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Treatment of *Chlorella* sp. extract on heat shock cluster (HSC) response from the tissue and bloodcells proliferation of *Epinephelus fuscoguttatus-lanceolatus* infected by Viral Nervous Necrosis

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Abstract. VNN infection can trigger an immune response, inflammation in fish cells and tissues such as Heat Shock Cluster (HSC). The objective of this study was to determine the presence of HSC responses in Cantang groupers (*E. fuscoguttatus-lanceolatus*) that infected by VNN and treated with *Chlorella* sp extract. The methods of the study included the preparation of *Chlorella* sp extract, infecting fish with VNN, testing the comparison among the treatments. The treatment of *C. vulgaris* was (K): grouper without treatment with both *C. vulgaris* and VNN, (V): treatment of VNN without *C. vulgaris*, (A1): treatment of *C. vulgaris* 17 g/ml, (A2): treatment of *C. vulgaris* 33 g/ml, (A3) treatment of *C. vulgaris* 50 g/ml, (AV1): treatment of *C. vulgaris* 17 g/ml and VNN, (AV2): treatment of *C. vulgaris* 33 g/ml and VNN, (AV3): treatment *C. vulgaris* 50 g/ml and VNN. The results analysis included blood cell and tissue observation using immunohistochemistry and immunoratio, and confirmation of HSC assay results with inhibition test of grouper cell blood labeled with anti-mouse antibody IgG, and secondary antibody IgG anti-mouse SA-HRP. The results showed that *Chlorella* sp extract administered to VNN-infected grouper and fish without VNN infection, significantly increase the HSC response.

1. Introduction

Viral Nervous Necrosis (VNN) disease is a serious problem of marine fish cultivation, especially grouper it can cause 50-100% death in larvae aged 10-20 days. Viruses that cause VNN generally infect the larval stage to juvenile and attack the eye and brain nervous system that was characterized by vacuolation, with symptoms that are quite specific because the fish shows abnormal swimming behavior and is still at the bottom of the tub. The symptoms that arise are with fish spinning or whirling, sleeping dead or fish are at the bottom like dead [1].

VNN infection can trigger inflammation in fish tissues. Inflammation is an important response given by the immune system that will ensure survival during infection and tissue injury, where this inflammatory response is very important for maintaining normal tissue homeostasis [2]. Inflammatory control caused by a virus derived from natural ingredients has not been widely developed. One of them is the use of bioactive compounds of microalgae *C. vulgaris*.

The general contents of *C. vulgaris* indicate in the crude extract. The crude extract is a constituent component of *C. vulgaris* that is a protein and pigment in the form of RNA or enzyme. Crude extracts can be developed and applied as an anti-inflammatory in groupers [3]. Crude extracts can suppress



inflammation that occurs in tissues when VNN infection occurs. Inflammatory reactions can also be marked by the expression of HSP [4]. HSP is a type of HSC that naturally present in fish bodies which are physiologically a means of adaptation for fish to deal with stress [5]. This study was conducted to determine the activity of crude extracts of marine microalgae *C. vulgaris* against HSC include a type of HSC like Heat Shock Protein (HSP) as anti-inflammatory due to the infection of VNN in Cantang grouper (*Epinephelus fuscoguttatus-lanceolatus*).

2. Methods

This research was conducted with experimental methods including:

2.1. *C. vulgaris* extraction

100 grams of *C. vulgaris* powder was macerated using 500 ml methanol 96% with a ratio of 1:5 for 2x24 hours while stirring. After the maceration, the solution is separated from the pulp by filtering it using a filter paper (Whatman No. 1), then the pulp was demobilized 2 times. Maceration results were concentrated by evaporating the solvent using a low-pressure vacuum rotary evaporator at 40°C, 60 rpm, and a pressure of 200 mBar until no more solvent dripped. Furthermore, the extract was weighed to determine the yield of the extract, as follows equation (1).

$$\text{Yield (\%)} = \frac{\text{rough extract (g)}}{\text{sample (g)}} \times 100\% \dots\dots\dots (1)$$

2.2. Phytochemical screening test

Preparation of test solutions for phytochemical screening (alkaloids, saponins, tannins, steroids, triterpenoids, glycosides) was conducted by dissolving microalgae extracts from *C. vulgaris* in methanol with a ratio of 1: 10. Alkaloid measurement was conducted by reacting the test solution to Dragendroff reagent and the test solution to Mayer reagent. The formation of orange deposits in the reaction with Dragendroff reagent and yellow precipitate in the reaction with Meyer's reagent showed the presence of alkaloids.

