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RESEARCH ARTICLE

AQUAHERB CONDITIONERS: THE SILVER BULLET FOR ASIAN SEABASS *Lates calcarifer* AND SILVER POMPANO *Trachinotus blochii* PROTECTION AGAINST VIBRIOSIS

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Abstract

Alternative strategies for minimizing the detrimental effects of bacterial infection and prevention of diseases in aquaculture are necessary since the ongoing efficacy of antibiotics is proving to be unsustainable. One of the most promising approach is the use of aqua herbal conditioners to stimulate the immune system of fish to allow them to fight off infections. In this study, the protective effect of aqua herbal conditioners produced from, mainly, mangrove and neem plant extracts in marine fish, was tested on Asian Seabass *Lates calcarifer* and Silver Pompano *Trachinotus blochii* at 8-10 g of weight size. Challenge tests were performed by immersion with two pathogenic bacteria: *Vibrio harveyi* and *Vibrio parahaemolyticus*, at a concentration of 10^5 cells ml^{-1} for 60 minutes after 12 h, 24 h and 36 h conditioning treatment. The experimental trial show that after 72 h, commercially available aqua herbal conditioners (AquaHerb) was able to significantly increase the percentage survival of *L. calcarifer* and *T. blochii* and reduces their susceptibilityto the *V.harveyi and V.parahaemolyticus*. Significantly higher leukocytesnumber, monocyte, neutrophil andphagocyticindexwere detected in all conditioning group for Silver Pompano and Asian Seabass. These results suggest that the combination of herbal extracts together with other trace elements contained in AquaHerb were able to act as immunostimulants and appear to improve the immune status and disease resistance of Asian Seabass and Silver Pompano.
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INTRODUCTION

Currently, disease outbreaks have become a major constraint to the sustainability of aquaculture production, especially in the marine fish farming system (Nagasawa and Cruz-Lacierda, 2004). Potential economic losses from disease outbreaks are significant, and can affect the production, profitability and sustainability of the aquaculture industry (Ruwandeepika, H.A.D, 2010). Many diseases that mostly caused by pathogenic or opportunistic bacteria (Bachère, 2003) are linked to the degradation of water quality and stress associated with

(super) intensive culture system (Sritunyalucksana, K. 2001), which often leads to high mortalities in nauplii or juvenile stages of aquatic organisms (Marques *et al.*, 2005).

Among the bacterial pathogens, vibriosis is a well-known cause of serious problems in the aquaculture industry with a worldwide occurrence (Toranzo & Barja, 1990). This disease has been described as vibriosis or bacterial disease, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis or red leg disease (Aguirre-Guzman *et al*., 2004). Several pathogens belonging to the *Vibrio* sp. have become a nightmare in aquaculture industry. One of the most important species that are associated with human and animal diseases is *Vibrio parahaemolyticus* (Ruwandeepika, H.A.D, 2010). This species occurs along marine coastal waters and is a commonly known worldwide as a leding causative agent of seafood-associated bacterial gastroenteritis (Levin, 2006; DePaola *et al*., 1990; Joseph *et al*., 1982) and diseases in Brine shrimp *Artemia* (Gunther & Catena, 1980; Puente *et al*., 1992; Rico-Mora & Voltolina, 1995; Orozco-Medina *et al.*, 2002) and *L. vannamei* with different characteristics *e.g.* gill necrosis, lethargy, and loss of appetite (Aguirre Guzman *et al*., 2010).

Until recently, conventional treatment used to control bacterial diseases in aquatic organisms was the administration of antibiotics and disinfectants (Brown, 1989). However, unresponsible use of antibiotics has stimulated the development of bacterial resistance (Defoirdt *et al.*, 2007; Subasinghe, 1997) and allergy in humans (Alderman & Hastings, 1998; Cabello, 2006). Therefore, it became clear that several alternative strategies to control the microbiota in aquaculture system are urgently needed.

Among other prophylactic approach (such as vaccines, immunostimulants and probiotics), the use of herbal biomedicines is receiving considerable attention to control the bacterial, fungal and viral diseases in vertebrates (Dhayanithi *et al.*, 2013; Shangliang *et al*., 1990; Novriadi and Haw, 2014; Zhang *et al.*, 2009) and invertebrates (Direkbusarakom *et al.*, 1998; Harikrishnan *et al.*, 2011). Interestingly, the use of those *prophylactic* approach including herbal remedies to the fish have provided several benefits such as reducing stress response, increasing the activity of innate parameters and improving disease resistance (Austin & Brunt, 2009; Hoffmann, 2009; Magnadóttir, 2010; Nayak, 2010). However, none of herbal biomedicines studies have provided sufficient information on the immune response activation of Silver Pompano *Trachinotus blochii* and Asian Seabass *Lates calcarifer* in the early period of grow out production.

