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Preventive and Curative Effects of Phyllanthus niruri Allium sativum Combination on Tiger Grouper Epinephelus fuscoguttatus Infected by Vibrio alginolyticus

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Abstract

The aim of this study was to evaluate the preventive and curative effects of supplementation of the combination of stone-breaker (*Phyllanthus niruri*) and garlic (*Allium sativum*) through the feed to control bacterial diseases on tiger grouper juvenile (*Epinephelus fuscoguttatus*) caused by *Vibrio alginolyticus*. The extract was prepared in five combination doses with two replications: 15+20, 15+25, 20+20, 20+25 and 25+20 g L^{-1}. These combination doses were tested at *in vitro* test to observe the antibacterial activity of medicinal plants against *V. alginolyticus* (10^4 CFU mL^{-1}, 0.1 mL). The *in vivo* test was conducted using CRD (completely randomized design) consisting of four treatments and three replications; negative control, positive control, preventive, and curative treatment. Tiger grouper juveniles (length 7.74±0.27 cm; weight 12.50±0.394 g) were reared in aquariums (60x30x30 cm^3) with a stocking density of 6 fish per aquarium. The fish were fed with the experimental feed using at satiation method three times a day.

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The challenge test was conducted by injecting 0.1 ml the suspension of *V. alginolyticus* (10^4 CFU mL^{-1}) to the fish (positive control, preventive and curative treatment), while the fish in negative control were injected with PBS (phosphate buffer saline). The combination of stone-breaker and garlic could inhibit the growth of *V. alginolyticus* with the largest inhibition zone obtained in a combination dose of 20+25 g L^{-1} (12.33 mm). The combination of stone-breaker and garlic has proven to play the role as the preventive and curative agent to control *V. alginolyticus* infection on tiger grouper juveniles with better survival rate values than positive control.

Keywords: Vibrio alginolyticus; Phyllanthus niruri; Allium sativum; tiger grouper.

1. Introduction

Groupers are the high value marine fish, especially in Southeast Asia, including Indonesia, Malaysia, Thailand, and Philippines [1]. One of major grouper species is the tiger grouper (*Epinephelus fuscoguttatus*). It is greatly valued because of its high quality flesh and high market price. This fish is one of the most exploited species in the live fish trade that makes it to be highly demanded. In order to alleviate the pressure on wild stocks, many countries have promoted aquaculture in the hopes of producing a more sustainable tiger grouper yield [2]. On the other hand, the cultivation of the tiger grouper has faced a critical problem caused by disease outbreak. Among the pathogens, *Vibrio* is the well-known genus causing serious problems in aquaculture. *Vibrio parahaemolyticus* and *V. alginolyticus* are the species which can cause hemorrhagic septicemia, especially in nursery and grow-out cage systems of groupers [3]. Furthermore, *V. alginolyticus* has been reported to be the causative agent of high mortality outbreaks related to abdominal swelling in larvae of several fish species [4, 5, 6]. This pathogen is able to produce extracellular toxic products which are dangerous for the specific organs of the tiger grouper, such as intestine and kidney. Hemolysin is one of several toxin secreted by *V. alginolyticus* which damages red blood cells and leads to physical injuries in mouth and fin tissues [7].

Chemotherapy methods such as the use of antibiotics and chemical materials are quite popular to treat or prevent the bacterial infections, but the long-term use of chemotherapeutic agents can lead to the development of drug-resistant strains [8, 9, 10], allergy and toxicity in human [10, 11] because of the accumulation in the fish products. Therefore, the prophylactic protocols, such as the use of medicinal plants and an immunostimulant for disease control are more preferable.

Stone-breaker (*Phyllanthus niruri*) and garlic (*Allium sativum*) are medicinal plants which have been used in several studies because of their antibacterial and immunostimulant properties. Flavonoids found in stone-breaker were indicated to have the potency as antimicrobial, immunostimulant, antioxidant, anticarcinogenic, hepatoprotectant, and anti-allergic molecules [12]. Garlic contains anti-bacterial molecules derived from allicin. Garlic has the ability to increase the welfare of fish and control the growth of pathogens, especially bacteria and fungi [13]. The combination of stone-breaker and garlic supplemented to the feed was effective to prevent bacterial infections and reduced the mortality rate on several aquatic organisms infected by bacterial disease. The feed mixed with stone-breaker and garlic given for 14 days resulted in 73.33% of survival rate in catfish challenged by *Aeromonas hydrophila* [14] and 83.33% of survival rate in Nile tilapia challenged by *Streptococcus agalactiae* [15]. Besides having the function as the preventive agents, medicinal plants also have