Saponin examination is done by observing foam formation after shaking. Foam as high as 1 -10 cm which is stable for not less than 10 minutes and not lost in the addition of 1 drop of 2N HCl indicates the presence of saponins [6]. Tannin examination was carried out by reacting 3 ml of the test solution to 5 drops of 1% NaCl and 3 drops of gelatin solution. If the precipitate is formed, the positive extract solution contains tannin [7]. Teroid and triterpenoid examinations are carried out by Lieberman Burchard's reaction. The formation of a brownish or violet ring on the boundary of the solution indicates the presence of triterpenoids, whereas if it appears a greenish blue ring shows the presence of steroids [8]. Examination of flavonoids against microalgae ethanol extracts was conducted by Taubeck test. The yellow fluorescent solution under 366 nm UV, intensely indicates the presence of flavonoids [6].

2.3. Uv-Vis (Ultraviolet Visible) spectrophotometer analysis

UV-Vis spectrophotometer was conducted by measuring the absorbance wavelength spectra of ultraviolet radiation the best fraction of *C. vulgaris* extract with a 1 nm resolution spectrophotometer (from 200-800 nm) in a normal quartz bowl with a trace length. The best fraction of column chromatography taken 3 ml was put into the cuvette, then scanning it with a wavelength of 200 nm.

2.4. Acclimatization of Cantang grouper

The animal model for analysis was the Cantang grouper fish with size from 13 to 15 cm, provided from UD. Giso Bangkit Situbondo. The newly arrived grouper fish seed was not directly fed, because it requires adaptation to the new maintenance media. Then the fish is fasted first so that its appetite was maintained. The feed was given after the fish looks healthy and aggressive. The feed used in the form of fresh mackerel was chopped up to a small size adjusted to the opening of the fish's mouth. Feeding is carried out at 2-3 days after the fish first entered. The feed was given ad libitum that is feeding little by little until the fish is full. The purpose of ad libitum feeding is to avoid the deposition of uneaten

food residues at the bottom of the tub. The aquarium will experience a decrease in water quality. Research treatments, include: K treatment (Control, without administration of VNN and *C.vulgaris*); V treatment (VNN without *C.vulgaris*); A1 treatment (*C.vulgaris* 17 µg/ml); A2 treatment (*C.vulgaris* 33 µg/ml); A3 treatment (*C.vulgaris* 50 µg/ml); AV1 treatment (*C.vulgaris* 17 µg/ml + VNN); AV2 treatment (*C.vulgaris* 33 µg/ml + VNN); AV3 treatment (*C.vulgaris* 17 µg/ml + VNN).

2.5. In-Vivo test of *C.vulgaris* crude extracts on Cantang grouper

In-vivo tests were conducted by the oral method (Sonde) with the help of feeding tube hose which was conducted for 3 times, namely on the 0, 5th and 10th days. The treatment doses [9], namely by giving 33 µg/mL (clinical trial results). In the study that will be conducted using different doses, namely by giving doses of 17 µg/mL, 33 µg/mL, and 50 µg/mL. The dosage of crude extract given is different to each round, by calculating the stock dosage of crude extract that had been obtained from spectrophotometric results compared to the weight of the fish. The application of crude extracts is adjusted to the fish's body weight. This dose calculation was done to get optimal results in conducting research. Fish that has been nurtured and treated are dissected to analyze their organs, namely Cantang grouper fish eye organs.