In marine fish, stimulation of innate humoral and cellular defense mechanisms and not the acquired immune response become the main platform to increase the resistance of host from infectious diseases. It is well known that fish are more depends on non specific defense mechanism than mammals (Anderson, 1992). Therefore, in order to observe the humoral and cellular components in fish. it is necessary to study a number of biologically relevant assays such as phagocytic cells similar to macrophages, neutrophils, and natural killer (NK) cells and also various humoral defense components, such as lysozyme production, natural hemolycin, transferin and C-reactive protein (CRP) (Secombes *et al*., 1996, Sakai, 1999).

In present study, the use of herbal biomedicines as an immunostimulant in combination with Vibrio parahaemolyticus and Vibrio harveyi for the challenge test was evaluated in a gnotobiotic culture system to study the effects of this prophylactic approach to enhance the immune system in marine fishes. For that purpose, commercially available herbal bio-medicines (Invertebrates Aqua Conditioner-AquaHerb) were offered to gnotobiotic Silver Pompano (*Trachinotus blochii*) and Asian Seabass (*Lates calcarifer*). The survival of *T. blochii* and *L. calcarifer* was observed during various challenge tests and number of cellular immune response were gathered after conditioning and challenge test. The results will provide us with the possibility of herbal aqua-conditioners to induce the immune response of marine fishes against *V. harveyi* and *V. parahaemolyticus.*

I. Material and Method

II.1 Experimental animal and culture tank

Juvenile of Silver Pompano (*Trachinotus blochii*) and Asian Seabass (*Lates calcarifer*) with single weight size (8-10 g) were collected from Batam Mariculture Development Center, Riau Island Province, Indonesia and maintenance of Silver Pompano (*Trachinotus blochii*) and Asian Seabass (*Lates calcarifer*) were done in experimental tanks. The site consists with spherical tank containing 175 L saline water (30 mg l^{-1}) with UV treated seawater and filtered by 1 mm cartridge filter. Internal aerator with flow rate of 0.3 l min^{-1} for all tanks was used. Parameters like dissolved oxygen, temperature and pH were monitored twice a day. Meanwhile, other parameters such as Ammonia (NH_3), Nitrit (NO_2), Nitrat (NO_3) and Posphate (PO_4) were monitored once a day. Fish were fed *ad libitum* three times per day with commercial feed only after conditioning period.

II.2 Experimental design

The eco-friendly herbal-based bioconditioners (AquaHerb) for Aquaculture (AquaHerb) were used to enhance and promoted several immune response in marine fishes. The 8-10 gr of Silver Pompano and Asian Seabass

were exposed to a concentration of 0.03 g L^{-1} of AquaHerb for 12 h, 24 h and 36 h conditioning treatment prior to feeding and challenge with *V. harveyi* and *V. parahaemolyticus*. The control consisted out of juvenile that were not exposed to anything. A washing step was performed to remove all remaining AquaHerb before transferring to experimental tanks and immersed in *V. harveyi* and *V. parahaemolyticus* suspension at a density of 10^5 cells.ml^{-1} for 60 minutes. All treatments were compared to a control group that was not exposed to the AquaHerb. The experiment was done with three replicates per treatment and survival as well as the number of immune response was determined for 72 hours after each conditioning period.

II.3 Bacterial culture and growth conditions

II.3.1 Bacterial strains

Two bacterial strains were examined as pathogens, namely: *Vibrio harveyi* and *Vibrio parahaemolyticus*. Pure cultures of the bacterial strains were obtained from the Brackishwater Research Centre Laboratory-Jepara. Both pathogens belonging to the *Vibrio harveyi* complex and were selected because they are known as virulent pathogens for aquatic organisms (Ruwandeepika, H.A.D, 2010)

