IN-VIVO TEST OF *Spirulina* sp AS INDUCER OF β-Actin IN CANTANG GROUPER (*Epinephelus fuscoguttatus-lanceolatus*) INFECTED BY VIRAL NERVOUS NECROSIS

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Abstract

Cantang grouper fish (*Epinephelus fuscoguttatus-lanceolatus*) is a result of hybridized fish between a female of tiger grouper fish (*Epinephelus fuscoguttatus*) and male of kertang grouper (*Ephinephelus lanceolatus*). In the development of cultivation, there are many problems, one of them infected with the class of virus Nodaviridae, namely Viral Nervous Necrosis (VNN). Fish had a defense against cellular immunity against the VNN virus by β-actin. The aims of this research to explore the crude extract of *Spirulina* sp as a β-actin inducer for the anti-inflammatory immune system in grouper fish against VNN attack. The method used in this research is experiment methods. Crude extracts of *Spirulina* sp (33 μg/ml) were conducted by feeding orally to groupers, and VNN infections were conducted by feeding the already positive VNN meat. Detection of VNN using RT-PCR, however β-actin detection using PCR, and IHC in the organ of Cantang grouper fish. The results showed that the percentage of DAB value of control fish (14.0%), fish treated with *Spirulina* sp (25.7%), fish treated with VNN (31.9%), and fish treated with *Spirulina* sp extract and VNN infection (32.4%). The percentage of DAB values were indicated by the detection of the target gene β-actin. Immunity in fish increases with the addition of *Spirulina* sp. The increased β-actin expression may also be used as an indicator of a grouper's body defense against VNN infection.

Keywords: *Epinephelus* sp, *Spirulina* sp, *Viral Nervous Necrosis*, *β-actin*

INTRODUCTION

Fish from subfamily *Epinephalinae (grouper)* had considerable economic value in the tropics and subtropics. In particular, live fish is very valuable in Southeast Asia [1]. The high price of grouper fish makes the entrepreneurs to increase cultivation [2]. One type of grouper that had economic value is Cantang grouper (Epinephelus sp). Grouper fish cantang is a hybridized fish between female tiger grouper with male kertang grouper [3].

Development of cultivation efforts was found many problems, one of which is the attack of disease or virus that can cause mass death [4]. The cultivation constraints on the Epinephelus group (Grouper) in Indonesia are the limited supply of seeds due to a pathogenic infection that causes deaths of more than 80%, even up to 100% [5]. The death of the seed was caused by Viral Nervous Necrosis attack. VNN leads to retinopathy and encephalopathy which have a wide range of hosts [6]. VNN attacks to show symptoms such as erratic swimming fish, fish always on the bottom of the water [7] and skin tone turns dark, loss of appetite and bladder is usually enlarged, besides the fish behaves abnormally [8-9].

Utilization of microalgae has been developed especially in pharmacology field. Microalgae had benefits as antioxidants for fish because microalgae contain vitamins, polysaccharides, and bioactive compounds [10]. The polysaccharide fraction of the microalgae has the potential to inhibit the production of retroviruses [11]. Fragment of protein pigment from *N. oculata* potentially as an anti-inflammatory at the time of ssRNA virus attack grouper [12]. In addition, microalgae also had potential as raw materials for biomass fuel (biomass fuel feedstock) [13].

One of the microalgae that can be used as an antiviral is *Spirulina* sp. *Spirulina* sp is not only able to support the growth of fish, but also can increase the immune system in fish because it contains high protein, vitamins, and

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minerals as well as biologically active compounds such as pigment, such as chlorophyll a and β-carotene [14], which can be used as antivirals, antioxidants, and antimicrobials. Carotenoid content also has a biological function that can be used as a precursor of vitamin A and can modulate the immune system [15].