curative properties due to the presence of various complex chemical substances, which are found as secondary plant metabolites [16]. The use of medicinal plants combination as the preventive and curative agent for bacterial infection has not been widely studied, especially for the disease caused by *V. alginolyticus* infecting tiger grouper. This study was performed to evaluate the preventive and curative effects of the supplementation of the combination of stone-breaker and garlic into the fish feed to control the bacterial disease caused by *V. alginolyticus* infecting tiger grouper

2. Materials and Methods

2.1 Identification of the Experimental Bacteria

The bacterial isolate used in this study was obtained from the culture collection of Brackish Water Culture Center, Situbondo, East Java, Indonesia, which was identified as *V. alginolyticus*. It was re-cultured on SWC (sea water complete) slant agar and was incubated at 28 °C for 24 hours. The isolate obtained was then re-identified to ensure that there was no contaminant in the isolate. The re-identification process was conducted through the physiological characterization and the biochemical test consisting of Gram staining, oxidative/fermentative test, motility test, catalase test, and oxidase test [17]. After it was confirmed that the isolate was *V. alginolyticus*, the isolate was then rejuvenated on SWC slant agar.

2.2 Virulence Enhancement of Experimental Bacteria

Before used in the next experimental steps, the experimental bacteria virulence was enhanced by the Koch's postulate method. Bacterial cells were inoculated into SWC broth (25 mL) and the bacterial suspension was incubated in water-bath shaker (28 ^{o}C; 150 rpm) for 24 hours. The bacterial suspension was then rinsed twice with PBS (phosphate buffer saline) solution. The suspension was injected to tiger grouper with a dose of 0.2 mL per fish using syringe via intraperitoneal route. The fish with clinical symptoms (hemorrhage on the mouth or reddish fins) were dissected to be isolated on TCBS (thiosulfate citrate bile-salt sucrose) plate agar. The plate was incubated at 28 °C for 24 hours. Each bacterial colony that grew on TCBS plate agar was re-isolated in SWC slant agar to obtain pure bacterial isolate. The isolate was then identified following the method by [17].

2.3 Determination of LD_{50} (Lethal $Dose_{50}$)

Tiger groupers were stocked randomly into six aquariums (10 fish per aquarium). The experimental bacterial suspension (10^5, 10^4 and 10^3 CFU mL^{-1}) was injected via intraperitoneal route. The observation of the number of alive and dead fish was conducted for seven days. The LD_{50} was determined following the method by [18]. The LD_{50} dose obtained in this study was 10^4 CFU mL^{-1}.

2.4 Production of Stone-breaker and Garlic Extract

The stone-breaker and garlic powder used in this study were obtained from the Research Center of Medicinal and Aromatic Plants, Bogor, West Java, Indonesia. The stone-breaker extract was produced by dissolving the stone-breaker powder in sterile distilled water and boiling it at 90 °C for 15 minutes. Garlic extract was

produced by dissolving garlic powder in sterile distilled water [15].

2.5 In Vitro Test

The antibacterial activity of the combination extract was observe using Kirby-Bauer method [19]. The stone-breaker+garlic extract was prepared in five combination doses: 15+20, 15+25, 20+20, 20+25 and 25+20 g L^{-1}, respectively. Each treatments were replicated twice. The bacterial suspension (0.1 mL) with LD_{50}-dose was spread onto SWC plate agar. Paper discs (d=0.5cm) were immersed in the extract for 5 minutes and were then placed on SWC agar. The plate was incubated at 28 ^{o}C for 24 hours. Inhibition zones formed around the paper discs were measured using a ruler (accuracy ± 1 mm). The combination dose that produced the largest inhibition zone would be used for *in vivo* test.

2.6 Preparation of Experimental Feed

The commercial feed with 45.11% protein content was used as the experimental feed material. The commercial feed was mixed and was repelleted with the stone-breaker and garlic extract. The experimental feed was divided into three groups; control (without any supplementation), preventive (the commercial feed supplemented with stone-breaker+garlic extract 20+25 g kg^{-1} of feed, based on the result of *in vitro* test), and curative (the commercial feed supplemented with stone-breaker+garlic extract 40+50 g kg^{-1} of feed). The dose for curative treatment was twice higher than preventive treatment [20]. All feed groups were also supplemented with 0.1% vitamin C.

