ISSN: 2347-5129
IJFAS 2014; 1(3): 73-78

www.fisheriesjournal.com
Received: 07-10-2013
Accepted: 20-11-2013

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Immunostimulation effects of herbal bio conditioners on tiger grouper (*Epinephelus fuscoguttatus*) against *V. parahaemolyticus* infection

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ABSTRACT
In the present study, the protective effect of herbal-based conditioners as an immunostimulants was tested on tiger grouper (*Epinephelus fuscoguttatus*) juvenile at various times of their culture period to enhance their resistance against bacterial infection. The trial comprised of a single formulation of herbal-based bio conditioners with scheduled water changes during the treatment. Three period of exposure (6 h, 12 h and 24 h) with herbal-based bio conditioners as well as a control are performed in completely randomized design of experiment followed by a challenge test using single pathogenic bacteria: *Vibrio parahaemolyticus* at concentration of 10^5 cells ml^{-1}. Percentage survival and host-pathogen interaction were determined at the end of exposure and challenge test. Various challenge tests showed that herbal-based bio conditioners (AquaHerb) significantly increase the percentage survival ($P<0.05$) of tiger grouper and improve their resistance against *V. parahaemolyticus* infection. Statistically, percentage of leukocytes, monocytes, lymphocyte and neutrophile treated with 24 h of AquaHerb immersion were higher than 6 h, 12 h and control group ($P<0.05$). In addition, tiger grouper immune system performance was found to be better than in the control group. Finally, by combining the positive impact of herbal-based Bio conditioners, this prophylactic approach can become a very effective alternatives to the use of antibiotics and other synthetic compounds.

Keywords: Herbal-based bio-conditioners, *V. parahaemolyticus,* Tiger grouper, Percentage survival, host-pathogen interaction.

1. Introduction
In tiger grouper (*Epinephelus fuscogutatus*) cultivation, disease outbreaks are being increasingly reported as a major constraint to the sustainable growth of production. Many diseases are linked to the stress conditions associated with the intensification systems of farming and the degradation of the environmental biome quality. Under poor conditions, small and adult fish of tiger grouper are often opportunistically infected by fungi, bacteria and viruses. Among the pathogens, vibriosis is a well-known cause of serious problems in the aquaculture industry with a worldwide occurrence [1] and cause the most severe economic losses worldwide [2]. *Vibrio parahaemolyticus* is a known agent that cause severe haemorrhagic septicemia and gastroenteritis syndrome [3]. Although chemotherapy is quite popular to treat or prevent the bacterial infections, frequent use of chemotherapeutic agents has allowed for the development of drug-resistant strains [4, 5, 6] and led to allergy and toxicity in humans [6, 7]. Therefore, the use of prophylactic protocols to stimulate and enhance the immune responses has now become urgent [8].

The possibilities for using herbalism as a prophylactic approach have gained much interest in the last years mainly due to the technology of this phytotherapy whose safety, efficacy and their application can be mastered [9]. Plants herbalism have several benefits for aquaculture, such as production of inhibitory compounds, sources of safer and cheaper chemical and they can also help to cure various diseases, as they contain active principles [10] and antimicrobiological activities due to the active principles such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils [11, 12, 13, 14, 15]. Bioactive substances obtained from natural sources such as mangrove, melaleuca and neem extracts is considered friendly to our environment. For centuries, these natural extract have been used in traditional medicine to treat

various disease [16] and capable to activate the immune system of several ornamental fish [17] and freshwater fish [18]. However, the efficacy of commercially available mangrove, melaleuca and neem extracts in controlling the Vibrio disease in fry tiger grouper are rarely discussed. Therefore, the purpose of this study was to examine the influence of commercially available mangrove, melaleuca and neem extracts (AquaHerb) to enhance the immune responses and percentage survival of Tiger grouper (*Epinephelus fuscoguttatus*) against *V. parahaemolyticus* infection.

2. Material and Methods

2.1 Animal

1-2 cm of Tiger grouper (*Epinephelus fuscogutattus*) obtained from Mariculture Development Centre, Batam were used as the test animals. The culture density was 30 fish/10 liter of water. Acclimatization process was conducted for 2 weeks preceding the experiment. 10-20% of water volume was replaced weekly.