The regulation of the immune system mechanism is very important, especially to protect against disease or virus. One of the proteins of the immune system that plays an important role is β-actin [16]. β-actin can be called as a housekeeping gene, in which the gene or protein is needed as a basis of cellular maintenance [17] against viral infection and β-actin acts as a facilitator.

β-actin has an important role as a key to maintaining cytoskeletal structures, cytokinesis, endocytosis, cell motility, and cell adhesion [18]. Then the β-actin expression will also increase when there are trigger factors according to the study [19], the utilization of protein pigment fragments from the *N.oculata* may increase β-actin expression. Where the function of receptors on cell walls such as antigen presentation receptors and signal detection will also increase.

This research was conducted to determine the benefits of crude extract *Spirulina* sp which is used as a β-actin inducer for anti-inflammatory in the immune system of Cantang grouper fish (*Epinephelus fuscoguttatus-lanceolatus*) against VNN attack.

MATERIALS AND METHODS

This research was conducted in Situbondo and Biotechnology Laboratory Faculty of Fisheries and Marine Science, Brawijaya University, Malang. The material used in this research is dried *Spirulina* sp in the dried form from BPBAP Situbondo and Cantang grouper from a hatchery in Situbondo.

The equipment used in this research are bucket as a place of maintenance media, aerator set, syringe, tray, thermometer, refractometer, DO meter and pH meter. The method used in this research is an experimental method.

Spirulina sp Extraction

Samples of *Spirulina* sp were extracted by maceration process using methanol PA solvent with ratio 1:5 for 24 hours. Then filtered using filter paper to remove the dregs to obtain the extract from the solvent. Furthermore, to obtain the extract, the solvents were removed by using a rotary vacuum evaporator with a temperature of 40°C, with a rotation of 60 rpm.

Phytochemical Test

Phytochemical screening test aims to determine the qualitative presence or absence of a class of bioactive compounds that have potential as antioxidants. Phytochemicals typically include alkaloids, phenolic compounds, triterpenoids, flavonoids, and saponins [20].

The phytochemical metabolite secondary analysis method partially refers to [21- 22]. Identification of flavonoids compounds was conducted using concentrated HCl reagents plus Mg, positive results indicate dark red or pink. Identification of alkaloids compounds was conducted using reagent Mayer, a positive result is white color precipitate. Identification of alkaloids compounds was conducted using Dragendrof reagents, a positive result is orange color precipitate. Identification of alkaloids was used Bouchardate reagent, a positive result is brown color precipitate. Identification terpenoid was conducted using bouchardat reagent, positive results there is a bluish green color which means contain terpenoid steroid type, there orange or orange color which means contain terpenoid, triterpenoid type. Identification of tannin was conducted using 1% $FeCl_3$ reagent, the positive result there is blackish brown color, blackish black, and green is blackish. Identification of saponins was conducted using hot water and shaken strongly, positive results form a permanent froth for not less than 10 minutes as high as 1-10 cm, and added concentrated HCl 1 drops, the positive result of permanent foam is not lost.

Analysis of UV-Vis (Ultraviolet-Visible) Spectrophotometer

Analysis of UV-Vis (Ultraviolet-Visible) Spectrophotometer was performed by measuring the wavelength spectra (nm) absorbance of ultraviolet radiation from *Spirulina* sp extracts with a spectrophotometer.

In-vivo Test

An animal model was used Cantang grouper fish (*Epinephelus fuscoguttatus-lanceolatus*). Cantang grouper fish used is measuring between ± 7-10 cm. Newly introduced groupers are not directly fed, as they require adaptation to new maintenance media. Types of feed use trash fish and given by adlibitum. Water quality maintenance is made homogeneous based on pH, temperature, salinity, and DO.