II.3.2 Bacterial Culture

Isolates of the two bacterial strains *Vibrio harveyi* and *Vibrio parahaemolyticus* that were previously stored in 30% glycerol at -80^0 C, were aseptically inoculated in 30 ml marine broth by incubation overnight at 25^0-28^0C with constant agitation. 150 µl was subsequently transferred and grown to stationery phase in 30 ml marine broth six hours before challenge. The bacterial densities were determined spectrophotometrically at an optical density of 550 nm. The bacterial densities were calculated using the equation: **Concentration (CFU/ml) = [1200*10^6*OD]** according to McFarland standard, (BioMerieux, Marcy L'Etoile, France), assuming that an **OD_{550}=1.000 corresponds to 1.2×10^9 cells/ml**

II.3.3 Bacterial Stock

1 ml of the bacterial colony was transferred and grown to stationery phase in 5 mL of *Difco™ Marine Broth 2216* by incubation overnight at 25^0-28^0 C with constant agitation. Bacterial suspensions were then transferred to centrifugation tubes and centrifuged at 4000 g for 5 minutes. The supernatant was discarded and pellets were resuspended in 7 ml filtered autoclaved sea water (FASW). The solution was homogenized and 3 ml of 30% Glycerol solution was added. 150 µl of each colony was distributed to the sterilized Eppendorf tube and stored at -80^0 C.

II.4 Cellular Immune Response and Analysis

Blood sampling was performed after on 12 h, 24 h and 36 h conditioning treatment and 72 h post-bacterial challenge test. Ten fish from each treatment and control were taken to determine the number and percentage of leukocyte count, neutrophile, monocyte and phagocytic based on Anderson and Sewicki (1993); Blaxhall PC (1972) and Wedemeyer and Yasutake (1977).

II.6 Percentage Survival of Silver Pompano and Asian Seabass

The survival (%) of Silver Pompano and Asian Seabass were determined according to procedures described by Marques *et al.* (2004). For this purpose, the number of live Silver Pompano and Asian Seabass was registered before conditioning or challenge with bacteria by counting with the naked eye. At the end of experiment, the number of swimming fish was scored and survival (%) was calculated according to the following equation :

Survival (%) = Final number of surviving animals / initial number of animals x 100%

II.7 Statistical Analysis

Survival data of Silver Pompano and Asian Seabass were arcsine transformed for statistical comparisons to satisfy normal distribution and homoscedasticity requirements. Survival data were subjected to one way ANOVA followed by Tukey's multiple comparison range using the statistical software SPSS version 21.0 to determine significant differences among treatments. All significance levels of the statistical analysis were set at $p<0.05$.

II. Results

Asian Seabass *Lates calcarifer* Bloch and Silver Pompano *Trachinotus blochii* are important aquaculture food fish species cultured in Indonesia. Asian Seabass culture commenced in Bandar Lampung in 1980 while Silver Pompano commenced in Batam in the beginning of 2005. With recent advances in culture technology, production of both species continued to increase and expected to reach 20% per year over the coming years (Mayunar, 1999). However, the dramatic increase in the production of Asian Seabass and Silver Pompano and constantly in contact with parasites, bacteria and viruses in (super) intensive culture system that commonly applied in Indonesia has led to the emergence of infectious diseases within these systems.

In the last two decades, antibiotics were widely used, not only for controlling aquatic diseases, but also for promoting the growth of fish (Cabello, 2006). This massive mis(use) of antibiotics has stimulated the emergence of bacterial resistance and challenged researchers to find alternative strategies (De Schryver *et al.,* 2009). Therefore, instead of antibiotics, increasing attention is being paid to the use of herbal medicine for the treatment and control of many diseases in marine fish farming (Duke, 1987). Herbal treatment has been recently shown to be instrumental as an anti-microbial activity, facilitate growth, and maturation of cultured species (Novriadi and Haw, 2014; Dhayanithi *et al.,* 2010; Shangliang *et al.,* 1990; Harikrishnan *et al.,* 2011a). Moreover, the administration of anti-stress characteristics of herbs through oral (diet) or injection route enhance the innate and adaptive immune response of fish and shellfish against bacterial, viral and parasitic diseases (Harikrishnan *et al*., 2011a) and rapidly degraded without posing any environmental hazard (Maqsood *et al.,* 2011).

