

IN-VIVO TEST OF *Chlorella vulgaris* EXTRACT AS HEAT SHOCK PROTEINS INDUCTION OF CANTANG GROUPER (*Ephinephelus fuscoguttatus-lanceolatus*) INFECTED BY VIRAL NERVOUS NECROSIS

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Abstract

Cantang Grouper fish (*Ephinephelus fuscoguttatus-lanceolatus*) has a high value both in international and local markets. However, the production of these fishes has decreased due to viral infection known as VNN (Viral Nervous Necrosis). In relation to viral infections, an induced Heat Shock Protein (HSP) by *Chlorella vulgaris* is an effort to be used as an anti-inflammatory agent because of VNN infection. This research aimed to know the potential of *C. vulgaris* extracts as an inducer of heat shock protein in Cantang Grouper fish. Method used in this research was experimental method using an *in-vivo* test treatment with 33 µg/ml of *C. vulgaris* extracts in Cantang grouper fish. There were four treatments applied, they were (A) control fish, (B) fish infected with VNN, (C) fish treated with *C. vulgaris* extracts, and (D) fish infected by the VNN and treated with *C. vulgaris* extracts. Based on the phytochemical test, *C. vulgaris* extracts contained alkaloids, terpenoids, and tannins. Control fish showed no apparent inflammatory reaction. Fish treated with 33 µg/ml of *C. vulgaris* extracts had low inflammatory response of HSP70. Fish infected by VNN and treated with 33 µg/ml of *C. vulgaris* extract strengthened its inflammatory response as for fish infected by the VNN but without being treated by the extract showed very strong tissue inflammatory response. Results in this study indicate that that *C. vulgaris* extracts can inhibit the development of the virus and is capable to reduce the inflammatory response to Cantang grouper infected by VNN.

Keywords: Cantang grouper fish, *Chlorella vulgaris*, Heat Shock Protein, Viral Nervous Necrosis

INTRODUCTION

Grouper fish is one of the leading commodities of Indonesian fishery that has high economic value and high price and is the commodity of export. One of the grouper fishes cultivated in Indonesia is a Cantang grouper fish. The cantang grouper is the result of a hybrid between Tiger grouper (*Epinephelus fuscoguttatus*) and Kertang grouper (*Epinephelus lanceolatus*) [1]. This type of grouper is a hybrid grouper that was first produced at the Sabah University Marine Research Institute and has now become a very popular fish for marine aquaculture in the Southeast Asian region. Cantang grouper has several types of benefits such as rapid growth, tolerance to low water salinity and low water pH [2].

Grouper fish has a high value both in International and local markets. However, the productivity of hybrid Cantang grouper decreases due to environmental pressures including poor water quality, which makes the grouper become susceptible to viral, bacterial, stress infections resulting in poor growth and end of death [3]. Virus is one of the problems faced in the cultivation process of Cantang grouper. Viral infection is a serious illness that can lead to an acute mortality. One of the viruses that infect groupers is VNN (*Viral Nervous Necrosis*). VNN is one of the most prevalent epidemic diseases worldwide as reported in OIE (Office International des Epizooties) [4]. Stadia larvae and juveniles are susceptible stadia infected with VNN causing 100% mortality [5]. VNN generally attacks the organ system of the eye and brain nerves with

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symptoms that are quite specific as the fish exhibits abnormal swimming behavior and generally the fish stay at the base. Some of the symptoms of VNN-infected fish such as swirling fish; swimming at the bottom and showing unusual behavioral symptoms [6].

HSP (*Heat Shock Protein*) is naturally present in fish bodies that is physiologically a way of adaptation for fish to deal with stress [7]. HSP expression is influenced by various cell and cell tissues, in response to both biological stressors such as infectious pathogens, as well as abiotic stressors such as heat and cold sting, and environmental contaminants. These proteins have been categorized into several families based on their molecular weight such as HSP90 (85-90 kDa), HSP70 (68-73 kDa), HSP60, HSP47, and small HSPs (12-43 kDa) [8]. The inflammatory reaction is one of the body's responses to viral infection. Inflammatory reactions can also be characterized by HSP expression [9]. Increased HSP levels serve to stimulate the inflammatory response due to viral infection. In the inflammatory pathway due to viral infection, the expression of HSP70 increases to provide cytoprotection by blocking the components of the inflammatory signal pathway, such as the NF- κ B transcription factor [10].