2.2 Bacterial Culture

Isolates of the bacterial strains *Vibrio parahaemolyticus* obtained from Brackishwater Research Centre, Jepara that previously stored in 30% glycerol at -80 ^{0}C, were aseptically inoculated in 30 ml marine broth by incubation overnight at 25-28 ^{0}C 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.

2.3 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 25-28 ^{0}C with constant agitation. Bacterial suspensions were then transferred to centrifugation tubes and centifugated 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.

2.4 Experimental Design

The objective of the experiment was to examine the effect of herbal-based bio conditioners (AquaHerb) on tiger grouper against *V. parahaemolyticus* infection. Challenge tests were performed with *Vibrio parahaemolyticus* at a density of 10^5 cells/ml after three different period of herbal immersion, namely: 6h, 12 h and 24 h. completely randomized design are performed with three repetitions and without water exchange during the therapy. The concentration of AquaHerb are equal to all period, namely: 1 gr/20 L. Marine water for AquaHerb administration are treated with mechanical, biological and UV filter prior to use to eliminate the number of bacteria. Normal feeding regime and water exchange was conducted after medication and during observation. During the observation, water quality was checked regularly with moderately-high aeration as to maintain the concentration of dissolved oxygen above 5 mg/L. The temperature, salinity, pH, nitrate, nitrite and ammonia levels were monitored in different tanks at regular intervals.

2.5 Survival (%) of tiger grouper

The survival (%) of *tiger grouper* were determined according to procedures described by Marques *et al.* (2004). For this purpose, the number of live tiger grouper was registered before herbal therapy and challenged with bacteria by counting with the naked eye. At the end of challenged test for each treatment, namely: 72 hours after medication, the number of live tiger grouper was scored and survival (%) was calculated according to the following equation :

$$\text{Survival (\%)} = \text{Final number of surviving } \textit{grouper} \text{ / initial number of } \textit{grouper} \times 100\%$$

2.6 Host-pathogen interaction analysis

The analysis for cellular immune response of tiger grouper after herbal medication was measured based on the number of leukocytes, monocytes, lymphocyte and neutrophile. The Percentage of host-pathogen parameters were determined according to procedures described by Anderson and Siwicki [19].

2.7 Statistical analysis

Data for Survival (%) of tiger grouper are presented as mean values followed by the standard deviation. Survival data and host-pathogen interaction of tiger grouper 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 $p<0.0$

3. Results

3.1 Main Composition of Chemical and Herbal-extracts of AquaHerb

Table 1: Chemical composition of AquaHerb

S. N	Chemical Name
1	Mangrove extracts
2	Neem Extracts
3	Ethanol
4	Inert Preservatives
5	Ferrous and Copper Sulphates
6	Dried Peat & Leaves, Saponins, Dried Root Barks Tannin
7	Flavonoids

Based on the composition of AquaHerb presented in Table 1 showed that mangrove, neem and melaleuca estracts together with dried peat, leaves saponin and roots tannin were identified as the main active agent of this commercial herbal-based bio conditioners (AquaHerb). This active substances are being used in a wide range of applications including to enhance the immune system of fish against firstly, Viral, Bacterial and Parasitic infections.

3.2 Percentage Survival

The results presented in Figure 1 indicate that immersion priming with herbal Bio conditioners (AquaHerb) enhanced

tiger grouper survival in comparison to the non-primed tiger grouper at every exposure time ($p<0.05$). In the presence of *V. parahaemolyticus*, significant improvements in tiger grouper survival were observed in the nauplii immersed with 1 gr/20 L from 6h to 24h post priming in comparison to the control treatment ($p<0.05$). the immersion of 1 gr/20 L for 24 h was able to significantly ($p<0.05$) induce higher percentage survival in comparison to other time of AquaHerb immersion and to the control (without AquaHerb medication). In addition, 12 h priming was better than 6 h of AquaHerb immersion ($P<0.05$). When looking at the results of the challenge test, we can see that in the presence of *V. parahaemolyticus*, the tiger grouper without any medication for 24 h had a significant lower survival (%) compared to other control and other treatments ($P<0.05$).

