Evaluation of traditional plant extracts for innate immune mechanisms and disease resistance against fish bacterial *Aeromonas hydrophila* and *Pseudomonas* sp.

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Abstract. The purposes of this study were to evaluate effect of ethanol herbal extracts of *Boesenbergia pandurata*, *Solanum ferox* and *Zingimber zerumbet* on Tilapia (*Oreochromis nilaticus*) innate immune mechanisms and disease resistance against *Aeromonas hydrophila* and *Pseudomonas* sp. Fish were intramuscularly injected with 0.1 mL/fish (10^{10} CFU·mL^{-1}) of each bacterium on the day 6th of post treatment using extract by several methods (injection, oral administration and immersion). The doses of extract were 600 ppm of *B. pandurata*, 900 ppm *S. ferox* and 200 ppm of *Z. zerumbet*. The percentage mortality, Relative Percent Survival (RPS) and innate immune response were assessed on weeks 1, 2, 3 and 4. All the methods were effective to enhance the immune parameters after 2 weeks application and the RPS of treatment reached more than 90 %. The results showed that the injection method of extracts was the most effective method to control *A. hydrophila* and *Pseudomonas* sp. The result indicated that all the doses of extracts could be significantly influence the immune response and protect the health status of tilapia against *A. hydrophila* and *Pseudomonas* sp. infections.

1. Introduction

Aeromonas hydrophila and *Pseudomonas* sp. are two bacterial pathogens that infect tilapia fish in aquaculture. *Aeromonas hydrophila* causes exophthalmia, fin root, darkened and ulcerative lesions on the body even severe bleeding [1, 2]. *Pseudomonas* sp. causes injury to internal organs such as changing the consistency of the kidneys and heart [3]. Herbal plants extract for disease control is highly recommended because besides safe for fish and the environment, the plant extract is also cheap and easy to application in aquaculture [4].

The plant extract effectiveness for disease control is very diverse. Feed application of *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa* ethanol or methanol extract increase the immune response and resistance of goldfish infected with *A. hydrophila* [5, 6]. Mangrove (*Avicennia marina*) leaves extract demonstrated inhibit the growth of bacteria *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *Vibrio anguillarum* isolated from sea water ornamental fish [7]. Furthermore, methanol extract of leaves of *A. marina* is capable to increase the survival of ornamental fish and also inhibits the growth of pathogenic bacteria in the fish body.

Extract plants that contain levamisole, flavonoids, steroids, carbohydrates have ability to inhibit the growth of pathogenic bacteria in fish [8], saponin [9] and glycyrrhizin exhibit as natural antibacterial properties. Some plant extracts have a antibacterial activity to inhibit the pathogen bacteria and increase the fish nonspecific and specific, such as *O. sanctum* [10, 11], azadirachtin [12], *Viscum*

album, *Urtica dioica* and *Zingiber officinale* [13], *Radix astragalin seuhedysari* and *Radix angelicae sinensis* [14, 15], *Astragalus radix* and *Scutellari radix* [16] and *Achyranthes aspera* [17, 18].

Administration of plant extracts through injection enable to stimulate immune system faster than oral administration, because the extract is slowly absorbed by the fish in the later administration [5]. Some plant extracts application through fed in fish were able to enhance the total leukocytes and phagocytic activity [7, 19, 20, 21, 22, 23]. Immersion method of *Azadirachta indica* leaf extract was effective to control the *A. hydrophila* infection in goldfish (*Cyprinus carpio*) [24]. Show that use of garlic extract (1,000, 2,000 and 3,000 ppm) to prevent *A. hydrophila* infection was effective with survival rate ranged 83 to 100 % [25].

In this research, we observed the immunostimulant activity of tree plant extracts *Boesenbergia pandurata*, *Solanum ferox* and *Zingimber zerumbet* to control *A. hydrophila* and *Pseudomonas* sp. bacteria pathogens in tilapia through three different methods: injection, immersion and oral administration.

2. Materials and Methods

This research was done in April to December 2016 at The Laboratory of Environmental Microbiology, Faculty of Fisheries and Marine Science and Laboratory of Forest Products Chemistry, Faculty of Forestry, Mulawarman University, East Kalimantan, Indonesia.