Inducer HSP genes derived from natural ingredients have not been developed, one of them the use of bioactive microalgae compounds. Microalgae have great potential as an important source of energy in the future because it is abundant and has a complex content [11]. Microalgae are a group of autotrophic organisms that are known as potential sources of raw materials because they have very complex chemical content. The chemical composition of microalgae biomass is well studied and contains proteins, carbohydrates, lipids, pigments, vitamins, antioxidants, and trace elements. Proteins can be the most dominant compounds in microalgae such as *Chlorella* sp. [12]. Several types of marine algae such as red algae, brown algae, and green algae are potential source of bioactive compounds from nature and are very beneficial to the development of

the pharmaceutical industry. Algae are capable of producing halogenated secondary metabolites that are as bioactive compounds [13].

C. vulgaris is one type of single-celled microalgae that can grow and be found in warm climates. *C. vulgaris* has many bioactive compounds include proteins, vitamins, minerals, carbohydrates, fats, chlorophyll and beta-carotene [14]. In some studies, it has been suggested that the protein pigments from *Chlorella* have many benefits. Some of the bioactive compound of them can be developed and applied as antibacterial [15] with measurable immune responses such as NF-kappa beta response [4] as well as antiviral and anti-inflammatory viral infections in the grouper [16]. The aims of this research to determine the effect of extract *C. vulgaris* with as an inducer of the HSP in VNN-infected Cantang grouper.

MATERIALS AND METHOD

Place and Time of the Research

The experiment and the analysis performed in this research were conducted in Situbondo Grouper Farming and Biotechnology Laboratory, Faculty of Fisheries and Marine Science, Malang. This research was conducted in February-May 2018.

C. vulgaris Extraction

Powder samples of *C. vulgaris* were collected from BPBAP Situbondo. *C. vulgaris* extraction processes were done by maceration process using methanol p.a with ratio 1:5 and incubate for 24 hours. Furthermore, the macerate samples were separated from the waste by filtering through a filter paper. Liquid phase of samples were concentrated by evaporating the solvent using a vacuum rotary evaporator with a low pressure at 40 °C.

Phytochemical Test

Phytochemical tests were conducted to determine the presence or absence of secondary metabolite content of *C. vulgaris* including flavonoids, saponins, tannins,

alkaloids, and terpenoids. Flavonoid screening was performed by dissolving the concentrated extract from *C. vulgaris* in hot methanol and added with 0.1 g of Mg powder and 5 drops of concentrated HCl. Formation of orange color indicates the presence of flavonoids.

Saponin screenings were conducted by dissolving *C. vulgaris* extract in 10 mL of hot water for 10 seconds. Stable froth formation indicates the presence of saponins. Tannins screenings were conducted by dissolving concentrated *C. vulgaris* extract in 10 mL of distilled water and filtrate was added with 3 drops of FeCl₃ 1%. The formation of brown-black color indicates the extract from *C. vulgaris* contains tannins.

Identifications of alkaloid compounds were used by Mayer, Wagner, and Dragendorff methods [17], 0.5 g of extract was added with 1 mL of 2M HCl and 9 mL of distilled water for 2 min., chilled and filtered. The filtrates were divided into 3 parts each added to the reagents of Mayer, Wagner, and Degendorff. If there are brown or red colored precipitate (Wagner), the white colored precipitate (Mayer) or orange colored precipitate (Dragendorff) indicates the extract is positive to contain alkaloids. Terpenoid screenings were taken to dissolve the concentrated *C. vulgaris* concentration in 0.5 mL of chloroform, then add 0.5 mL acetic anhydride and split the mixture with 2 mL of concentrated H₂SO₄ through the tube wall. The formation of a reddish brown color indicates the presence of terpenoid compounds.

UV-Vis Tests

UV-Vis test was conducted by inserting solution in the form of a solution of *C. vulgaris* extract into cuvet. Following by measuring uptake at a maximum wavelength, the absorbance range read on the spectrophotometer should be between 0.2 – 0.8 or 15% to 70% when reading as transmittance. Then record the results obtained. Furthermore, samples rate was calculated by entering the absorption price on the standard curve line equation.

