*Fin rot*	*Darkness*	*Exophthalmia*
Control	-	-	-	-	-	-	-	-	-
Boesenbergia pandurata	+	+	+	+	+	+	+	+	-
Solanum ferox	+	+	-	+	+	-	+	+	-
Zingiber zerumbet	+	+	-	+	+	+	+	+	+
Aeromonas hydrophila	++	++	++	++	++	++	++	++	++
Pseudomonas sp.	++	++	+	++	++	+	++	++	+

Note : (-) none, (+) mild, (++) moderate.

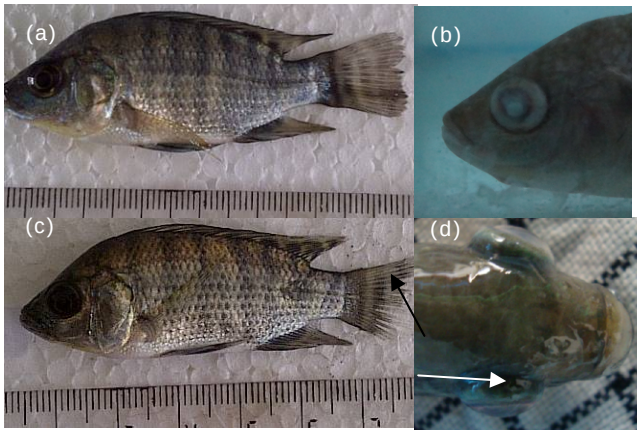

Figure 2. Fish pathology after injection with extract and bacteria pathogen. (a) normal fish, (b) fin rot, and (c & d) exophthalmia.

Bacteria growth. The growth of *A. hydrophila* in fish body was decreased on *B. pandurata* and *Z. zerumbet* extract administrations (Figure 3). Similarly phenomenon was also observed in *Pseudomonas* sp. bacteria growth in fish post-treated with *S. ferox* extract where injection was the best method in decreasing the bacteria growth in infection, while the feeding method appears more effective compared to bath immersion method as recorded in this study (Alambra et al 2012; Hardi & Saptiani 2015; Hardi et al 2016b; Harikrishnan & Balasundaram 2008; Harikrishnan et al 2010a; Harikrishnan et al 2009).

Total of leukocyte. Total leucocyte of tilapia which injected by three ectracts showed that the total leucocytes were increased after 7 days after injection, incrementally enhancing until the day 14 (Figure 4). Total leukocytes are considered as an indicator of fish health status (Bridle et al 2011; Zokaeifar et al 2012). This is an indication of increase of immunity system of the experimental fish. The average of total leukocyte in fish fed extract of *B. pandurata* gradually increased from day-0 to day-14. This finding is in accordance with several previous studies reported by Lawhavinit et al (2011), Alambra

et al (2012), Harikrishnan & Balasundaram (2008), and Harikrishnan et al (2009) that ethanol plant extract boosted immune system of the fish. Therefore, this study revealed that the extracts utilized have the immunostimulating effects on fish target. Immunostimulatory compound was not directly affecting immune memory cells, as activation and differentiation of memory cells. However, they are specific in that immunostimulants enhance particular immune responses against pathogens. The immunostimulating activities of the extract were through enhancing phagocytic activities. This is supported by Tan & Vanitha (2004) who reported that gingerols extract from *Zingiber officinale* induces the activity of IL-6, a potent B cell stimulant.