2.6. In-Vivo VNN test on Cantang grouper

The in-vivo test of VNN was conducted on the 5th and 10th days. VNN treatment was conducted by cutting small pieces of fish that have been infected with VNN as much as 5 grams per fish and given orally. Then observations are made, this observation aims to see changes in fish behavior from normal to abnormal or specific symptoms such as irregular swimming.

2.7. Hematological analysis (Erythrocytes, Leukocytes, Hematocrit, Hemoglobin)

The total of erythrocytes was calculated by equation (2) [10]:

$$\sum \text{Erythrocyte} = \text{Mean of counted erythrocyte cells} \times \frac{\text{diluent}}{\text{volume}} \dots\dots\dots (2)$$

The total of Leukocytes was calculated by equation (3) [10]:

$$\sum \text{Leukocyte} = \text{Mean of counted leukocyte cells} \times \frac{\text{diluent}}{\text{volume}} \dots\dots\dots (3)$$

2.8. Determine of hematocrit value

The hematocrit values were calculated by the microhematocrit method. Bertarine microhematocrit is inserted into collected blood samples until the blood fills up to about three-quarters (3/4) of the capillary pipe. In addition, one end of the capillary pipe is clogged by plugging it in the clogging wax. Then centrifuged for 5 minutes using a microhematocrit centrifuge with a speed of 1,500 rpm. Besides that, it is read by using a hematocrit reader and the results are expressed in% [11].

2.9. Determine of hemoglobin value

Hemoglobin value can be calculated by the following formula. The measurement of hemoglobin value is by the way the sample blood is sucked with a Sahli pipette up to a scale of 20 mm³ or on a scale of 0.2 ml. Then the tip of the pipette is cleaned with tissue paper. The blood in the pipette is transferred to a Hb-meter tube that has been filled with 0.1 N HCl to a scale of 10 (red). The blood is then stirred with a stirring rod for 3-5 minutes. The distilled water is added to the tube until the color of the blood is like the color of the standard solution in the Hb-meter. The hemoglobin scale can be seen on the gr% (yellow) path scale. The amount of hemoglobin in grams per 100 ml of blood [12].

2.10. Immunohistochemical analysis

The process of water withdrawal from the network (dehydration) is carried out using alcohol with concentrations ranging from 80% to 100% and purified with xylol (clearing) before being planted in paraffin (embedding). The network in paraffin blocks was sliced serially using a rotary microtome with a thickness of 5 µm, attached to an object glass coated with 70% alcohol or 0.2% Neofren® in

toluene, then stored in a 40°C incubator for 24 hours. The preparation is then colored with various coloring procedures according to the purpose.

Immunohistochemistry staining has 3 steps that must be done, namely the preparation of glass objects used for attaching preparations or histological preparations, making neufren (attaching agent) to help process the preparation of the glass into the object and the immunohistochemical staining procedure itself. Immunohistochemistry staining involves several stages of preparation, including preparation of glass objects, coating from glass objects of neufren (attaching agent), attachment of sliced preparations to glass objects and immunohistochemical staining procedures themselves.

In immunohistochemistry staining, a positive reaction is indicated by the appearance of brown color in the cell that has specificity with the primary antibody used. The primary antibody used for immunohistochemistry staining is the anti-HSP fish primary antibody.

3. Results and discussion

3.1. Phytochemical analysis

In testing phytochemical compounds, the results of the reading of color stains appear on silica gel. The results of the positive compounds only 3 of the 5 compounds tested, namely alkaloids, terpenoids, and tannins. This negative compound contained are flavonoid and saponin compounds.

Table 1. Phytochemical analysis results.

Phytochemical compound	Reactor	Results	Annotation	Phytochemical analysis
Flavonoid	Concentrated HCL + Mg	No color	- (Negative)	Flavonoid
Alkaloid	Bouchardat	Brown precipitation color	+ (Positive)	Alkaloid
Terpenoid	Bouchardat	Brownish orange precipitation color	+ (Positive)	Terpenoid
Tannin	FeCl ₃ 1%	Black brown color	+ (Positive)	Tannin
Saponin	Water+ Concentrated HCL	No permanent foam	- (Negative)	Saponin

This is different from other studies which stated the phytochemical test results of *C.vulgaris* extracts contained flavonoid and saponin group compounds [13]. Differences in content in the same species can occur. The content of phytochemical compounds is influenced by various factors, namely species, varieties, conditions of growth, season variation, processing and storage methods [14].