2.7 Preparation of Experimental Fish and Medium

The experimental fish used in this study were tiger grouper juveniles (average weight: 12.50±0.39 g; average length: 7.74±0.27 cm) obtained from Kepulauan Seribu, Jakarta, Indonesia. The fish were acclimatized in the experimental environment for 1-2 weeks. The fish were then stocked randomly into aquariums (60x30x30 cm^3) with a stocking density of 6 fish per aquarium. The aquariums were set with a single recirculation system. Water quality parameters were monitored every day and were maintained within the following ranges: dissolve oxygen at 4.5-8.4 mg L^{-1}, ammonia content at 0.02-0.09 mg L^{-1}, pH at 7.72-8.30, salinity at 33-36 ppt, and temperature at 30-34 °C.

2.8 In Vivo Test

The experimental feed was given using at satiation method three times a day (morning, noon, and afternoon). This test was conducted using completely randomized design (CRD) consisting of four treatments with three replications as follows:

Negative control : The fish were fed with control feed for 14 days. The fish were then injected with PBS (0.1 mL per fish) via intraperitoneal route. The fish were fed with control feed for 7 days after injection.

Positive control : The fish were fed with control feed for 14 days. The fish were then injected with the

suspension of *V. alginolyticus* (10^4 CFU mL^{-1}; 0.1 ml per fish) via intraperitoneal route. The fish were fed with control feed for 7 days after injection.

Preventive: The fish were fed with preventive feed for 14 days. The fish were then injected with the suspension of *V. alginolyticus* (10^4 CFU mL^{-1}; 0.1 ml per fish) via intraperitoneal route. The fish were fed with control feed for 7 days after injection.

Curative : The fish were fed with control feed for 14 days. The fish were then injected with the suspension of *V. alginolyticus* (10^4 CFU mL^{-1}; 0.1 ml per fish) via intraperitoneal route. The fish were fed with curative feed for 7 days after injection.

Paramaters observed in the *in vivo* test were feeding response, survival rate, changes in external and internal organs. The feeding response was observed daily by comparing the total of feed eaten with the biomass of the fish. The survival rate was calculated daily and at the end of *in vivo* test. Changes in the morphology of external and internal organs were observed at the end of *in vivo* test. All data were collected for analysis.

2.9 Data Analysis

The collected data were analyzed by analysis of variance (ANOVA) and Duncan's test to determine significant differences among treatments using SPSS 16. The inhibition zone diameter, feeding response and survival rate were analyzed by quantitative statistics, while the morphology of external and internal organs was described qualitatively.

3. Results

The combination of stone-breaker and garlic showed the largest inhibition zone (12.33 mm) at a combination dose of 20+25 g L^{-1}. This dose showed a significant difference ($P<0.05$) among other combination doses (Table 1).

Table 1: The diameter (mm) of inhibition zone formed by stone-breaker+garlic at different combination doses against *Vibrio alginolyticus*

Code	Doses of stone-breaker+garlic (g L^{-1})	Replication			Average
		1	2	3	
A	15+20	7	7	7.6	7.20^{a}
B	15+25	8	8	8.5	8.17^{b}
C	20+20	7	7.5	7.8	7.43^{ab}
D	20+25	12	13	12	12.33^{d}
E	25+20	10	11	10	10.33^{c}

Different superscript letters indicate significant difference results ($P<0.05$)

The feeding response showed similar and stable values on day 1-14 (before the challenge test). The feeding response values declined after the bacterial injection and began to increase on day 2 after the bacterial injection, with the highest value obtained in negative control, followed by the preventive treatment. The lowest value of feeding response was obtained in positive control (Figure 1).

The injection of *V. alginolyticus* suspension to tiger grouper juveniles caused the significant decrease in the survival rate of the fish on day 2-5 after the challenge test. Furthermore, the survival rate of tiger grouper juveniles tended to be stable on day 6-7 after the challenge test (Figure 2). The oral supplementation of stone-breaker and garlic to tiger grouper juveniles showed significant effects ($P<0.05$) on the survival rate of the fish at the end of the challenge test. The highest survival rate (100±0%) was found in negative control, followed by preventive, curative, and positive control: 83.33±16.67%, 61.11±9.62%, and 38.89±9.62%, respectively (Figure 3).