In-vivo Test of Crude Extract of *C. vulgaris* in Cantang Grouper Fish

In-vivo tests were performed on Cantang grouper with length of 7-11 cm. Their tests were performed by an oral method with the aid of feeding tube hose performed for three different days: 0 days, 5th day, and 10th day. This research conducted using a dose of 33 µg/mL, where is referred to the previous clinical test [16] that is by using protein purification *C. vulgaris*.

In-vivo Test of VNN in Cantang Grouper

In-vivo tests using VNN was performed on the 5th and 10th days. VNN infection analysis was given by cutting small fish meat that was positively infected by VNN through oral method estimated to weigh 5 g per fish.

Detections of Heat Shock Protein (HSP) using Immunohistochemistry (IHC)

Detections of Heat Shock Protein (HSP) were conducted by using Immunohistochemistry (IHC) with the preparation of brain organ of fish treatment which was given HSP specific antibody. The organ tissues of the grouper fish were incubated in xylol for 5 min. The organs were rinsed twice with alcohol in serial concentrations of 90 %, 80% and 70% for 5 min in each process. The tissues slide preparations were rinsed with ddH₂O for 5 min., 3 times, and the network slide preparation was stored in the refrigerator at 4 °C for overnight. The tissue preparation was washed with Phosphate Buffered Saline (PBS) pH 7.4 for 5 min., three times and incubated again with 3% H₂O₂ for 10 min. Blocking of non-specific proteins was performed by incubation in 5% PBS or with 1-2% BSA. Tissue samples were incubated with anti-HSP70 IgG anti-mouse primary antibody with a concentration of 1:1000 overnight, 40 °C. The result was washed with PBS pH 7.4 for 5 min, three times, followed by incubation in an anti-IgG anti-rat secondary antibody with a 1:200 ratio for 1 h. Tissue preparations were rinsed again with PBS pH 7.4 for 5 min., three times and incubated in 1:500 SA-HRP (Streptavidin-Horseradish Peroxidase) for 40 min. The incubation was then washed with

PBS pH 7.4 for 5 min., three times and added DAB (3,3'-diaminobenzidine) chromogen for 20 min. Samples were washed with PBS pH 7.4 for 5 min., three times. After cleansing, it was rinsed with dH₂O for 5 min., three times, then added counterstain with main hematoxyline (HE), 10 min. The final preparation step was washing the tissues under running water for 5 min, repeated three times. After the prepared slide was dried, it was then observed under a microscope [16].

RESULTS AND DISCUSSIONS

Extract of *C. vulgaris*

The result of the process of extraction of *C. vulgaris* by maceration process showed the form of a paste with 14 g of weight. For the percentage analysis, the yield of the extract can be calculated based on the final weight ratio (weight of the extract produced) with the initial weight (cell biomass weight used) multiplied 100% [18]. The yield of *C. vulgaris* extracts using methanol solvents were 7%.

Phytochemical Test

The bioactive compounds contained in *C. vulgaris* extracts were flavonoids, saponins, tannins, alkaloids, and terpenoids. Identification results of the extract from *C. vulgaris* were shown in Table 1.

Table 1. Phytochemical Test Results of Crude Extract of *C. vulgaris*

Compounds	Reagent	Result	Conclusion
Flavonoid	Concentrated HCL + Mg	Without color	- (Negative)
Alkaloid	Bouchar dat	Brown colored precipitate	+ (Positive)
Terpenoid	Bouchar dat	Reddish brown colored precipitate	+ (Positive)
Tannins	FeCl ₃ 1%	Brown-black color	+ (Positive)
Saponins	Concentrated water+ HCL	Without stable froth formation	- (Negative)

The results of color stain were observed on silica gels. Phytochemical tests results showed that *C. vulgaris* extract had only three positive compounds from five compounds including alkaloids, terpenoids, and tannins. However, *C. vulgaris* extracts did not contain flavonoid and saponins. In contrast to other studies reporting that *C. vulgaris* extract contained flavonoids and saponins [19]. Differences in the content of the same species may occur. The content of phytochemical compounds is influenced by various factors, i.e. species, varieties, growth conditions, seasonal variations, processing methods and storage [20].