In this study, commercially available herbal medicines AquaHerb were chosen because of their recorded ability to enhance the activity of the marine fish immune system. AquaHerb specially developed from combination of highly purified mangrove and neem extracts, immune system promoters, essential osmoregulatory salts and trace elements. Today, mangrove that have been used to treat various diseases for centuries are being applied in a wide range of aquaculture activity to control the bacterial, fungal and viral diseases (Kathiseran, 2000; Valentin *et al.,* 2010; Dhayanithi *et al.,* 2012). The second highly purified plant extract, neem *Azadirachta indica* has been known as an antibacterial and express good *in vitro* activity against *Aeromonas hydrophila*, *Enterobacter* sp., *Streptococcus* sp., *E. coli*, *P. aeruginosa*, *Proteu*s sp., *Vibrio cholerae*, *V. alginolyticus*, *V. parahaemolyticus* and *Yersinia enterocolitica* (Dhayanithi *et al.,* 2010).

The results from this study presented in **Figure 1** indicated that AquaHerb conditioning was able to significantly increased the percentage survival of Asian Seabass and Silver Pompano survival challenged with *V. harveyi* and *V. parahaemolyticus* at a density of 10^5 cells/ml in comparison to control ($p<0.05$). In contrast, both marine fish species that were not primed with AquaHerb and fed solely with commercial fed could not resist this pathogen and showed low survival at different time points. After challenged with *V. harveyi* and *V. parahaemolyticus* survival of fish treated with AquaHerb was improved when compared with the control group. It is possible that the higher survival is the result of enhancement of some components of non-specific immune system of the marine fish by AquaHerb. Moreover, at 24 h and 36 h of AquaHerb conditioning group provided better survival than the 12 h conditioning with and/or without challenged. These survival results suggest that the efficacy of 0.03 g L^{-1} of AquaHerb lasted for long term of conditioning in the presence of pathogens. This in line with the study from Yin *et al.,* (2008) and Ramamurthy *et al.* (2014) who stated that the combination of purified plant extract and long term of herbal application were able to enhanced the survivability of treated fish.

The results from this study also showed that AquaHerb were able to modulate some parameters of the non-specific immune functions of Asian Seabass and Silver Pompano. The non-specific defence mechanisms of fishes include leukocyte function (Govind *et al.,* 2012), neutrophil activation, production of peroxidase and oxidative radicals, together with initiation of other inflammatory factors (Ellis, 1977; Ainsworth *et al.,* 1991). In this study fish conditioned with AquaHerb showed an elevated leukocyte number over the whole period of the experiment in comparison to control (Table 1). In the presence of *V. harveyi* and *V. parahaemolyticus*, 24 h of AquaHerb conditioning treatment were able to significantly improved the fishes survival in comparison to other conditioning treatment. Interestingly, the leukocytes level of control group were also increased in the presence of pathogens. However, the number it self is still lower than that of the AquaHerb treated group. The high number of leukocytes in the presence of pathogen could be due to the main function of leukocytes as one component of non-specific immune system that will localize and eliminate pathogens through phagocytosis (Sukenda *et al*., 2008)