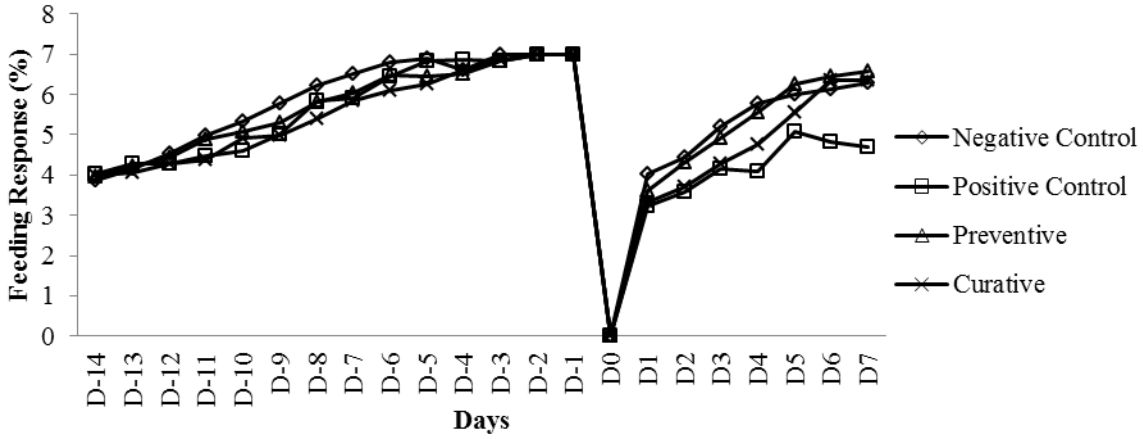


Figure 1: The feeding response during *in vivo* test

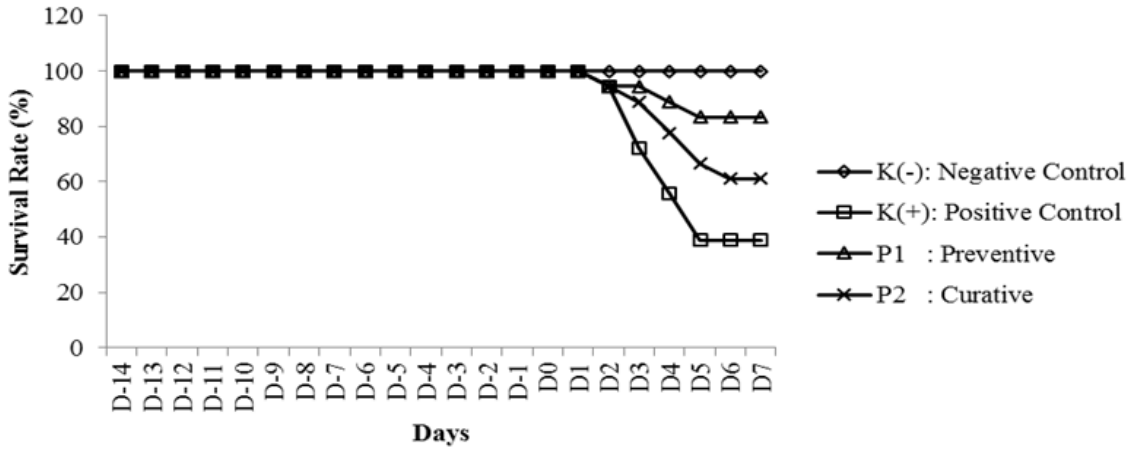


Figure 2: The daily survival rate of tiger grouper (*Epinephelus fuscoguttatus*) juveniles during *in vivo* test

