The effect of Dunaliella salina extract on NFkB expression in Cantang Grouper (Epinephelus fuscoguttatus x E. lanceolatus) exposed by Viral Nervous Necrosis

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Abstract : VNN is one of the viruses that can cause death in cantang grouper hatchery in Southeast Asia including Indonesia. The purpose of this research was to find out the expression of NFkB in cantang grouper brain which was exposed by VNN and treated by D. salina extract. Fifty hybrid groupers with 7-9 cm length were stocked in 5 containers with a density of 10 fish/container. Five treatments applied with different D. salina extracts are 0, 250, 300, 350 and 400 mg/kg feed. Feed treatment was given twice a day for 10 days before being challenged with VNN. The expression of NFkB was detected with immunohistochemistry tests and analyzed by immunoreaction software. The result showed that the highest NFkB expression is in treatment 0 mg/kg feed with a percentage of 66%, while the lowest expression is shown in 400 mg/kg feed with a percentage of 31.2%. Different dosages of D. salina have a significant effect on NFkB expression so that it can be concluded that D. salina has the potential as a VNN antiviral candidate in the grouper.

1. Introduction
Grouper production in Indonesia reached 8,972 tons in 2015 and increased to 15,089 tons in the third quarter of 2016 [1]. Cantang grouper is one of the hybridized species that has some advantages when compared to others, which are faster growth with a low FCR [2] and only takes 7 months to reach a weight of 500-700 g/fish [3]. In the same case with groupers in general, the diseases that often attack cantang grouper are Viral Nervous Necrosis (VNN) caused by Betanodavirus, the Nodaviridae family. Betanodavirus has a spherical shape with a diameter of 25-34 nm, non-enveloped and has a genome consisting of two single-stranded positive-sense RNA molecules [4]. Betanodavirus was very resistant in aquatic environments and able to survive long in seawater at low temperatures [5]. In Indonesia, snapper and grouper seed infected with VNN in East Java in 1997 and then spread to the Ambon waters [6]. The main target of VNN is the nervous system of the brain and eye, they will actively be replicating in these organs, causing extensive vacuolation on the tissue of organs [7]. VNN showed abnormal behavior such as circling or whirling, decreased appetite and sleeping at the bottom to cause death [8]. VNN causes a decrease in the immune system of both cellular and humoral, innate and adaptive systems as well as a decrease in gene expression [9].

Efforts to prevent infection VNN in cantang grouper is using D. salina that contains chlorophyll-α, violaxanthin, and veuchexanthin which improves the immune system of fish [10]. D. salina also contains carotene 12.6% of the total dry weight that consist of β-carotene (60.4%), astaxanthin
(17.7%), zeaxanthin (13.4%), lutein (4.6%) and cryptoxanthin (3.9%) [11]. Zeaxanthin, violaxanthin, antheraxanthin, and also including the carotenoids lycopene being owned by D. salina which serves to enhance the immune system from pathogens [12]. D. salina extract at a dose of 300 mg/kg feed resulted in the highest SR value is 33% which is shown to inhibit the death of fish and shrimp from a virus [13]. The fat contained in D. salina serves as energy reserves as much as 9 kcal in 1 gram, keeps the stable temperature in the fish body and prevents inflammation in case of infection due to viruses or other pathogens. β-carotene is also a source of vitamin A and vitamin C of 352,000 IU to boost immunity [14]. β-carotene and antioxidants superoxide pressing function by stabilizing cellular compounds [15].

Detailed knowledge of the molecules associated with the immune system can be used for aquacultures such as biomarkers and vaccines. Nuclear Factor-kappa Beta (NF-kB) is found in all types of animal cells related to cellular responses to stimulation. NF-kB is a protein heterodimer composed of different combinations of the Rel family of transcription factors [16]. NF-kB controls the transcription of DNA that plays an important role in regulating the immune response to infection [17]. NF-kB also acts to control cell survival in the face of oxidative stress, cytokines, free radicals, ultraviolet radiation and regulate the immune response against the virus [18].

NF-kB can be activated by inflammatory cytokines such as TNF (Tumor Necrosis Factor) or IL-1 (Interleukin-1), lymphokines, free radicals, viral or bacterial infections, radiation and activation of B or T cells [19]. If there are VNN, NF-kB will be active then going towards the nucleus and induces the proteins of the immune system against viral infections [20]. The expression of NF-kB involves two major signaling pathways, canonical and non-canonical (alternative), both essential to regulate immune and inflammatory responses [5]. Functionally, the canonical NF-kB is involved in almost all aspects of the immune system, while the non-canonical NF-kB evolved as additional signals in cooperation with the canonical pathway in regulating the function of the adaptive immune system [21].