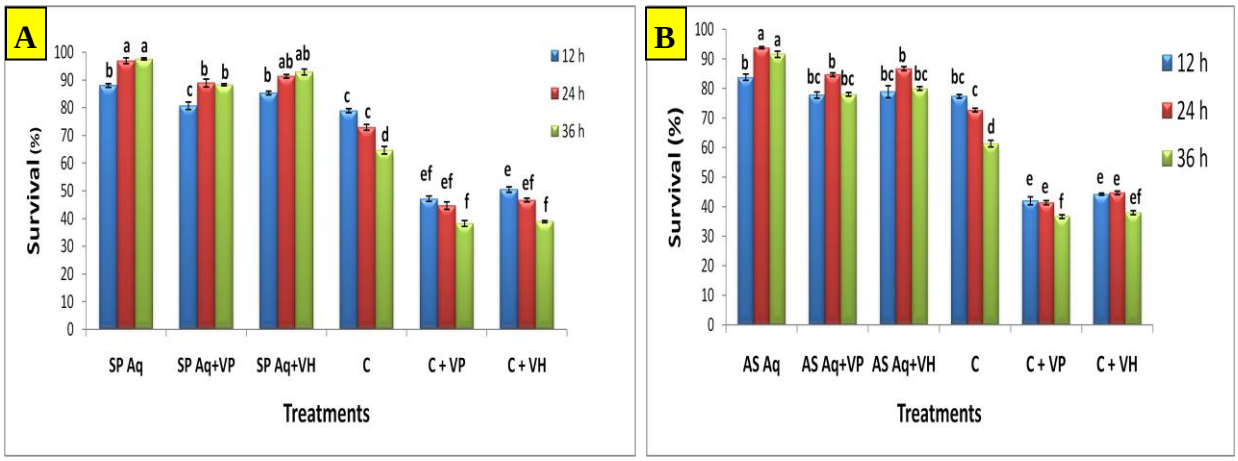

Figure 1. Histogram of the mean survival (%) of Silver Pompano *T. blochii* (**A**) and Asian Seabass (**B**) exposed to 0.03 g L^{-1} of AquaHerb for 12 h, 24 h and 36 h conditioning treatment prior to feeding and challenged. Control indicates no treatments. SP corresponds to silver Pompano; AS corresponds to Asian seabass. Aq corresponds to AquaHerb; Aq+VP corresponds to AquaHerb and challenged with *V. parahaemolyticus*; Aq+VH corresponds to AquaHerb and challenged with *V. harveyi.* Survival was scored at 72 h after challenge. Significant differences between the treatment and control are indicated by different letters (*p<0.05;* n=3).

No	Treatments	Number of leukocytes (10^3 cell ml^{-1})					
		Silver Pompano (A)			Asian Seabass (B)		
		12 h	24 h	36 h	12 h	24 h	36 h
1	AquaHerb	109±1,9	112.3±1,8	105.3±1,2	93.4±1.7	107.5±2.2	98.1±1.2
2	AquaHerb+ VP	128.4±1.2	134.1±2.2	129.7±0,8	123.1±0.8	133.2±0.9	126.4±1.5
3	AquaHerb + VH	130.3±0.3	137.5±0.7	125.9±0.4	123.1±1.8	138.3±0.7	127.2±0.9
4	Control	58.2±1.7	55.4±1.9	52.7±1.8	53.3±1.5	46.2±1.1	45.1±1.9
5	Control + VP	78.5±1.9	79.3±2.1	56.7±0.8	76.3±2.1	78.3±2.9	79.4±1.5
6	Control VH	81.2±2.2	78.4±0.9	59.3±0.3	77.8±1.1	79.6±1.7	80.5±1.9

Table 1. Leukocytes number (average±std.deviation) of Silver Pompano *T. blochii* (**A**) and Asian Seabass (**B**) exposed to 0.03 g L^{-1} of AquaHerb for 12 h, 24 h and 36 h conditioning treatment prior to feeding and challenge with *V. harveyi* (VH) and *V. parahaemolyticus* (VP).

In this study, AquaHerb conditioning treatment had increased total percentage of Asian Seabass and Silver Pompano monocytes in the presence or absence of pathogen. As fish are infected, monocytes which are the main cells of the non-specific defense system shifts from blood cells into tissue and become macrophages that are capable to kill a variety of pathogens including bacteria (Wijendra and Pathiratne, 2007). The result presented in **Figure 2** showed that the overall monocytes number of the challenged fishes were significantly higher in AquaHerb group in comparison to control (*P<0.05*). In the absence of pathogen, AquaHerb was able to improve the monocytes number equal to the control group challenged with pathogen. This noted phenomenon indicates a positive prophylactic response by Aquaherb.