3.2. Uv-Vis Spectroscopic analysis results

The results of Uv-Vis spectroscopic analysis was shown in Table 2.

Table 2. Uv-Vis spectrophotometer analysis results of *C.vulgaris* extract.

Wavelength (nm)	Absorbance	Description
663.1	0.346	Chlorophyll
423.0	0.690	Chlorophyll
341.0	0.387	Tannin
232.0	1.021	Alkaloid
207.0	3.421	Terpenoid

There is a wavelength that appears at 663.1 nm which is thought to be identified as a chlorophyll a compounds. Maximum absorption by chlorophyll an occurs to two wavelength peaks at 430 and 660 nm [15]. At a wavelength of 423.0 nm, it is also suspected chlorophyll pigment compounds, this is described by [16] that the chlorophyll pigment has a maximum wavelength in the blue area of 423 nm.

In the picture above there is also a wavelength of 341 nm, the wavelength is thought to be the wavelength of the tannin compound. In the UV-Vis test, tannin compounds appear wavelength 340 nm-347 nm [17]. There is also a wavelength at 232.0 nm. This wave was thought to be the wavelength of alkaloid compounds. Alkaloid compounds to appear at 237.0 wavelengths in the test using UV-vis [18]. There are also wavelengths of 207.0 nm and 204.0 nm. The wavelength is thought to be the wavelength of the terpenoid compound. In another study, wavelengths above 200.80 nm also included bonds between terpenoids [19].

3.3. Hematological status of Cantang grouper

The hematological status of Cantang groupers included the number of erythrocytes, leukocytes, hemoglobin levels, and hematocrit values, shown in Figure 1.