Alkaloid tests results were observed brown colored precipitate to indicate positive tests of the alkaloids class. Alkaloids are one of the secondary metabolisms found in plants. Alkaloids can triggers the nervous system, raising blood pressure, reducing pain, antimicrobials, sedatives, heart disease drugs and others [21].

Terpenoid test result was observed by the presence of brown orange colored precipitated on the tested solution. The terpenoids are cyclic or acyclic and often have alcohols, aldehydes, or carboxylic acids. Plants containing terpenoid compounds have ecological value because they work as anti-fungus, insecticides, anti-predator, anti-bacterial and anti-virus [22].

Tannin compounds showed a change in the color of brownish black colored that indicates a positive test on tannins groups. Tannins are a polyphenol compound having a large molecular weight comprising hydroxyl and carboxyl groups [23]. Tannins have antibacterial property through reactions to cell membranes, enzyme inactivation, and destruction or inactivation of genetic material functions [24]. Tannins also have a complex biological role ranging from protein precipitation to chelating metals. Tannins can also function as biological antioxidants [25].

UV-Vis Test

UV-Vis test results of *C. vulgaris* extracts were shown maximum peak at 663.1 nm. Interesting peaks were also detected at

232.0 nm, 207.0 nm, and 204.0 nm. These wavelengths are meant for a detection of terpenoid compound. Terpenoid compounds have maximum wavelength uptake at 225 nm and 272.5 nm [26]. In other study, wavelengths above 200.80 nm also included bonded terpenoid compounds [27]. The wavelength indicates electron transition $\pi-\pi^*$ which is a typical UV spectral absorption for a triterpenoid compound having a chromophore of a non-conjugated double bond.

The highest wavelength result of 663.1 nm indicated a presence of chlorophyll a. Maximum absorption by chlorophyll a occurs at two wavelengths bands, *i.e.* 430 and 660 nm [28].

Tabel 2.Uv-Vis Test Results of *C. vulgaris* Extract

Wavelength (nm)	Abs
663.1	0.346
423.0	0.690
341.0	0.387
232.0	1.021
207.0	3.421
204.0	3.377

In-vivo Test of *C. vulgaris* Extract to Cantang Grouper using Immunohistochemistry (IHC)

Extracts of *C. vulgaris* were able to suppress inflammation at the time of VNN virus infection occurred. The responses were shown by detecting the induced heat shock proteins identified through immunohistochemistry methods. HSP expressions analysis was focused on target organs of the brain. Immunohistochemistry is able to visualize cell components, such as proteins or other macromolecules in tissue samples. Immunohistochemistry (IHC) analysis using immunoRatio-derived software resulted in a different percentage of DAB between the control fish group and the other three treatment fish.

Control Fish

The results of observation on the control fish using immunohistochemistry and continued analysis using immunoRatio obtained DAB percentage of 21% (Figure 1).

This showed that the target gene (HSP) presented in the control fish was expressed by 21%. HSP remained expression even under normal conditions. Under normal conditions HSP is a protein that plays a role in the formation of protein structures, this is called protein folding [29]. In the observation of damage level in brain organ of control fish, found no damage either necrosis, vacuole or hypertrophy

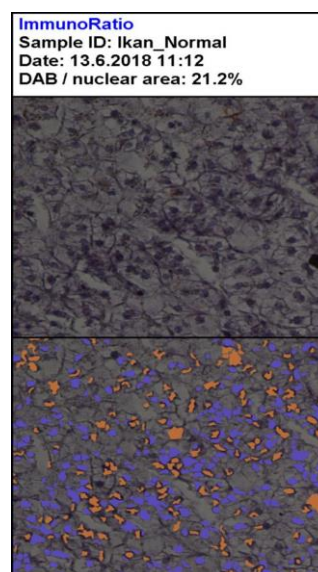


Figure 1. Immunohistochemistry Results of Cantang Grouper Brain (Control Fish)

Fish with VNN Treatment

Immunohistochemistry results of fish brain with VNN treatment were obtained DAB percentage value of 63.7% (Figure 2). It shows that the target gene (HSP) was presented in VNN-treated fish expressed by 63.7%. High HSP expression values were indicated that the cell is stressed due to VNN infection. VNN is one of the triggers that affect HSP expression. When the cell has stress, the HSP will experience an increase in expression because the HSP is trying to protect the cell to avoid changes in protein structure formation [29].