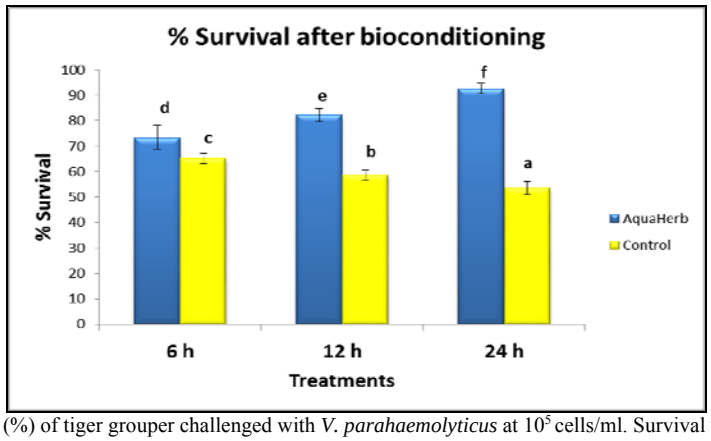

Fig 1: Histogram of the mean survival (%) of tiger grouper challenged with *V. parahaemolyticus* at 10^5 cells/ml. Survival was scored at 72 hours after AquaHerb immersion and the addition of *V. parahaemolyticus* for the challenge. Each experiment was repeated twice and similar results were observed. Significant differences among the treatments and control at corresponding time of herbal exposure are indicated by different letter ($p< 0.05$).

3.3 Host-pathogen interaction analysis

Table 3: Host-pathogen interaction expression of *tiger grouper* either immerse with 1 g/20 L of AquaHerb for 6,12 and 24 hours (AHI = After herbal immersion) and subsequently challenged once with *V. parahaemolyticus* at a density of 10^5 cells/ml. Each treatment results was compared to the control. Significant differences among the treatments and control at corresponding time of herbal exposure are indicated by different letter ($p<0.05$).

No	Treatments	Parameters							
		Leukocytes (10^3 cells/mL)		Monocytes (%)		Lymphocyte (%)		Neutrophile (%)	
		AHI	API	AHI	API	AHI	API	AHI	API
1	AquaHerb 6 h	81.2^c	92.8^c	9.8^c	11.1^a	78.2^b	81.9^a	8.1^b	9.6^b
2	Control 6 h	58.9^d	53.8^d	8.2^d	8.9^d	69.1^c	67.8^c	7.4^c	6.9^c
3	AquaHerb 12 h	92.5^b	96.6^b	10.6^b	11.9^a	81.3^a	89.7^a	8.4^b	9.9^b
4	Control 12 h	57.7^d	$45,3^e$	8.1^d	8.5^d	69.7^c	63.1^c	7.6^c	6.8^c
5	AquaHerb 24 h	95.3^a	107.1^a	11.6^a	12.4^a	87.9^a	92.4^a	9.1^a	10.2^a
6	Control 24 h	58.1^d	41.8^e	$7,9^e$	8.2^d	66.1^c	59.8^d	7.6^c	6.1^c

3.4 Water quality

Table 2: Range and average of various water quality parameters in different AquaHerb treated tank.

No	Treatments	Parameters					
		pH	Salinity (‰)	NO_2 (mg/l)	NO_3 (mg/l)	NH_3 (mg/l)	PO_4 (mg/l)
1	AquaHerb 6 h	7,93±0,01	31±1	0,1±0,1	0,1±0,1	0,07±0,03	0,1±0,1
2	Control 6 h	7,81±0.02	31±1	0,3±0,2	0,1±0,1	0,04±0,04	0,1±0,1
3	AquaHerb 12 h	8,11±0.05	31±1	0,5±0,1	0,4±0,2	0,09±0,01	0,3±0,1
4	Control 12 h	7,65±0.04	31±1	0,3±0,1	0,3±0,1	0,08±0,02	0,1±0,1
5	AquaHerb 24 h	8,24±0.03	31±1	0,6±0,1	0,5±0,2	0,17±0,02	0,3±0,1
6	Control 24 h	7.59±0.05	31±1	0,3±0,2	0,5±0,2	0,09±0,01	0,2±0,1

3.5 Water quality

Table 4: Range and average of various water quality parameters during Challenge test with *V. parahaemolyticus* at a density of 10^5 cells/ml.