2.1. Fish and bacteria

Oreochromis nilaticus were purchased from Kutai Kartanegara fresh water hatchery. The fish with 10.1 ± 3.2 g (mean ± SD) in weight were used in trials. The *A. hydrophila* (EA-01) and *Pseudomonas* sp. (EP-01) isolates were obtained from Laboratory of Environmental Microbiology, Faculty of Fisheries and Marine Science. The bacteria was cultured in BHI (Brain Heart Infusion Broth, DIFCO®) and BHIA (Brain Heart Infusion Agar, DIFCO®) media for 24 h at 30 °C and the density of bacteria for injection test was 1010 CFU·mL^{-1} [26].

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

Herbal materials were collected from traditional market in Samarinda (figure 1). The ethanol extract of plants were prepared according to previous study by [8, 27]. For the treatment, extract concentrations 600 mg·L^{-1} of *B. pandurata*; 900 mg·L^{-1} of *S. ferox* and 200 mg·L^{-1} of *Z. zerumbet* with three replicates and three administration methods i.e. injection, immersion and oral administration [8 26]. The extracts dosages in this research were the best dosages as antibacterial and imunostimulant in tilapia against *A. hydrophila* and *Pseudomonas* sp. infection.

2.3. Experimental design

The tilapia fish were injected intramuscularly with *A. hydrophila* and *Pseudomonas* sp. (1.6 10^{10} CFU·mL^{-1}) and then at 6th days post injection, tilapia ware treated by using the extract through several methods (injection, immersion and oral). The fish group infected with *A. hydrophila* was treated with *B. pandurata* and *Z. zerumbet*. While, fish group infected with *Pseudomonas* sp. was treated with *S. ferox* extract.

Administration of extract by injection was done by injecting the tilapia with extract intraperitoneally (0.1 mL/fish) and the fish is reared for four weeks.

For oral administration, the extracts were incorporated in pellet feed by mixing 500 mL herb extracts with 1–2 % yolk egg and adding to 1 kg of commercial fish feed. Tilapia was fed with the pellet fed twice a day for 14 days, and reared for four weeks. Immersion administration was done by immersing tilapia in extract solution for 30 min and rearing for four weeks.

This experiment consists of six groups: 1) negative control, the fish was not treated with extract and not injected with bacteria; 2) the fish was injected with *A. hydrophila* and treated with *B. pandurata* extract; 3) the fish was injected with *Pseudomonas* sp. and treated with *S. ferox* extract; 4) the fish was injected with *A. hydrophila* and treated with *Z. zerumbet* extract; 5) the fish was injected

with *A. hydrophila* and not treated with extract; and 6) the fish was injected with *Pseudomonas* sp. and not treated with extract.

2.4. Observation

The observation parameters in this research were abnormality swimming response (gasping, weakness and aggressive); anatomy pathology (fin root and darkness exophthalmia) and total bacteria count in the fish blood and RPS (relative presentation survival). Total of leukocyte and phagocyte index were observed every week until 4th weeks.

3. Results and Discussion

The result showed that the three extracts effectively enhance the fish immune system and increasing the recovery after bacterial infection in the fourth week.

(a) (b) (c)

Figure 1. Herb plants used in the research, a) *Boesenbergia pandurata*, b) *Zingimber zerumbet* dan c) *Solanum ferox*.

3.1. Fish infection

Aeromonas hydropila and *Pseudomonas* sp. infection caused upnormal swimming such us gasping, weakness and aggressive, while the fish were given extracts *B. pandurata* and *Z. zerumbet* showed normal swimming begun in the 2nd weeks until fourth weeks post-treatment through three methods. The fish treated with extract through the injection method recovered quickly, and also exhibited normal pattern of fish swimming and gross pathology. In detail can be seen in figure 2 and 3.