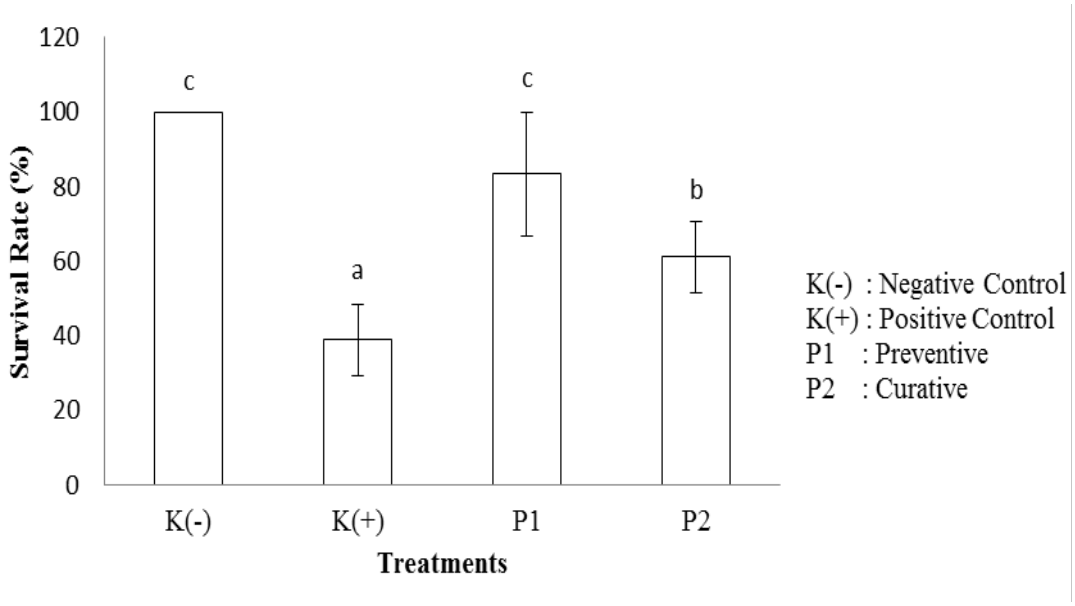


Figure 3: The survival rate of tiger grouper (*Epinephelus fuscoguttatus*) juveniles after the challenge test with *Vibrio alginolyticus*. Different superscript letters on each bar indicate significant different results (P<0.05)

The infected fish showed changes in external organs, such as hemorrhage on pectoral fin and under the mouth, ulcers on pectoral fins; the base of caudal fin and the body; rotten dorsal, anal, and caudal fins (Figure 4). The infected fish also showed dis-colorization and inflammation in the internal organs (Table 2) with changes in the form and the color (Table 3).

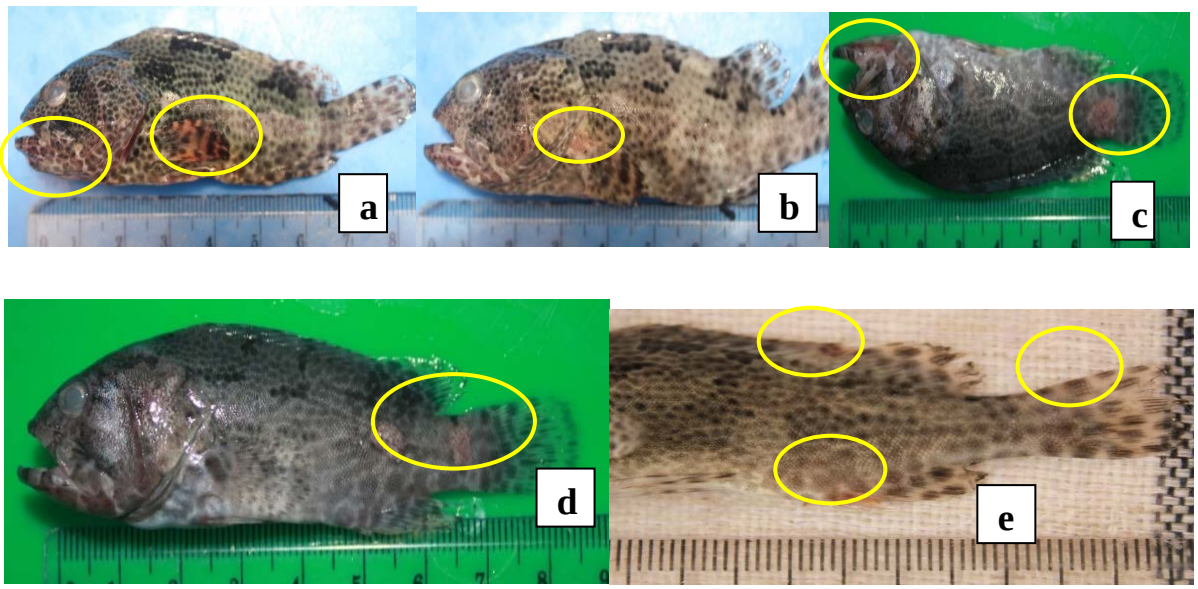


Figure 4: Changes on external organs (marked by yellow circles) of the tiger grouper (*Epinephelus fuscoguttatus*) juvenile after the challenge test with *Vibrio alginolyticus*; a. Hemorrhage on the pectoral fin and under the mouth, b. Ulcers on pectoral fins, c. Ulcers on the base of caudal fin and hemorrhage under the mouth, d. Ulcers on the body and the base of caudal fin, e. Rotten dorsal, anal, and caudal fins