No	Treatments	Parameters					
		pH	Salinity (‰)	NO_2 (mg/l)	NO_3 (mg/l)	NH_3 (mg/l)	PO_4 (mg/l)
1	AquaHerb 6 h	8,11±0,2	31±1	0	0,4±0,1	0	0
2	Control 6 h	8,03±0,4	31±1	0	0,4±0,2	0	0
3	AquaHerb 12 h	8,01±0,3	31±1	0	0,5±0,1	0	0
4	Control 12 h	8,09±0,4	31±1	0	0,4±0,1	0	0
5	AquaHerb 24 h	8,09±0,3	31±1	0	0,3±0,1	0	0
6	Control 24 h	8,02±0,4	31±1	0	0,4±0,1	0	0

4. Discussion

Tiger grouper is one of high-value finfish in aquaculture industry and become an important activity for the small scale fish farmers throughout the Asia-Pacific region [20]. In the past, Limited supply of 'seed stock' has been identified as the major constraint for the expansion of tiger grouper. However, nowadays, bacterial infections have become a chronic problem in tiger grouper hatchery operations and causing monetary loss and may increase fish mortality rate up to 80% [21].

In the present study, herbal immersion were able to increase the number of tiger grouper percentage survival (%) against *V. parahaemolyticus* infection in comparison to control ($p<0.05$). This in line with the study from Dhayanithy [17, 22] stated that neem and mangrove extract dissolved in ethanol significantly reduce the *V. parahaemolyticus* number and their infection in marine ornamental fishes. The values presented in Figure 1 indicated that the AquaHerb immersion for 24 h without any exchange of water during the treatment have been successfully provide a better resistance and survival, 92,67±1%, in comparison to 6 h (73,5±2,2%) and 12 h (82,2±1,2%) of immersion. According to these results, to induce the activation of innate immune system and provide a better protection against *V. parahaemolyticus* in tiger grouper, time period of immersion is one of the critical factor. The present finding is in agreement with the results of Harikrishnan [23] stated that the administration of herbal Bio conditioners within 6 and 3 day prior to a challenge significantly increased the survival rate in the tested fish.

Significant correlations were found between the percentage survival with host-pathogen analysis, including: monocytes, leukocytes, lymphocyte and neutrophile at the end of the experiments. In general, the immersion of AquaHerb influenced the increase number of leukocytes. Where 24 h of immersion produce higher leukocytes if it was compared to control, 12 h and 6 h of immersion. It meant that the longer the fish immersed in herbal Bio conditioners capable to produce higher number of leukocytes. In fish immune system, leukocytes play an important role against infectious pathogen [24] and the immersion with AquaHerb definitely helped to enhance the immune system of Tiger grouper.

Similar trend was also observed in the percentage of monocyte when 1 g/20 L of AquaHerb was used to induced tiger grouper defence system. Table 3 also indicated that the 24 h of immersion produced the highest monocyte after immersion and challenged with pathogen ($P<0.05$). The increase of monocyte during the infection will facilitate and strengthen the monocytes ability to destroy bacteria [25]. Monocytes play an important role in non-specific immunity and the inflammatory response as the precursors of macrophage. After being activated, macrophage will had stronger phagocyte activity. However, this ability are still lower than granulocyte [26]. The increasing number of monocytes was also triggered by the pathogen infection. Therefore, as shown in Table 3, the number of monocytes is higher after *V. parahaemolyticus* infection compared to the monocytes number after AquaHerb immersion. In addition, the number of monocytes in the control treatment was also increased post infection. However, in general, this monocytes number are still lower in the control groups in comparison to the herbal-based conditioners therapy.