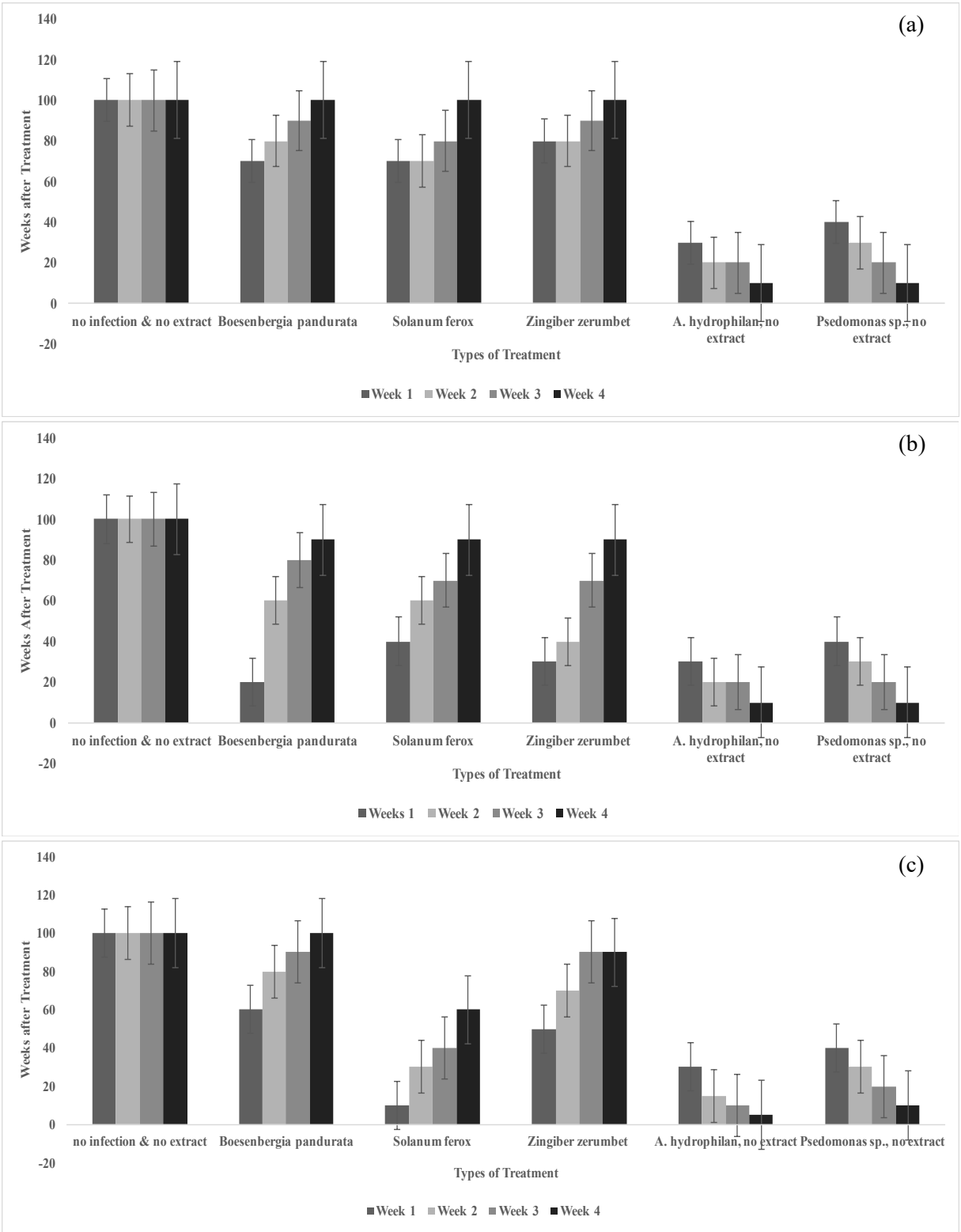


Figure 2. Fish swimming recovery (gasping, weakness and aggressive) of treated fish with three extract and three methods. a) Immersion, b) oral administration and c) injection.