Table 2: The internal organs profile of tiger grouper (*Epinephelus fuscoguttatus*) juvenile in the end of the challenge test

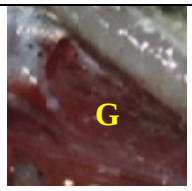
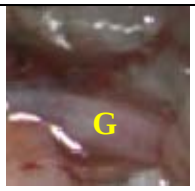
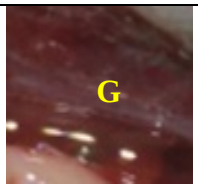
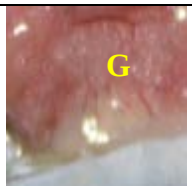
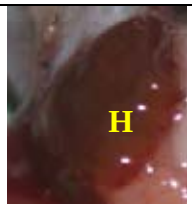
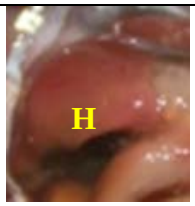
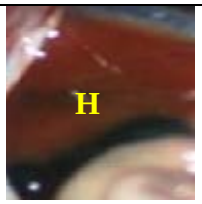
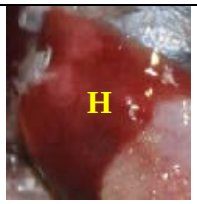
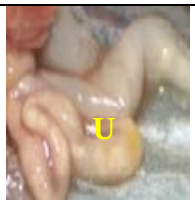
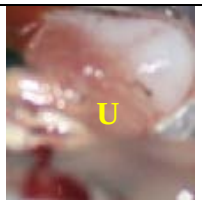
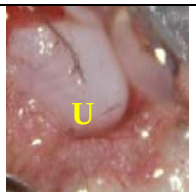
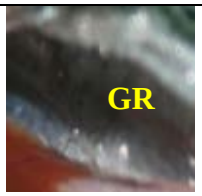
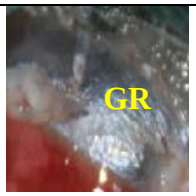
Internal Organ	Negative Control	Positive Control	Preventive	Curative
Kidney (G)				
Liver (H)				
Intestine (U)				
Swim Bladder (GR)				

Table 3: Changes in internal organs of tiger grouper (*Epinephelus fuscoguttatus*) juvenile in the end of the challenge test

Internal Organ	Form/Color	Negative Control	Positive Control	Preventive	Curative
Kidney (G)	Form Color	Normal Red-brown	Normal Pale pink	Normal Red	Inflammation Pink
Liver (H)	Form Color	Normal Red-brown	Smaller size Pale pink	Normal Red-brown	Enlarged size Red-brown
Intestine (U)	Form Color	Normal Pink	Smaller size Pale pink with yellow liquid	Normal Pink	Normal Pink
Swim Bladder (GR)	Form Color	Normal Transparent white	Normal Transparent white	Normal Transparent white	Inflammation Transparent white

4. Discussion

The result of *in vitro* test showed that the combination of garlic and stone-breaker produced an inhibition zone towards *V. alginolyticus* with an optimum dose of 20+25 g L^{-1}. This indicates that the inhibition of the growth of *V. alginolyticus* requires a higher dose of garlic than stone-breaker, as stated by [21] that a higher level of allicin derived from garlic are more favorable for medicinal applications. Allicin is an organosulphur compound contained in garlic extract [22]. The report by [23] stated that allicin was able to inhibit the synthesis of RNA and lipid, followed by the inhibition of the protein synthesis and the formation of bacterial cell wall. The stone-breaker contains the most promising secondary metabolites, such as alkaloids, flavonoids, and tannins [24], which are known to have the medicinal activity [25].