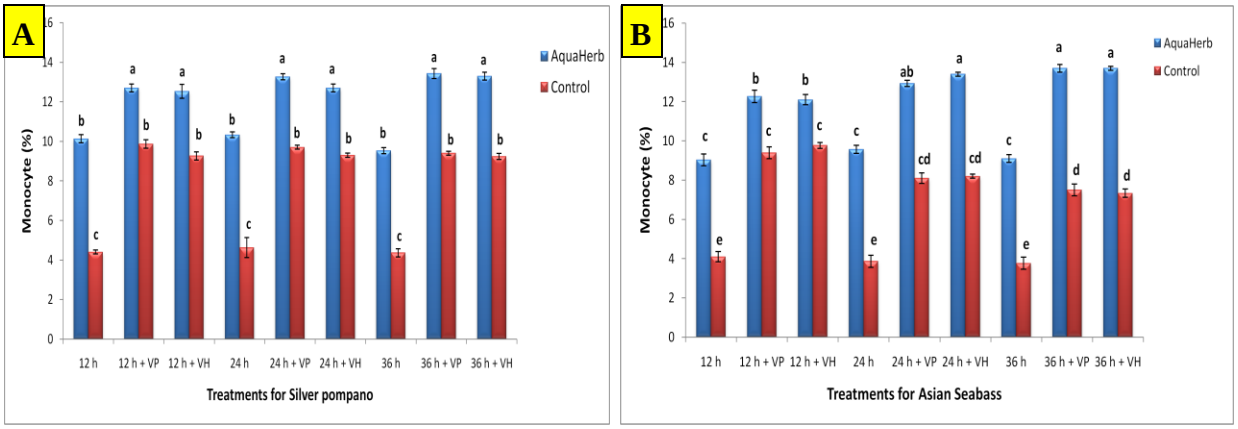

Figure 2. Histogram of the mean monocytes (%) of Silver Pompano *T. blochii* (**A**) and Asian Seabass (**B**) exposed to 0.03 g L^{-1} of AquaHerb for 12 h, 24 h and 36 h conditioning treatment prior to feeding and challenged. Control indicates no treatments. SP corresponds to silver Pompano; AS corresponds to Asian seabass. Aq corresponds to AquaHerb; Aq+VP corresponds to AquaHerb and challenged with *V. parahaemolyticus*; Aq+VH corresponds to AquaHerb and challenged with *V. harveyi.* Survival was scored at 72 h after challenge. Significant differences between the treatment and control are indicated by different letters (*p<0.05;* n=3).

The total percentage of neutrophils were also affected by the administration of AquaHerb conditioning. In the absence of pathogen, significant increase of monocytes was observed in 12 h, 24 h and 36 h conditioning treatment in comparison to control. In the presence of pathogen, neutrophile (%) of Silver Pompano and Asian Seabass without any addition of AquaHerb were able to significantly improve neutrophil (%) in comparison to the treatments that did not receive the addition of pathogen. However, overall result showed that the combination of AquaHerb and pathogens were able to significantly induce higher percentage of neutrophile than in other treatment group (*P<0.05*). Interestingly, there is no significant difference between 24 h and 36 h of conditioning treatment. Neutrophils are essential innate immune cells which determine the host's resistance against various bacterial and fungal infections (Kumar and Sharma, 2010). In addition to exhibiting phagocytic activity against bacteria, neutrophils also form neutrophil extracellular traps (NETs) to regulate severity of infection (Papayannopoulos and Zychlinsky, 2009) and kill bacteria extracellularly due to their high serine protease content (Brinkmann *et al.*, 2004). According to Dellman and Brown (1989), during a bacterial infection, normally the number of neutrophils in blood will increased significantly. This phenomenon is mainly caused by lymphoid that need to release leukocytes against infection. The enhanced neutrophil activity in the present study were logically reflected in the percentage survival of Asian Seabass and Silver Pompano in the challenge test where AquaHerb were found to be effective in inducing disease resistance against *V. harveyi* and *V. parahaemolyticus.*