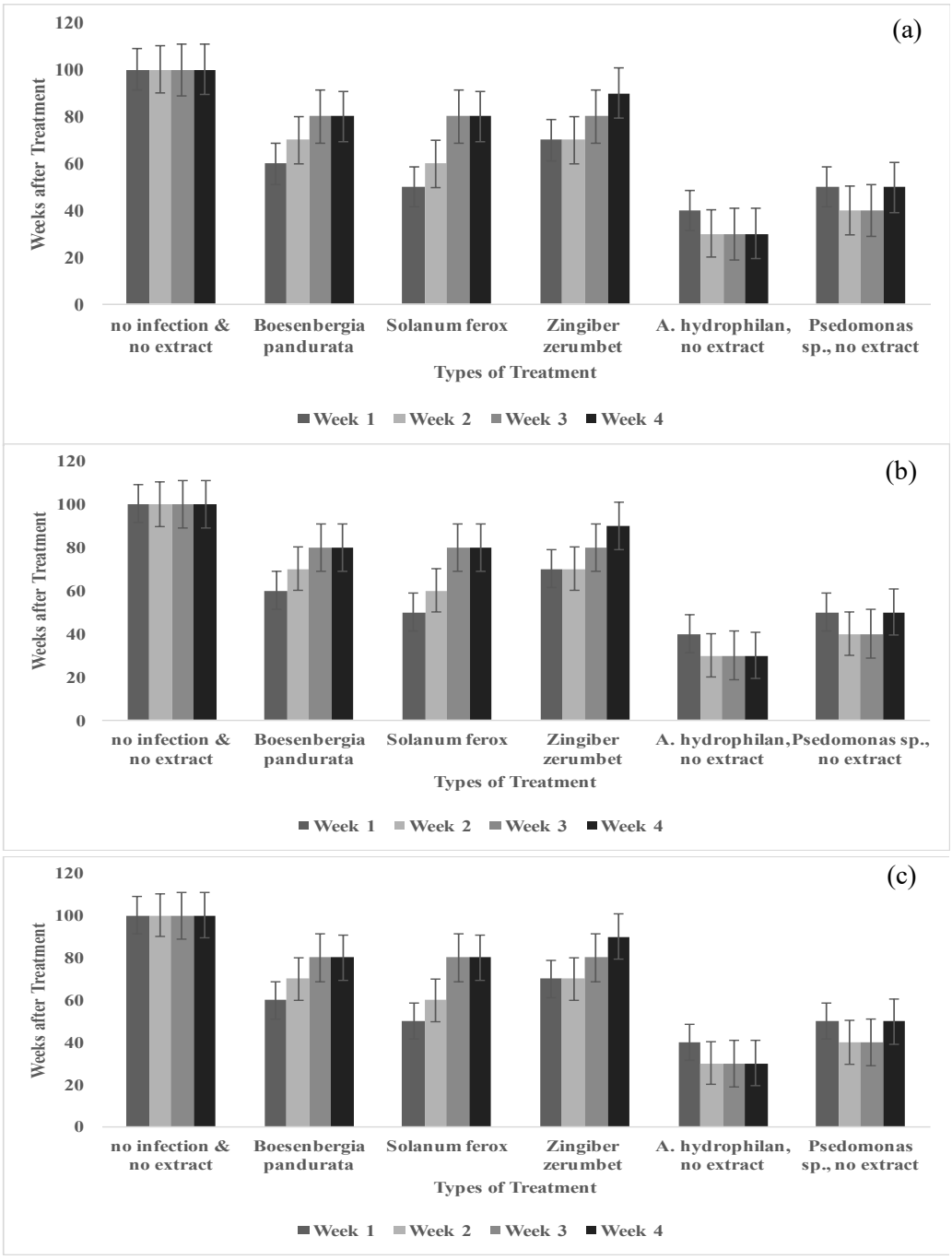


Figure 3. Anatomy pathology (darkness, exophthalmia and fin rot) of treated fish with three extracts and three methods. a) Immersion, b) oral administration and c) injection.