The in vivo test of crude extract from *Spirulina* sp in Cantang grouper fish was performed using orally method and dosage refer to [23], ie by administering 33 μg / ml (PCP purified protein clinical test results). While in this research using crude extracts from *Spirulina* sp. *Spirulina* sp extracts were given three times on day 0 day, 5th day, and 10th day. VNN infection in Cantang grouper fish was conducted by feeding VNN positive fish and given for two times on 5th day, and 10th day. After that, on the 15th day, all the fish was harvested and the target organ was isolated, ie the eyes.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was conducted to detect β-actin. IHC staining refers to Khan et al. [24] and Yanuhar and Khumaidi [19], using the VECTASTAIN® ABC kit (Vector Laboratory). Diaminobenzidine tetrachloride (Chromogen) was used to detect biotin. Tissue or sample was rehydrated using alcohol, then cleaned using xylene. Furthermore, cooled the endogenous peroxidase with 3% hydrogen peroxide in methanol for 30 minutes at room temperature, and should not be exposed to light (must be in the dark). Microwave antigen was taken with 0.01 mol/L sodium citrate buffers (pH 6). Subsequently, it was incubated with a monoclonal antibody β-actin (AC-15) at 4 °C, for 16 hours, with 1:1000 dilution and added secondary antibodies biotin-conjugated anti-IgG at room temperature for 30 minutes.

RESULT AND DISCUSSIONS

Extraction of *Spirulina sp*

Extraction of *Spirulina* sp was obtained as much as 21 grams of pasta. The percentage analysis of the yield can be calculated based on the ratio, ie the weight of the resulting extract (final weight) by the weight of cell biomass used (initial weight), then multiplied by 100% [25]. Rendement of *Spirulina* sp extracts with methanol PA solvent was 4.2%.

Phytochemical Screening Test

Phytochemical results of crude extract of *Spirulina* sp were showed on Table 1.

Tabel 1. Phytochemical Results of *Spirulina* sp.

Phyto-chemical	Reagent	Result
Flavonoid	Concentrated of HCl + Mg	+
	Mayer	+
Alkaloid	Dragendrof	-
	Bouchardat	+
Terpenoid	Bouchardat	+
Tannin	$FeCl_3$ 1%	+
Saponin	Water + Concentrated of HCl	+

The result of the phytochemical test of crude extracts of *Spirulina* sp was showed that *Spirulina* sp has active compound content which is indicated by certain color change on specific reagent. Flavonoids tests on a crude extract of *Spirulina* sp using concentrated HCl reagent + Mg was showed a change of color of the solution to redness, indicates *Spirulina*

sp positive contains flavonoids and the pink indication contains flavonoids [26]. The flavonoid content of *Spirulina* extracts had activity as an antioxidant [27].

An alkaloid test by a reagent mayer was indicated that *Spirulina* sp positively contains alkaloids. The same is also shown in the alkaloid test by using bouchardat reagent was showed positive results in the presence of brown precipitate, however, the alkaloid test by using dragendrof reagent was showed different results ie *Spirulina* sp no contain alkaloids. The alkaloid compound of *Spirulina* extract had an antioxidant activity such as anticancer [21].

The terpenoid tests were conducted Buchardate reagent was showed the presence of orange-colored sediment which means *Spirulina* sp positive contains terpenoid. The terpenoid content of crude extracts of *Spirulina* can be utilized as an antioxidant [21]. In addition, several studies on the content of flavonoids, alkaloids, and terpenoids found in crude extract *Spirulina* sp had various biological activities such as anticancer, antiproliferation, and antioxidants [28].

Tannin tests were conducted with 1% $FeCl_3$ reagent also showed a positive result that indicated a change of color to blackish brown, blackish black, and blackish green. The yellowish green color indication on the tannin test also indicates that the extract is positive for tannin, then the tannin compound can be utilized as an antioxidant [26].

Saponin tests were conducted using with Water + concentrated HCl reagent also showed the presence of permanent foam that is not lost indicating that the crude extract *Spirulina* sp positively contains saponins. The content of saponins in the crude extract of *Spirulina* can potentially be antiradical and antioxidant [22].