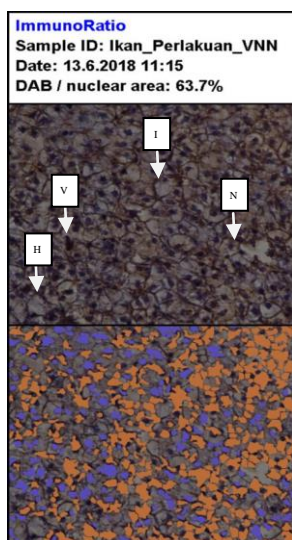


Figure 2. ImmunoRatio Results of Cantang Grouper Brain with VNN Treatment. (H) Hypertrophy, (N) Necrosis, (I) Inclusion body, (V) vacuole

The observation on brain organ of the VNN-infected Cantang grouper showed that VNN infection causes the cells to changed in shape and size. Cells with hypertrophy are described by increasing volume (cell magnification) (H). There is also a damaged cell (necrosis) (N) causing the cell to be destroyed, furthermore, it remains as an empty space in the brain tissue of VNN-infected groupers. Necrosis is the occurrence of cell death that suspected as a result of systemic infection, *i.e.* through the bloodstream then reaches to the brain and inflicts damage to the organ's composing tissue [30].

In the brain organ of Cantang grouper treated with VNN infection showed inclusion body (I). Inclusion body is characteristic of virus infection in target organs. Inclusion body is an early process of necrosis cells with black spots on the cytoplasm [31]. There is also damage to the brain organ with marked vacuole (V). Vacuoles appear as a result of cell swelling that indicates cell degeneration [32]. On observation of damage level of brain organ of VNN-treated fish as a whole, it is seen that there is hollow tissue. This indicates the fatal damage caused by infection of VNN.

Fish with Treatment of Extract of *C.vulgaris*

Immunohistochemistry results of brain organ fish treatment extract from *C. vulgaris* obtained DAB percentage value of 44.6% (Figure 3). This suggests that the target genes (HSP) present in fish treated with *C. vulgaris* extract were 44.6%. and on observation of the damage levels of brain organ on *C. vulgaris* treatment fish, found no damage such as necrosis, inclusion body, and vacuole.

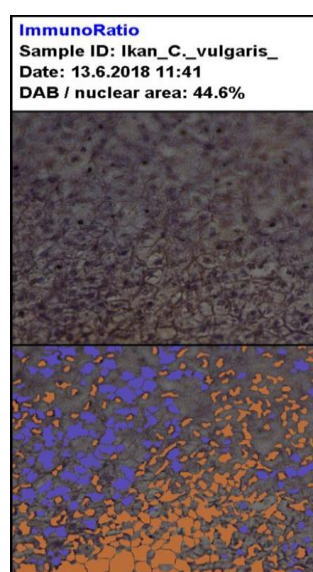


Figure 3. ImmunoRatio Results of Cantang Grouper Brain with Treatment of Extract of *C.vulgaris*

Fish with Treatment of Extract of *C.vulgaris* and VNN

Immunohistochemistry of fish brain treated with *C. vulgaris* and VNN obtained the value of DAB percentage 60.4% (Figure 4). This suggests that the target genes (HSP) in fish treated with extracts from *C. vulgaris* and infected by the VNN were expressed by 60.4%. This result was lower compared to the percentage of DAB fish infected by the VNN but without extract treatment. The level of stress on fish was lower due to the administration of *C. vulgaris* extract that could improve the immune system of the Cantang grouper. Condition of the immune system of fish infected by VNN increased due to the application of *C. vulgaris* extract,

furthermore, the body was ready and in good condition when the VNN started to infect the fish.