Treatment using extract through feed method showed increase in fish recovery after being infected. The recovery occurred as early as in 1-week to the 4-week reaching 90 % recovery rate with *Z. zerumbet*, and 80 % with *S. ferox* and *B. pandurata*. Immersion method was effective, but the recovery process was slower than by injection and oral administration. This might be having correlation with the absorption process of extract [5]

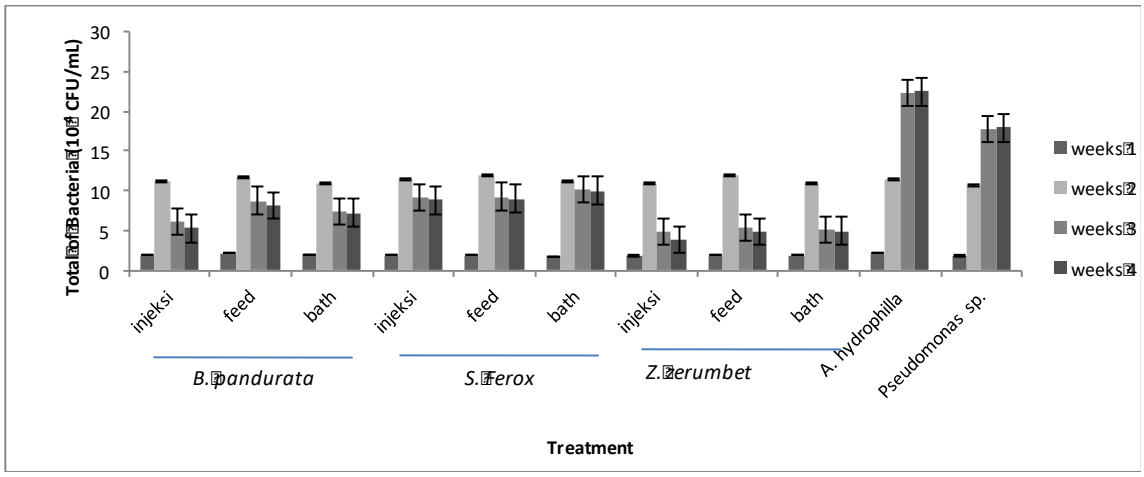
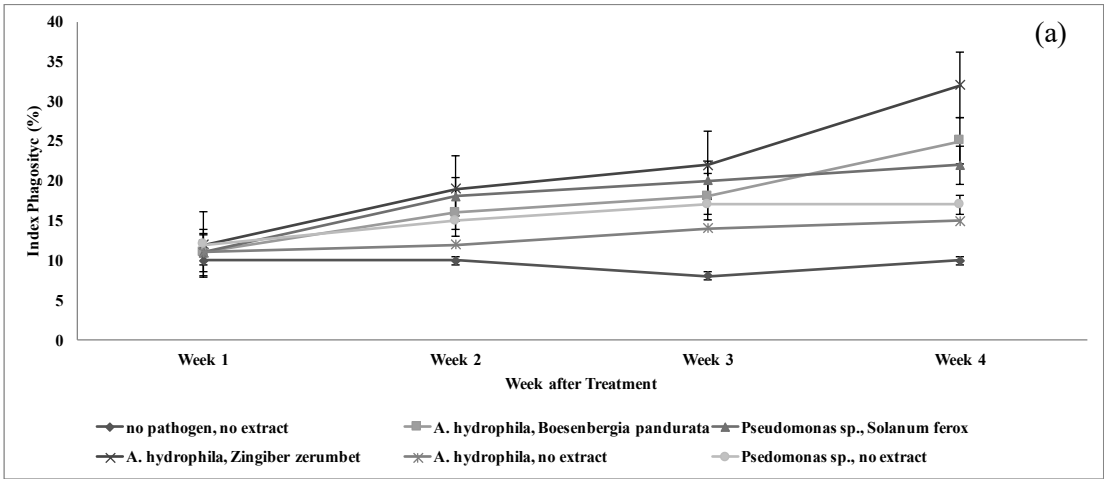
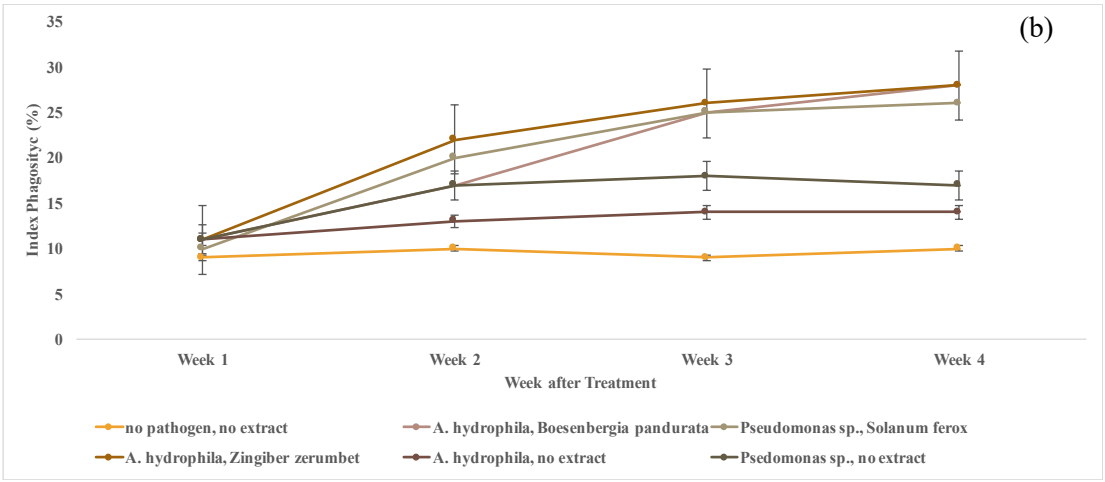