Analysis of UV-Vis (Ultraviolet-Visible) Spectrophotometer

The result of analysis of UV-Vis spectrophotometer was showed crude extracts of *Spirulina* sp had biological compound such as chlorophyll a, flavonoids, alkaloids, terpenoids, and saponins were shown in Table 2.

Tabel 2. Results of Analysis of UV-Vis Spectrophotometer of *Spirulina* sp.

Wavelength (nm)	Absorbance	Description
664.9	0.199	Chlorophyll a
401.0	0.634	Flavonoid
371.0	0.578	Flavonoid
269.0	1.109	Alkaloid
266.0	1.102	Alkaloid
258.9	1.115	Terpenoid
239.0	1.642	Terpenoid
236.0	1.781	Terpenoid
229.9	2.101	Terpenoid
207.0	3.583	Saponin
204.0	3.535	Saponin

The results of analysis of UV-Vis spectrophotometer of *Spirulina* sp (Table 2), was showed the wavelength of *Spirulina* sp. start from 204.0 to 664.9 nm. The highest wavelength of 664.9 nm with abs 0.199 was indicated *Spirulina* sp had chlorophyll a. Several studies like [29] and [30] explain that *Spirulina* sp contain chlorophyll a. In addition to the wavelength of 401.0 nm and 371.0 nm indicates that *Spirulina* sp contains flavonoid compounds. Flavonoids had antioxidant activity as a free radical catcher [31].

The alkaloid content of *Spirulina* sp was showed the wavelength of *Spirulina* sp. start from 269.0 nm and 266.0 nm. These alkaloids had the ability to increase antioxidant activity [32]. Then the most terpenoid contents with a wavelength of 258.9 nm, 239.0 nm, 236.0 nm, and 229.9 nm [33], terpenoid type compounds produced by green algae had activity as antimicrobial, anti-inflammatory, and antiviral. *Spirulina* sp extracts also contain saponins seen at wavelengths of 207.0 and 204.0, the saponin contents having activity as an antioxidant [34].

In-vivo Test of Cantang Grouper Fish

In-vivo tests of *Spirulina* sp extracts on Cantang grouper fish was conducted for 15 days. *Spirulina* extracts were given by oral method in control fish included under normal conditions because of the activity of

swimming, body color is bright and responsive to feeding.

Fish with *Spirulina* sp extracts was no difference in activity in fish, but the color was slightly changed. It is indicated treatment by the oral method will make fish stress [35], which explains that stress is a form of animal defense against the cause of stress (stressor).

Fish with the treatment of VNN was showed visible changes in fish swimming activity and a slightly darker body color. VNN-infected fish will exhibit abnormal swimming behavior and generally fish dwell on the bottom [7]. While in fish with *Spirulina* sp and VNN extract, the fish condition is still normal. This can be seen from the state of the fish when swimming, the color of the body is gray-black, although the fish is less responsive to feeding.

β-actin Expression of Control Fish

β-actin expression analysis results of eyes in fish control are the state of cells and tissue looks normal and no damage (Fig. 1).

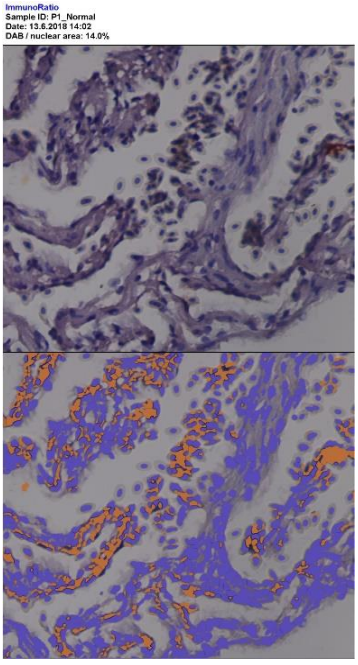

Figure 1. ImmunoRatio Result on Eye of Cantang Grouper Fish

β-actin is one of the proteins that play an important role in cells [16] especially in the immune system, and as an internal cell regulatory gene [36]. ImmunoRatio analysis results were showed a DAB value of 14.0% (Fig. 1). The percentage of DAB value of control fish was indicated 14.0% detected the target gene (β-actin) and characterized by a brownish yellow color on the IR result.