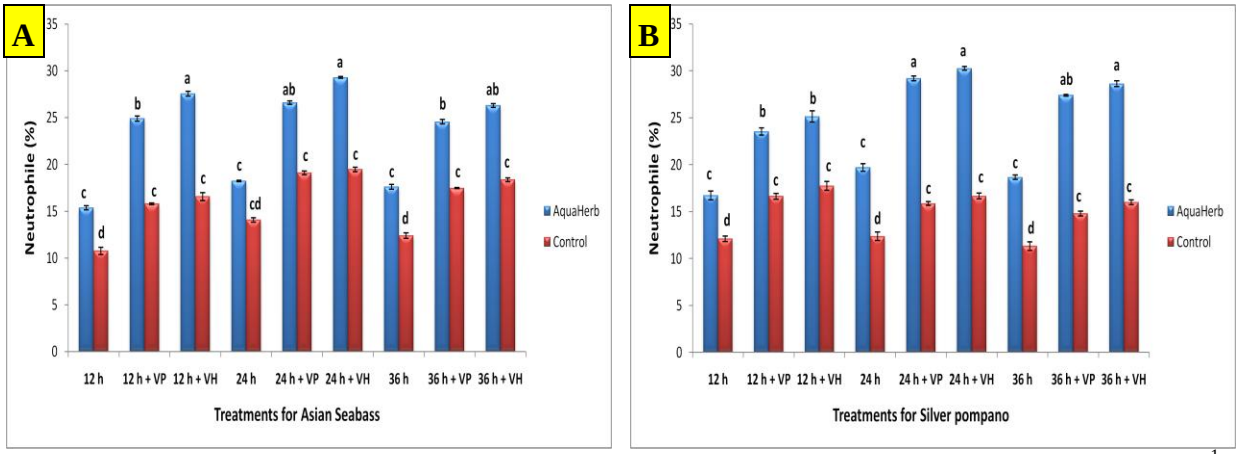

Figure 3. Histogram of the neutrophil (%) of Asian Seabass (**A**) and Silver Pompano (**B**) exposed to 0.03 g L^{-1} of AquaHerb for 12 h, 24 h and 36 h conditioning treatment prior to feeding and challenged. Control indicates no treatments. SP corresponds to silver Pompano; AS corresponds to Asian seabass. Aq corresponds to AquaHerb; Aq+VP corresponds to AquaHerb and challenged with *V. parahaemolyticus*; Aq+VH corresponds to AquaHerb and

challenged with *V. harveyi.* Survival was scored at 72 h after challenge. Significant differences between the treatment and control are indicated by different letters (*p<0.05;* n=3).

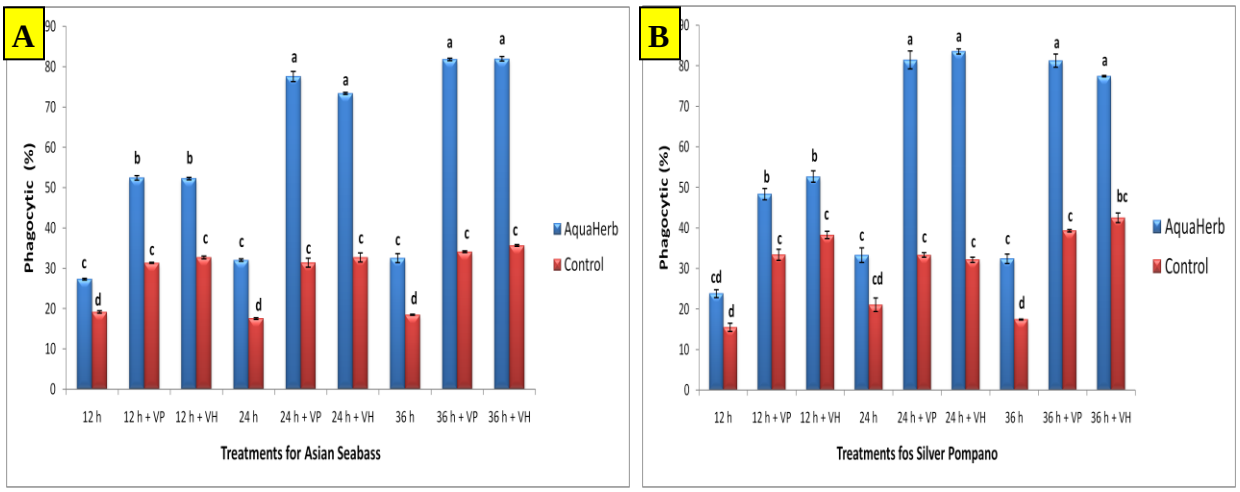

Figure 4. Histogram of the phagocytes activity (%) of Asian Seabass (**A**) and Silver Pompano (**B**) exposed to 0.03 g L^{-1} of AquaHerb for 12 h, 24 h and 36 h conditioning treatment prior to feeding and challenged. Control indicates no treatments. SP corresponds to silver Pompano; AS corresponds to Asian seabass. Aq corresponds to AquaHerb; Aq+VP corresponds to AquaHerb and challenged with *V. parahaemolyticus*; Aq+VH corresponds to AquaHerb and challenged with *V. harveyi.* Survival was scored at 72 h after challenge. Significant differences between the treatment and control are indicated by different letters (*p<0.05;* n=3).

Obviously, all animals possess phagocytic cells that will attack invading microorganisms. In our study, the purified extracts of mangrove and neem labelled as AquaHerb increased the phagocytic activity of Asian Seabass and Silver Pompano. In the absence of pathogen, there is no significant differences between 12 h, 24 h and 36 h conditioning treatment but still higher in comparison to control (*P<0.05*). Interestingly, in the presence of pathogen, the group of 24 h and 36 h induce a higher phagocytes activity compared to 12 h of conditioning treatment. In line with AquaHerb treatment, the control group also showed a significant elevated of phagocytes activity when challenged with pathogens. Corroboration for our results come from Dügenci and colleagues. (2003) who have reported that the addition of plant extract, such as ginger increased the phagocytic capability of the cells in rainbow trout. Furthermore, the combination of four Chinese herbs (*Rheum officinale*, *Andrographis paniculata*, *Isatis indigotica*, *Lonicera japonica*) was able to enhance the phagocytosis of Crucian Carp (Chen *et al.*, 2003).