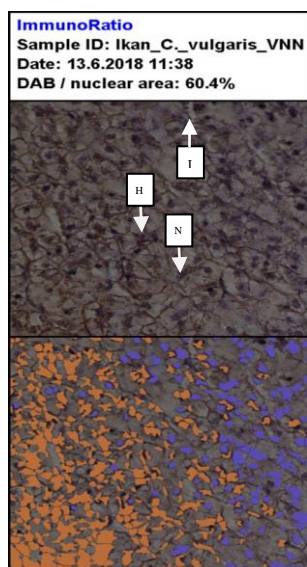


Figure 4. Immunohistochemistry Results of Cantang Grouper Brain with Treatment of Extract *C.vulgaris* and VNN. (I) Inclusion body, (N) Necrosis, (H) Hypertrophy

Damage levels of the brain treated with *C. vulgaris* extract and VNN infection showed inclusion body, necrosis, and hypertrophy, however, the damage was smaller compared to those infected by VNN infection but without extract treatment (Figure 4). In the observation of brain organ, there were some black spots detected indicating inclusion body (I), there was also necrosis or cell death indicated by arrow (N) with the empty space in brain organ. In this treatment also appeared to occur hypertrophy (H); it was a swelling or addition of volume cells due to viral infection. From the above data, there is a significant difference between the HSP profile on the control fish group and to the three fish with the treatment.

VNN is one example of the RNA genome virus that is composed of two molecules (RNA1 and RNA2) [33]. VNN infects the brain organ of grouper fish in an instant by directly attacking the fish receptors. VNN is a virus that does not have an envelope, then the virus spreads to the brain through the blood circulation.

Infection by this virus triggers the occurrence of inflammation in the fish tissue. Inflammation occurs in response to infection, heat stroke or cellular stress. VNN will trigger active immune cells to protect tissues and cells in fish. The cells in the fish body will experience stress which will cause the response to stress in the form of heat shock response (HSR). Under stressful conditions, HSP creates protein aggregation, refolds damaged proteins and lowers irreversible proteins. As a pro-inflammatory mediator, HSP can release necrotic, non-necrotic substances into the extracellular environment, resulting in various immune and inflammatory responses, including activation of immune system effector and cytokine release [9].

Extracting marine microalgae *C.vulgaris* with a dose of 33 µg/ml can inhibit the development of the virus qualitatively by being able to reduce the inflammatory response from tissue in the Cantang groupers. The compounds in *C.vulgaris* extract have the ability to block the adhesion of pathogens. The general mechanism of antiviral activity of the microalgae active compound against the wrapped virus may be based on a positively charged shield of the viral glycoprotein, when the virus attaches to the cell, the active compound in the microalgae can against with the viral amino acid glycoprotein and block the viral adsorption process [34].

The active compounds in *C. vulgaris* extract can also be used as an anti-inflammatory in VNN-infected grouper shown by heat shock protein expression response (HSP). Based on the results from control fish with labeling using IgG anti HSP 70 anti-mouse inflammatory responses do not appear the reaction to inflammation, in fish treatment of extract of *C. vulgaris* 33 µg/ml HSP-70 responses to low inflammatory responses, and treatment of extract of *C. vulgaris* 33 µg/ml and VNN inflammatory response strengthened and tissue inflammatory response with VNN treatment alone is very strong.

As an anti-inflammatory molecule, the expression infection the Heat shock protein 70 (HSP70) increases to provide cytoprotection by inhibiting components of the inflammatory signal pathway, such as the transcription

factor NF- κ B. Increased HSP levels serve to stimulate an inflammatory response to a type of Toll receptor 2 (TLR2)/TLR4/CD14 based on the mechanisms that lead to NF- κ B, TNF- α , IL1- β and IL-6 production activities. HSP can block the NF- κ B pathway by increasing the I- κ B α gene expression. Knowlton explained that HSP can increase immune modulation by providing a regulation target of T cell-specific [35].

CONCLUSIONS

Present study has the same result as the previous one showing that *C. vulgaris* extract may improve the induction response from heat shock proteins as anti-inflammatory markers for VNN-infected grouper fish tissue. The responses were expressed by the expression of HSP 70 both qualitatively and quantitatively. Morphology analysis results showed necrosis, tissue vacuolation, and tissue inclusion due to VNN antigen virus infection. It is important to perform a study on the purification of *C. vulgaris* extracts to obtain natural compounds that can be clinically anti-inflammatory as a result of VNN RNA virus infection.

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