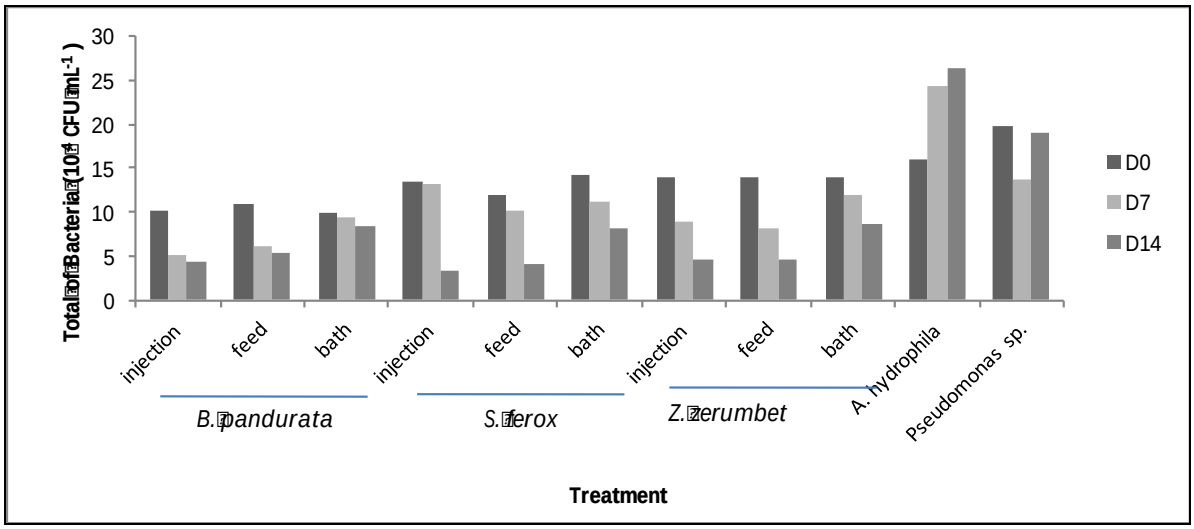

Figure 3. The bacteria density of *Aeromonas hydrophila* and *Pseudomonas* sp. after administration with *Boesenbergia pandurata, Zingiber zerumbet,* and *Solanum ferox* in vivo test.

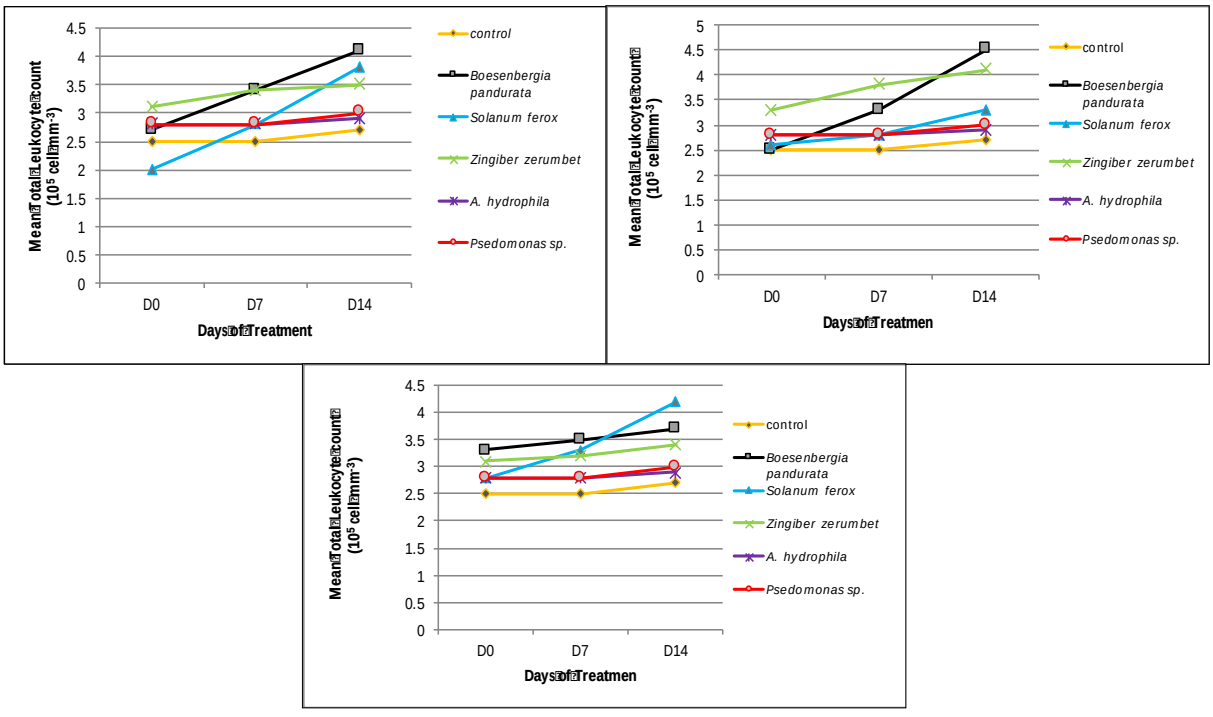

Figure 4. The average of leukocyte of infected fish treated with the *Boesenbergia pandurata*, *Solanum ferox,* and *Zingiber zerumbet* extracts through (a) injection, (b) feeding, and (c) immersion methods.

Conclusions. The extracts of *B. pandurata* and *Z. zerumbet* have an effective influence on preventing *A. hydrophila* infection by the feeding method, while *S. ferox* extract has the significant effect on preventing *Pseudomonas* sp. infection through bath immersion.

Further research needs to be conducted to evaluate respiratory burst of the infected fish treated with both extracts.

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