A. Water Quality Analysis Before Conditioning

No	Parameters	Silver Pompano			Asian Seabass		
		12 h	24 h	36 h	12 h	24 h	36 h
1	pH	7.76±0.73	7.86±0.81	7.87±0.62	7.71±0.85	7.73±0.61	7.82±0.77
2	Salinity (‰)	31±1	31±1	31±1	31±1	31±1	31±1
3	NO_2 (mg/l)	0.01±0.01	0.02±0.01	0.02±0.01	0.01±0.01	0.03±0.01	0.02±0.01
4	NO_3 (mg/l)	0.37±0.11	1.52±0.79	1.48±0.33	0.41±0.15	1.62±0.88	1.73±0.31
5	PO_4 (mg/l)	0.09±0.02	0.12±0.03	0.18±0.03	0.08±0.02	0.17±0.04	0.22±0.05

B. Water Quality Analysis After Conditioning

No	Parameters	Silver Pompano			Asian Seabass		
		12 h	24 h	36 h	12 h	24 h	36 h
1	pH	7.91±1.22	8.11±1.13	8.14±1.17	8.04±0.99	8.19±0.76	8.18±0.83
2	Salinity (‰)	31±1	31±1	31±1	31±1	31±1	31±1
3	NO_2 (mg/l)	0.06±0.02	0.11±1.2	0.17±0.05	0.07±0.04	0.09±0.03	0.08±0.02
4	NO_3 (mg/l)	0.44±0.16	1.67±0.85	1.73±0.91	0.51±0.22	1.77±0.55	1.54±0.11
5	PO_4 (mg/l)	0.14±0.05	0.24±0.03	0.19±0.06	0.18±0.04	0.15±0.49	0.23±0.33

C. Water Quality Analysis During 72 h of Observation

No	Parameters	Silver Pompano			Asian Seabass		
		12 h	24 h	36 h	12 h	24 h	36 h
1	pH	7,82±0.93	7.81±0.85	7.88±0.87	7.92±0.11	7.85±0.57	7.89±0.87
2	Salinity (‰)	31±1	31±1	31±1	31±1	31±1	31±1
3	NO_2 (mg/l)	0.02±0.01	0.01±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
4	NO_3 (mg/l)	0.29±0.23	0.18±0.11	0.27±0.09	0.25±0.08	0.26±0.11	0.35±0.12
5	PO_4 (mg/l)	0.03±0.03	0.07±0.01	0.08±0.02	0.05±0.03	0.09±0.02	0.04±0.03

Table 2. Monitoring of water quality (A) before conditioning, (B) after conditioning and (C) during 72 h of observation. 12 h, 24 h and 36 h correspond to period of conditioning and AquaHerb treatment.

Results for the various water physico-chemical parameters recorded for the AquaHerb treatment at three different point of observation are presented in **Table 2.** The results revealed that several parameters including *nitrite, nitrate* and *posphate* were affected by the addition of AquaHerb during the conditioning treatment. During conditioning, mean water pH of 24 h and 36 h was slightly higher than 12 h both on Seabass and Pompano rearing tank. This might be due to the impact of trace elements contained in AquaHerb that dissolved in the water. However, the range of pH still agreed with the range recorded by Sirajuddin (2009). In addition, the mean of nitrite, nitrate and posphate at 24 h and 36 h also showed an elevated concentration compared to 12 h of conditioning treatment both in Asian Seabass and Silver Pompano tank. This is mainly due to the influence of mangrove and neem extracts that contributed to the high inputs of nitrogen dissolved in the water. However, the value of *nitrite*, *nitrate* and *phosphate* are still within the accepted range (Sirajuddin, 2009) and also act as the "hospital for sick fish" just like mangrove ecosystem in marine environment.

Based on the results of experiments described above, it was obvious that the combination of herbal extract are more effective as an immunostimulant against bacteria from the *Vibrio* genus that are most frequently associated with mortalities in marine fish (Liu *et al*., 2003; Yii *et al*., 1997). The unique composition of AquaHerb added to rearing water act as immunostimulants and appears to improve the immune status and disease resistance of fish.

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