The supplementation of garlic and stone-breaker also affected the feeding response. Garlic is popular as a growth promoter [26, 27] and also affects feed intake [28]. This was indicated by the value of the feeding response on the supplemented fish that was higher than positive control. Furthermore, the values of feeding response were decreased after the injection supposed to be caused by the stress after the injection called as anorexia [29].

The supplementation of garlic and stone-breaker also affected the survival rate of tiger grouper juveniles. There was no mortality before the challenge test. This result indicated that the supplementation of garlic and stone-breaker was safe for the fish and had no toxic effect. However, there were significant changes on the survival rate after the challenge test. The highest survival rate after the infection was obtained in the preventive treatment, followed by the curative treatment and positive control. The study by [30] reported that garlic has a function as an immunostimulant, which can boost immune system to prevent pathogen infections. Generally, garlic facilitates the function of phagocytic cells and increases their bactericidal activities. The study by [31] verified that the addition of garlic to fish diets increased the erythrocytes count, hemoglobin content, hematocrit, leukocytes count, and thrombocytes count. The increase in leukocytes count will accelerate phagocytosis by activating macrophages [32]. Garlic can also stimulate natural killer cells, complement, lysozyme, and the antibody response of the fish. The activation of the immunological function is associated with the increase in the protection against infectious disease. To increase its medical function, garlic can be supplemented to the fish in the combination with other medicinal plants, because garlic can work synergistically with other medicinal plants [30]. The combination between garlic and stone-breaker was expected to have the preventive function against *V. alginolyticus*. The contents of stone-breaker are able to stimulate the activity of immune cells for a better performance [33]. Stone-breaker contains alkaloid, terpenoids, steroids, tannin and flavonoid which are responsible for the strong antibacterial activities [24, 34]. Stone-breaker also contains saponin which can be used to stop bleeding in treating of wound and ulcer, because it can help in red blood cell coagulation. Besides having a disease preventive effect, medicinal plants also have a curative effect. According to [20], the optimal dose for curative treatment is twice higher than preventive treatment. The survival rate of the fish treated with the curative feed was lower than the preventive treatment, but the supplementation of these medicinal plants as curative agents is still possible, because the survival rate of curative treatment was significantly higher than positive control. The lower survival in the curative treatment was asummed to be caused by the decrease of the feed intake after the pathogen injection. Based on the result of daily survival rate, it could be known that the

mortality of tiger grouper juveniles occurred on day 2-5 after the challenge test. This result is in line with the previous study by [35] who reported that the mortality caused by the infection of *V. alginolyticus* on groupers occurred on day 1-4 after the challenge test.

After the challenge test was conducted, the experimental fish in positive control, preventive, and curative treatment showed several clinical symptoms or even the mortality. The clinical symptoms appeared on the external organs of the infected fish were hemorrhage, ulcers, rotten or damaged fins. The findings by [36] verified that the natural disease caused by *V. alginolyticus* led to the following symptoms: septicemia, hemorrhage, dark skin, and ulcers on the skin surface. The infection of *V. alginolyticus* caused the color and morphological changes on the internal organs of the infected fish compared with the normal fish in negative control. Normal internal organs have several characteristics: the normal size of liver and kidneys, the red-brown liver, the normal size of intestines and swim bladder. The abnormalities of internal organs were found in the positive control fish, such as the pink kidneys, a smaller pale-pink liver containing yellow fluid, and pale intestines. Internally, the infected fish accumulated fluid in the peritoneal cavity and expressed some cases of hemorrhagic in livers [37, 38]. The study by [39] also reported the similar abnormalities in the infected fish, such as the pale liver, enlarged kidneys, damage intestines, and yellow fluid in the body cavity. Those damages are caused by the release of proteinases, proteases, and other extra-cellular enzymes produced by bacteria [40, 41]. These hydrolytic enzymes have been considered to be the major virulence factors, which are responsible for several symptoms displayed by the infected fish. However, other supplementation methods, such as immersion and injection should be studied in the next studies to complete the information in the use of the combination of garlic and stone-breaker.

5. Conclusion

The combination of stone-breaker and garlic could inhibit the growth of *V. alginolyticus.* The largest inhibition zone was obtained in a dose of 20+25 g L^{-1} (12.33 mm). The combination of stone-breaker and garlic has proven to play the role as the preventive and curative agent to control *V. alginolyticus* infection on tiger grouper juveniles. The preventive and curative treatment showed better survival rate values than positive control.

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