β-actin Expression on Eyes of Fish with Treatment of Extract of *Spirulina* sp

The results of β-actin expression analysis on eyes of fish with *Spirulina* sp extract were different with results of control fish. In this treatment looks a lot of brown ambers and indicates that there is a bond between the antigen and antibodies. The ImmunoRatio analysis was showed a DAB value of 25.7% (Fig. 2), and the percentage of DAB value of fish with *Spirulina* extracts of 25.7% detected the target gene (β-actin). *Spirulina* sp is one of the natural ingredients that can be utilized in the health field. This is in accordance with the research Yanuhar [12] that using protein pigment fragments from the *N. oculata* microalgae may increase the immune system in the Humpback grouper fish.

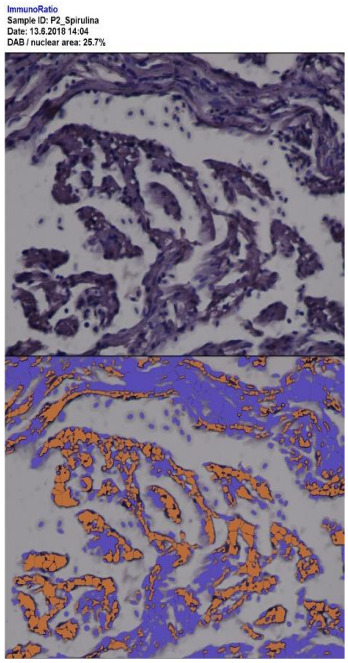

Figure 2. ImmunoRatio Result on Eye of Cantang Grouper Fish with Treatment of Extract of *Spirulina* sp

Spirulina sp extracts are capable of acting as a modulator for increased β-actin expression of the cell. The increased β-actin expression will also increase immunity in fish. This result was indicated by the presence of the active compound (terpenoid). *Spirulina* sp extracts as an inducer, thus triggering β-actin to proliferate, and when β-actin proliferates more, will increase the response in the cell. β-actin contained within the cell will function as a facilitator and play a role in cellular maintenance [17].

β-actin Expression on Eyes of Fish with Treatment of VNN Infection

The results of β-actin expression analysis on eyes in fish with VNN treatment were different from others, as in fish control and fish with *Spirulina* sp treatment. The results of ImmunoRatio analysis were shown in Fig. 3, the percentage value of DAB β-actin by 31.9%. ImmunoRatio analysis in this research was showed that VNN attacks of Cantang grouper fish can cause cell and tissue damage.

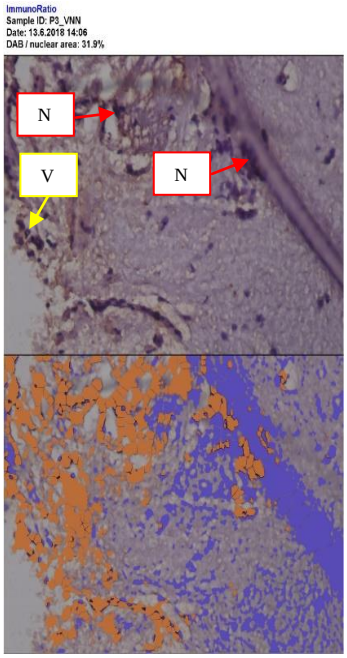

Figure 3. ImmunoRatio Result on Eye of Cantang Grouper Fish with Treatment of VNN Infection. Necrosis (N), Vacuolization (V).