2. Materials and methods
2.1 Sampel collection
Animal tests used in this study was cantang grouper size of 7-9 cm were obtained from BPBAP Situbondo. Before exposed with VNN, cantang grouper was fed with pellets that contain extracts of D. salina with dose of 0, 250, 300, 350 and 400 mg/kg feed for 10 days. To know that VNN has an infected fish test, the PCR test was performed. The brain of cantang groupers either healthy or infected organs was taken, then placed in a tube and given a 10% formalin for 2-3 days to be tested NFkB.

2.2 SDS-page testing
Protein extracts that have been obtained were analyzed using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method using electrophoretic gel 12.5%. The sample buffer is first added to the protein sample (1:1) in Eppendorf, heated at 100 °C for 5 minutes in 5 mm Tris HCl solvent at pH 6.8; 5% 2-mercapto ethanol; 2.5% w / v sodium dodecyl sulfate, and 10% v / v glycerol. Protein samples were cooled at 20 °C. Analyzed was carried out at a constant speed of 20 mA for 40-50 minutes or tracking dye 0.5 cm from the base of the gel. After that, the protein samples were stained with brilliant blue comas, then destaining to remove the colorant gel and suppress the protein band.

2.3 Expression test NFkB
Fixed brain organs are cut with a thickness of 2-3 mm, then inserted into a water bath and taken with glass object smeared by albumin glycerin. Staining procedures begins with heating the sample to a glass object coated with Poly L-Lysine in an incubator at 80 °C for 1 hour. Next, deparaffinization with xylol I, II, III (15 minutes each). Slide inserted into absolute ethanol, alcohol 90%, and 80% respectively for 15 minutes, then rinsed with distilled water. After that, the sample was put into a 1.6% H2O2 in methanol for 20 minutes. Samples were retrieval antigens by immersing, heated in the
decloaking chamber, then cooled at room temperature for 30 minutes and rinsed with distilled water. The samples were stored in PBS, dropped by primary antibody and incubated one night. Slide dropped by simple stain MAX PO incubated for 1 hour, dropped with DAB incubated 10 minutes, then washed with running water 5-7 minutes. After that, slides stain with mayer hematoxylin 2-3 minutes and soaked in saturated carbonate lithium for 3 minutes. The last stage is direct observation through a microscope and counted using methods immunoreaction.

2.4 Data analysis
Immunoratio data analysis using software that used to calculate the amount of expression of NF-kB contained in the nucleus, NF-kB was expressed to be brown, the value displayed in the form of % (percent) [22].

3. Results and discussion
3.1 SDS-PAGE
The purpose of the SDS-PAGE test is to determine the molecular weight of proteins that can induce VNN. Based on the fluorescent marker and the sample infected by VNN, known that they can infect cantang grouper on molecular weight 35 kDa and 50 kDa. At the A2 and A1 organs obtained molecular weight 37.65 kDa; 43.40 kDa; 45.42 and 48.42 kDa (Figure 1).

![Figure 1. The results of SDS-PAGE analysis cantang grouper: (A1) The organs of the brain, (A2) Organ eye.](image)

3.2 NFkB test
Test expression of NFkB is one method that can be used to detect a virus-infected cell. NFkB expression was infected with the virus will show a brown color, whereas uninfected are blue. The more the brown color is obtained, showing how large a viral infection of cantang grouper. The part that will be seen is the NFkB expression in the brain and eye regions of cantang grouper. Based on the research that has been done, D. salina extract can lower NFkB expression in the brains of fish as in Figure 2 and Table 1.
Table 1. NF-kB Test Results Brain Cantang Groupers

| D. salina extract dose (mg / kg feed) | The expression of NF-kB brain (%) |
|--------------------------------------|----------------------------------|
| normal fish                          | 23                               |
| 0                                    | 66                               |
| 250                                  | 59.7                             |
| 300                                  | 50.7                             |
| 350                                  | 48.2                             |
| 400                                  | 31.2                             |

VNN can infected fish in three ways: through the cells of the digestive tract epithelia, through the axons that exist on the surface of cells and through the bloodstream. Groupers injected by VNN in the intramuscular can replicate in the cytoplasm or nucleus. VNN infection in fish through intramuscular very quickly spread to infect the host through the peripheral nerves found in the muscle, and then into the central nervous system, causing loss of balance fish swim. VNN spread and replicate in the peripheral nervous system in the muscle tissue edges, then transported through the axon to the network spine and VNN will go directly into the central nervous system (brain) through the blood circulation [23]. Stimulation of virus infection can be inflammatory signals that activate NF-kB.