Figure 4. Total bacteria count of treated fish with three extract and three administration methods.

Total bacteria count in treated tilapia was almost lower than control group. Generally, application of the *Z. zerumbet* extract was able to reduce the number of *A. hydrophila* at week 2 to week 4 either through injection, oral administration or immersion and resulted the total bacteria count lower than that of fish treated with extract *B. pandurata*. Treatment of *S. ferox* extract through oral administration and injection were able to suppress the growth of bacteria *Pseudomonas* sp. up to 5.5 10^4 CFU·mL^{-1} while through immersion, TPC decreased to 10^5 CFU·mL^{-1}.

The phagocytic index (leucocyte) of extract treatment have increased since week 2. The *Z. zerumbet* treatment were highest increase compared *B. pandurata* and *S. ferox*.





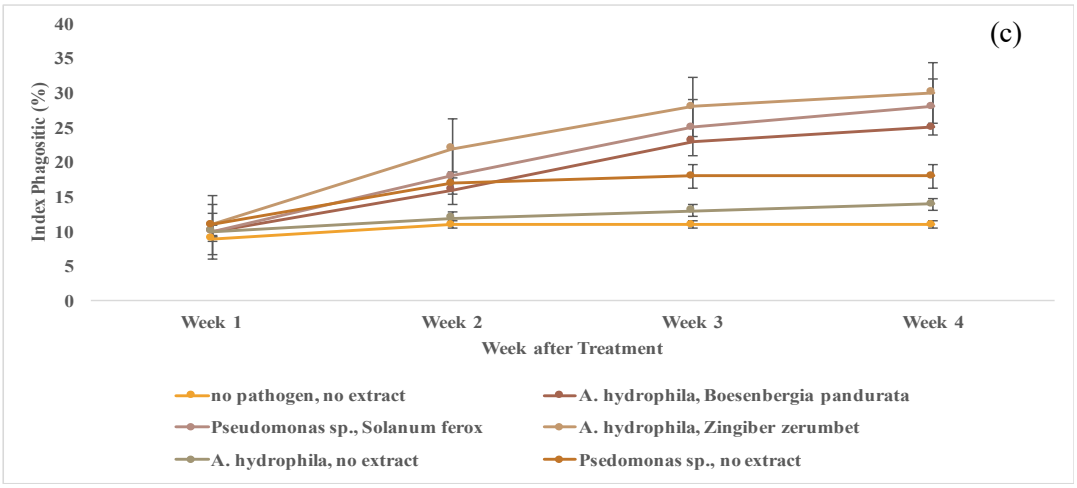


Figure 5. Phagocytic activity of treated fish three extract and three methods: (a) Injection, (b) oral administration and (c) immersion.