VNN is a spot virus and belongs to a type of virus of the class (ssRNA +) [38]. VNN will attack the nervous organs especially the brain and eyes [6], therefore the impact of this VNN attack cause cell or tissue damage, can even cause mass death. Figure 3 was showed some cell damage due to VNN attacks. Damage was caused by VNN infection includes vacuolization (V) and necrosis (N), according to the statement [37] which explains that VNN attacks on fish can cause cell or tissue damage. In addition, VNN infection can also cause deaths of up to 100% in juvenile stadia [5].

β-actin Expression on Eyes of Fish with Treatment of Extract of *Spirulina* sp and VNN Infection

The results of β-actin expression analysis on eyes of fish with the treatment of extract of *Spirulina* sp and VNN infection were showed different from others. ImmunoRatio analysis results were showed DAB value of 32.4% (Fig. 4), and the percentage of DAB value indicates detection of the target gene is β-actin. In this treatment, an increase in the immune system of grouper fish was shown by increasing the percentage of DAB value and by normal cell shape, although cell damages such as necrosis (N) and vacuolization (V) but the damages were not too severe (Fig. 4).

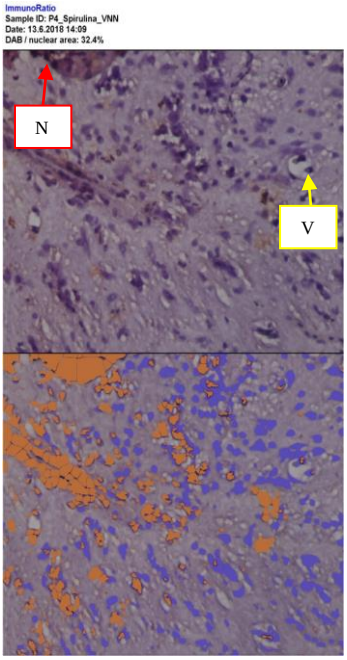

Figure 4. ImmunoRatio Result on Eye of Cantang Grouper Fish with Treatment of Extract of *Spirulina* sp and VNN Infection. Necrosis (N), Vacuolization (V).

Spirulina sp extracts were used as an immune system inducers in groupers can activate specific genes such as β-actin. *Spirulina* sp extracts can increase adapter molecules and transcription factors in the cells. The proliferation of β-actin is also increased due to trigger factors such as the active compound content of *Spirulina* sp. This was related to the role and function of β-actin in cells as cellular maintenance [17], the response within the cell increases with increasing β-actin expression. Increased expression of β-actin also affects the working system of receptors in the cell, such as recognition receptors (PRRs, TLRs, APC, and MHC). β-actin was acted as a facilitator and will help to process the signal faster while pathogens were detected. In addition, β-actin is also capable of activating T cells by giving mechanical forces in cell polarization and assembling or transporting molecules [39], when there is viral or pathogen infection in the cell, the process for pathogen deregulation is also faster, this pathogen or viruses do not damage cells or tissues.

Figure 4 was showed the cell shape is still in normal condition, though there is some cell damage such as necrosis (N) and vacuolization (V), cell damage is not too much. This was caused by immunity in fish had increased, furthermore, VNN infection occurs in the defense mechanism against pathogens or viruses is faster and VNN infections do not destroy all cells. In-vivo tests results were showed *Spirulina* sp had been able to increase the immune system in Cantang grouper fish though VNN infection, in addition to the fish are also able to survive. This is supported by research Yanuhar and Khumaidi [19] on the utilization of natural ingredients of *N. oculata* marine microalgae capable of enhancing the immune system in Humpback grouper fish.

CONCLUSION

In-Vivo test results in Cantang grouper fish with *Spirulina* sp extracts treatment and VNN infection treatment was showed crude extracts of *Spirulina* sp was increase β-actin expression. Increased β-actin expression indicated the immune response in the Cantang grouper fish is also increased, furthermore fish can or capable to survive when a pathogen or viral attack occurs.

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