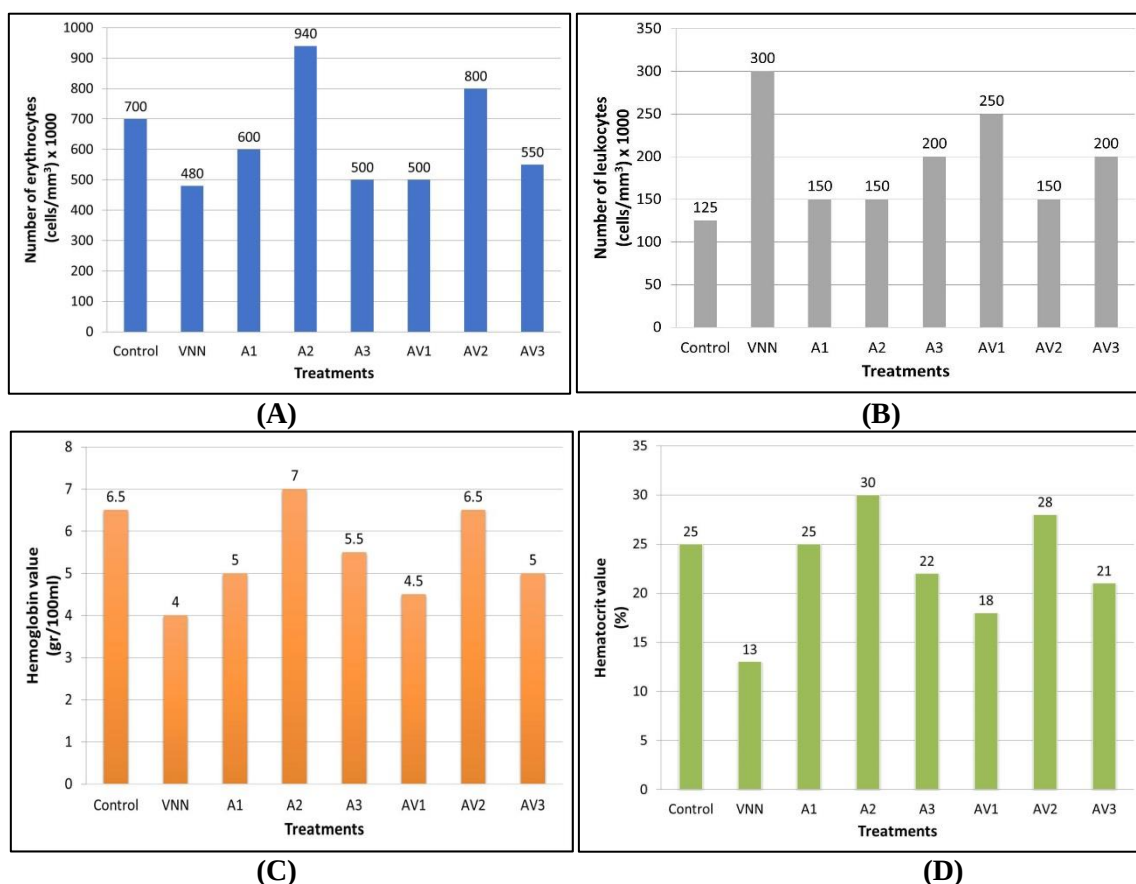


Figure 1. Hematological status of Cantang grouper: (A) total of erythrocytes cells; (B) total of leukocytes cells; (C) hemoglobin value; (D) hematocrit value

The erythrocytes observation shows that the highest value of grouper with A2 treatment is 940,000 cells/mm³, while the lowest number of erythrocytes in VNN treatment is 480,000 cells/mm³ (Figure 1A). Low erythrocyte levels indicate anemia and high levels indicate that fish is in a state of stress [19]. Erythrocytes of fish have nuclei and the number of erythrocytes varies depending on species, stress conditions, and ambient temperature, but the range of erythrocytes generally ranges from 1.05 to 3.0×10^6 cells/mm³ [21].

The leukocyte observation shows that the highest leukocyte count in the treatment of VNN obtained leukocyte count of around 300,000 cells/mm³, while the lowest leukocyte count in the control treatment of leukocyte counts around 125,000 cells/mm³ (Figure 1B). The factors that influence the number of leukocytes are the condition of the water and the health of the fish's body. Leukocytes help

cleanse the body of foreign objects, including invasion of pathogens through immune response systems and other responses. Diseased fish will produce many leukocytes to phagocytize bacteria and synthesize antibodies [22].

The hemoglobin observation shows the highest hemoglobin level was found in grouper with A2 treatment with a concentration of 7 gr/100ml and the lowest hemoglobin level was found in V treatment with a concentration of 4 gr/100ml (Figure 1C). The low Hb level causes the metabolic rate to decrease and the energy produced becomes low [22]. This makes the fish become weak and has no appetite and is seen to be still at the bottom or hanging below the surface of the water. Normal hemoglobin levels in the fish range of 5.05 to 8.33 grams / 100 ml of blood or gram /%. If the Hb level is low, it will have an impact on the low amount of oxygen in the blood.

The hematocrit observations show that the highest hematocrit value was obtained in grouper with A2 treatment with a value of 30%, whereas the lowest hematocrit values in VNN treatment was 13% (Figure 1D). Hematocrit values in teleost fish ranged between 20-30%, and in some marine fish species around 42% [24]. This statement is reinforced by the statement of Dellman and Brown (1992) [25] reporting that when infected, fish appetite will decrease and blood hematocrit values will decrease. In cases such as microcytic anemia, the number and size of red blood cells are reduced, so the hematocrit level is also low.

3.4. HSC expression on tissue

Expression of the tissue of Cantang grouper in the treatment was analyzed with immunohistochemistry that shown in Figure 2.