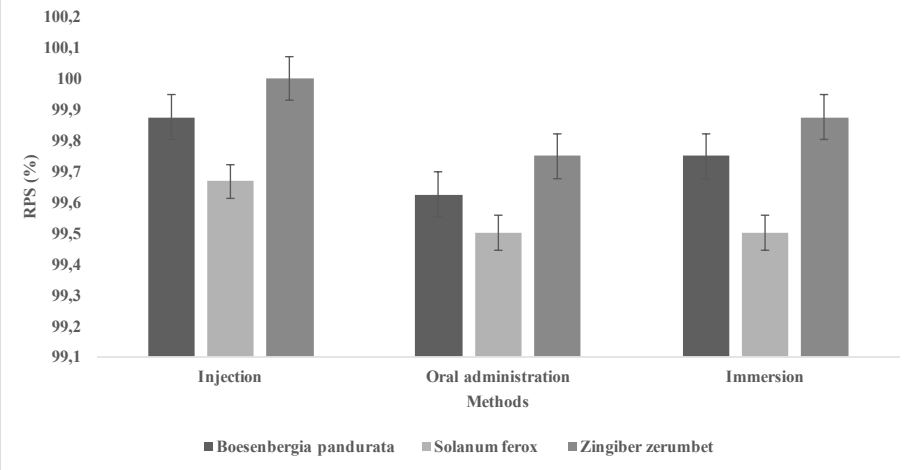


Figure 6. Effect of different extract administered on the Relative Percent Survival (RPS) (%) in Tilapia.

After challenge with *A. hydrophila* and *Pseudomonas* sp., all treated groups showed lower mortality compared to the control treatment. The best survival rate was observed in the group treated with *Z. zerumbet* extract (100 %) through injection. All extract were effective to control diseases caused *A. hydrophila* dan *Pseudomonas* sp. The extract administration as a immunostimulan increase the fish nonspecific immune system [23, 24, 28]. Fish innate immune response enhance the ability to eliminate the pathogen, and increase the fish survival rate after infection [29, 30, 31].

Administration of plant extracts as an immunostimulatory agent in fish maight be through injection, feed (oral administration) and immersion. The later method has advantages and disadvantages, but still effective [23]. Utilization plant extracts as antibacterial and immunostimulant in fish have been developed, but still limited information in the most effective method, dose and mechanisms in vivo are available. The results showed that the ethanol extract of *Z. zerumbet*, *S. ferox* and *B. pandurata* were able to increase fish phagocytic activity after two weeks of immersion and oral administration. Injection gave faster effect in one week.

The oral administration of *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa* administration in goldfish (*Carassius auratus*) increase the leucocyte phagocytic activity in two weeks administration until fourth week [5]. Ginger extract in fish feed increased the total protein level in blood plasma of fish and the highest level of plasma proteins was observed in the group fed with 1 % ginger extract [13]. The plant extract contains components that increase the phagocytic activity [13, 21, 32]. Phagocytic cells are important cells that play a role in the defense mechanism of fish [33].

After challenging with *A. hydrophila* and *Pseudomonas* sp. untreated tilapia showed high mortality (80 %) in week 4, while fish treated with the extract showed low mortality (10–20 %) in the end of experiment in the injection and oral administration methods [32, 34]. Injection of *O. sanctum* and *Nyctanthes arbortristis* in *Oreochromis mossambicus* reduced the mortality after *A. hydrophila* infection [5, 12]. The survival rate of tilapia fed with *Rosmarinus officinalis* extract in combination with *Astragalus membranaceus* and *Lonicera japonica* extract increase after *A. hydrophila* infection in 4^{th} weeks [35, 36].

4. Conclusion

Extracts of *B. pandurata, S. ferox* and *Z. zerumbet* with the concentrations 600, 900 and 200 ppm can be used in the treatment of *A. hydrophila* and *Pseudomonas* sp. infection in tilapia. This extracts improve the phagocytic index, recovery process from the infection. Injection method is more effective method to treat *A. hydrophila* infection by using *Z. zerumbet* and *B. pandurata* than oral administration and immersion method. Similar conclusion is also obtained for *S. ferox* extract in the treatment of *Pseudomonas* sp. infection.

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