In the present study, the percentage of neutrophile was higher at 24 h of post immersion and challenged in the herbal-based conditioner group ($P<0.05$). Moreover, the percentage of neutrophile was also increased in the control treatment post infection. Although in general, the number is still lower compared to therapy group. Corroboration for our results comes from the work of Nathan (2006) who have reported that the neutrophile was increased during bacterial infection and act as first innate immune cells which migrate to the infection site to destroy the foreign material through the phagocytic process.

Besides activating the immune system of tiger grouper, AquaHerb also slightly affected the water quality during the immersion period. The extended period of therapy influenced the increase of water pH as well as other parameters such as nitrite (NO_2), nitrate (NO_3), ammonia (NH_3) and phosphate (PO_4) that dissolved in the water. Interestingly, the water pH of fish without any medication has a downward trend and getting worse at 24 hours without water exchange. Gradual changes in pH are stressful to fish and might be the main reason for loss of appetite and low survival percentage in the control group, especially for 24 h without any exchange of water. Moreover, the increasing level of NO_2, NO_3, NH_3 and PO_4 caused by herbal medication also effect the appetite, but the mortality level are still lower compared to non-treated tank. From the experiment observation, after herbal medication, fish seemed to feel very hungry and consume a lot of feed at the beginning of feeding period. In contrast, although fasted for the same time, control fish did not have similar response to the administration of feed. Moreover, non-treated tank has a pungent odor due to the excessive release of mucus during treatment as the impact of stress condition.

Today, the application of herbal in aquaculture have an important role to control the fish disease and have produced satisfactory results [27]. This was possible because herbal as a natural products have the potential to be a rich source of active substances for immunomodulation [28]. Traditional medicines are not only safe for consumers but also has been used for generations as an alternative medicine for human medication. As an herbal, mangrove extract have an ability to control the bacterial, fungal and viral diseases. Traditionally, they have been used to treat various diseases for centuries [29] and swamps are filled with "herbal-broth", well known 'recuperation house' for sick fish.

The beneficial effects of these commercially available herbal Bio conditioners (AquaHerb) was also strengthened by the presence of dried peat, dried Leaves Saponin and dried roots tannin in their composition. Even saponin have been reported to have damaging effect on the fish respiratory epithelia [30], but in low concentration, saponin based adjuvants have the unique ability to stimulate the cell-mediated immune system and to enhance the antibody production [31]. This is confirmed by Chavali [32] reported that saponins either as crude mixtures or as a purified compounds was able to increase the immune cell proliferation. Moreover, the administration of saponin to the infected fish have been found to have a detrimental effect to protozoa [33], mainly due to the virucidal activity [34] and antifungal activity [35, 36] contained in their active substance. Our results are in accordance with the recent findings that indicated the prior administration of saponin contained in the AquaHerb before challenged with *V. parahaemolyticus* was able to enhance the tiger grouper immune system in comparison to control. In addition to saponin, the use of dried tannin contained in AquaHerb was also useful for the prevention of piscine diseases and off-flavor in fish [37] and did

not harm the productive performance [38]. Moreover, based on the field observation, the complementary of tannin and saponin will enhance their immune system and the appetite of fish after medication. However, all this benefits effects of this active substances will be only obtained if given in the appropriate dose.

5. Conclusion

Provision of Herbal Bio conditioners (AquaHerb) can enhance the survival percentage and hematological parameter between total leukocytes, monocytes, neutrophile, limphocyte of tiger grouper. An immersion of 1 g/20 L AquaHerb for 24 h was able to provide a better survival (%) and better immune response in comparison to 6 and 12 h of AquaHerb immersion. It is suggested to conduct further research by performed an analysis at molecular and protein level for several immune related genes of tiger grouper fish as the effect of AquaHerb immersion.

6. Acknowledgement

This research was financially supported by Batam Mariculture Development Centre through engineering fund for 2013. The author would like to thank to all colleagues in Batam: Sri Agustatik, Hendrianto, Ahmad Zaeni, Antin Sri Lestari and Anggit.

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