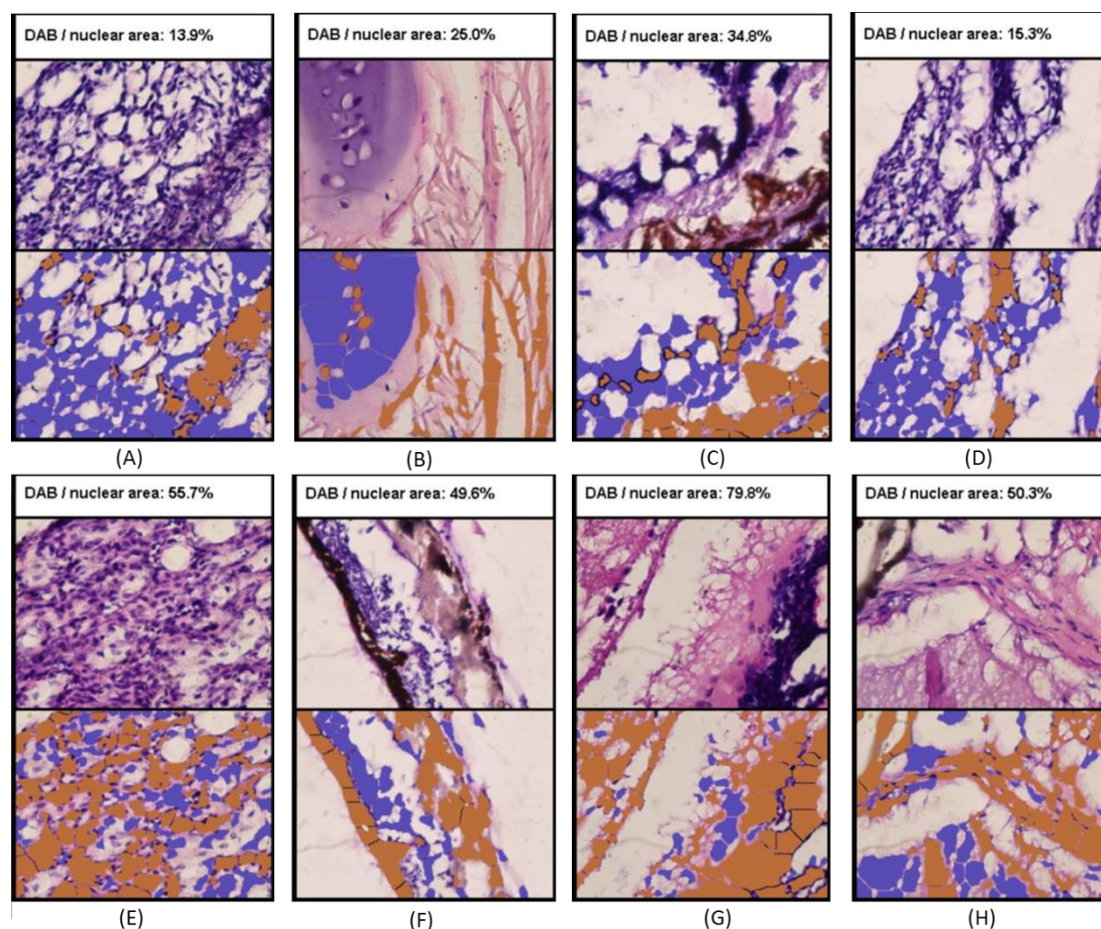


Figure 2. Immunohistochemistry results from each treatment; (A) Control (13.9%); (B) A1 (25.0%); (C) A2 (34.8%); (D) A3 (15.3%); (E) V (55.7%); (F) AV1 (49.6%); (G) AV2 (79.8%); (H) AV3 (50.3%)

The treatment of K is a control fish or fish without treatment and is used as a comparison of the treatment to be used. HSP expression was observed in control fish eye organs that show normal cells or tissues and no damage occurs (Figure 2). Fish's eye organ K obtained DAB with the percentage of 13.9% (Figure 2A). The DAB value is the percentage value of the expression of the HSP70 gene on the crunchy grouper brain organs. This value is fairly small because the HSP70 gene will only be active or express when the cell is in danger. HSP70 is a strongly induced protein after stress [26]. Once the body is in a normal condition without stress, the HSP70 gene will be expressed in small amounts.