Figure 2. The expression of NF-kB cantang grouper fed with D. salina extract with various doses of treatment. NFkB expression is shown of a brown color, while uninfected blue. (A) The fish control, (b) Dose 0 / kg of feed, (c) 250 mg / kg feed, (d) 300 mg / kg diet, (e) 350 mg / kg diet, (f) 400 mg / kg.
Based on the above table it is known that *D. salina* extract at a dose of 400 mg/kg of feed is best able to express NFkB, which amounted to 31%. Figure 2. The expression of NF-kB drops due to the extract of *D. salina* has a phenol group compounds capable of acting as an anti-microbial [24]. NF-kB can be activated by exposure to signals from LPS (*lipopolysaccharides*) or inflammation (*cytokines*) such as TNF or IL-1, a growth factor, a specific viral or bacterial infection [16]. NF-kB serves as a target TNF inhibition. Inhibition of TNF expression by phenolic compounds occur in the transcription process and simultaneously inhibits the expression of NF-kB [25]. Phenolic compounds block the expression of NF-kB induced by LPS, so that NF-kB will be restricted to replicate [26]. The expression of NF-kB can induce overexpression of pro-inflammatory genes in the nucleus, where NF-kB activated it will activate proteins that respond to inflammation. Phenol compounds can inhibit inflammation by inhibiting the expression of NF-kB followed by iNOS and COX-2 [27].

General mechanisms of microalgae as an antiviral active compound will react against viruses based on virus glycoprotein load and when the virus attaches to the cell. They can again against the virus with the amino acid glycoprotein virus and block the virus adsorption process [28]. Phenol compounds are antiviral compounds that can act to block the protein kinase thereby preventing overexpressed NF-kB [29]. Tannins are one polyphenol compounds that have a high molecular weight consisting of hydroxyl and carbohydrate groups. Tannins have a role as an antibacterial, antiviral and anti-inflammatory with NF-kB immune responses [30]. Tannin basically is derived from plants and can be hydrolyzed to glucose and gallic acid which is composed of flavonoids [31]. Tannins can against virus penetration into nuclei. The mechanism of tannin inhibits the growth of viruses by destroying the cell wall cytoplasm that causes structural damage virus to become easily damaged. Tannins also inhibit hydrolytic enzymes such as proteases and inhibit the transport of viral proteins [32]. Flavonoids are another type of polyphenol that has been known as antimicrobial, antioxidant, hypo-allergenic, antiviral and anticancer [32]. Flavonoids affect enzyme function, immune system which can inhibit the inflammatory process by a viral infection [33]. Flavonoids function as antimicrobial, antiseptic and disinfect. Flavonoids inhibit viral reverse transcriptase enzyme that can’t be synthesized viral RNA into cDNA and the virus can’t replicate [34]. The antiviral mechanism of flavonoids to inhibit the binding of the viral polymerase and nucleic acids (viral protein) so that the virus does not divide or replicate [35].

NF-kB that bound to the antibody expressed NF-kB which is a polymer labeled NF-kB (*mouse monoclonal* IgG) which is a combination of amino acid polymer, peroxidase, and rat primary antibodies (mouse antibody) are stored in a buffer of MOPS (3-Morpholinopropanesulfonic acid) pH 6.5 and antibacterial substances [36] [33]. Enzymatic activity of these components produces sediment color so that when the brain samples of cantang grouper given antibody NF-kB then tissue samples will be bound by these antibodies and a brown color which means that there are NF-kB are expressed and unexpressed are blue [37].

4. Conclusion
Based on the research, it is known that the addition of *D. salina* extract to feed can reduce the percentage of NF-kB expression of the brain of the cantang grouper that has been infected with VNN. At a dose of 400 mg/kg of feed, the highest percentage of NF-kB expression was reduced compared to other treatments.

5. References
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