Immunohistochemical analysis on fish eye organs with the treatment of A1, A2, and A3 results in DAB value of 25.0%, 34.8%, and 15.3% (Figure 2B, Figure 2C, and Figure 2D), respectively. The results of the percentage value of DAB in the A1 treatment tend to be low because in this treatment only the addition of *C.vulgaris* extract which is not harmful to the body of the fish so that the expression of HSP70 tends not to increase. The percentage value of DAB treatment A2 tends to be the same as the results in treatment A1 because the treatment is only given the addition of *C.vulgaris* extract which is not harmful to the body of fish so that the expression of HSP70 tends not to increase. The percentage value of DAB treatment of A3 tends to be the same as the results of treatment A2 because in this treatment only the addition of *C.vulgaris* extract is given which is not harmful to the body of fish so that the expression of HSP70 tends not to increase. HSP70 is not expressed in every cell, because this protein will be expressed when the cell experiences severe stress. This protein plays a huge role in the time of translation, translocation, proteolysis and folding protein [27].

Treatment V was the fish that was treated with VNN infection. Immunohistochemistry results on VNN treatment of eye fish obtained a percentage of 55.7% (Figure 2E). HSP70 expression values tend to be quite high, this indicates that cells experience stress due to VNN infection. VNN is a stressor that affects the expression of HSP70. When the cell experiences stress, the HSP will experience an increase in expression because the HSP tries to protect the cell that there is no change in the formation of the protein structure [27]. Immunohistochemistry results from AV1 treatment of eye fish organs obtained percentage value of 49.6% (Figure 2F). Immunohistochemical results from AV2 treated eye organs were 79.8% (Figure 2G) and AV3 treated eye organs were 50.3% (Figure 2H). The percentage value of DAB tends to be the same as the results from treatment A3 because this treatment is the addition of *C.vulgaris* extract that is not harmful to the body of the fish so that the HSP70 expression do not tend to increase. This value has increased considerably when compared with the five previous treatments, such as the results of the treatments K, V, A1, A2, and A3. This is due to the infection of VNN in this treatment that can activate HSP70 expression. Administration of *C.vulgaris* extract with a dose of 17 µg/mL can further increase the expression of HSP70 to perform its role as an agent that has cytoprotection properties [28]. This value has increased considerably when compared with the six previous treatments, such as the results of the treatment of K, V, A1, A2, A3, and AV1. This is due to the infection of VNN in this treatment that can activate HSP70 expression. Administration of *C.vulgaris* extracts with a dose of 33 µg/mL can further increase the expression of HSP70. When compared with the same AV1 treatment, infecting VNN and giving *C.vulgaris* extract were the same, in this AV2 treatment the DAB value was higher, it is possible that the administration of *C.vulgaris* extract with a dose of 33 µg/mL had a better effect in increasing HSP70 expression in the body of the clumpy grouper. This value has increased compared to the six previous treatments, such as the results of treatment K, V, A1, A2, A3, but decreased compared to AV1 and AV2. This is due to the presence of VNN infection and the administration of *C.vulgaris* extract in this treatment, which can activate HSP70 expression. However, when compared with the DAB AV1 and AV2 values, in this treatment decreased HSP70 expression, it is possible the addition of *C.vulgaris* extract with a dose of 17 µg/mL and 33 µg/mL was better compared than a dose of 50 µg/mL.

4. Conclusion

Based on the analysis of the hematological status and the results of the IHC analysis that was conducted by Cantang grouper infected by VNN virus with *C.vulgaris*, it was concluded that the hematological status of Cantang grouper was treated with V (VNN without *C.vulgaris*) in unhealthy

conditions. The cause of changes in the hematological status of Cantang grouper as in red blood cells, white blood cells and others due to infection in grouper fish is VNN. Different *C.vulgaris* doses have a significant effect on the immune system of the Cantang grouper.

5. References

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