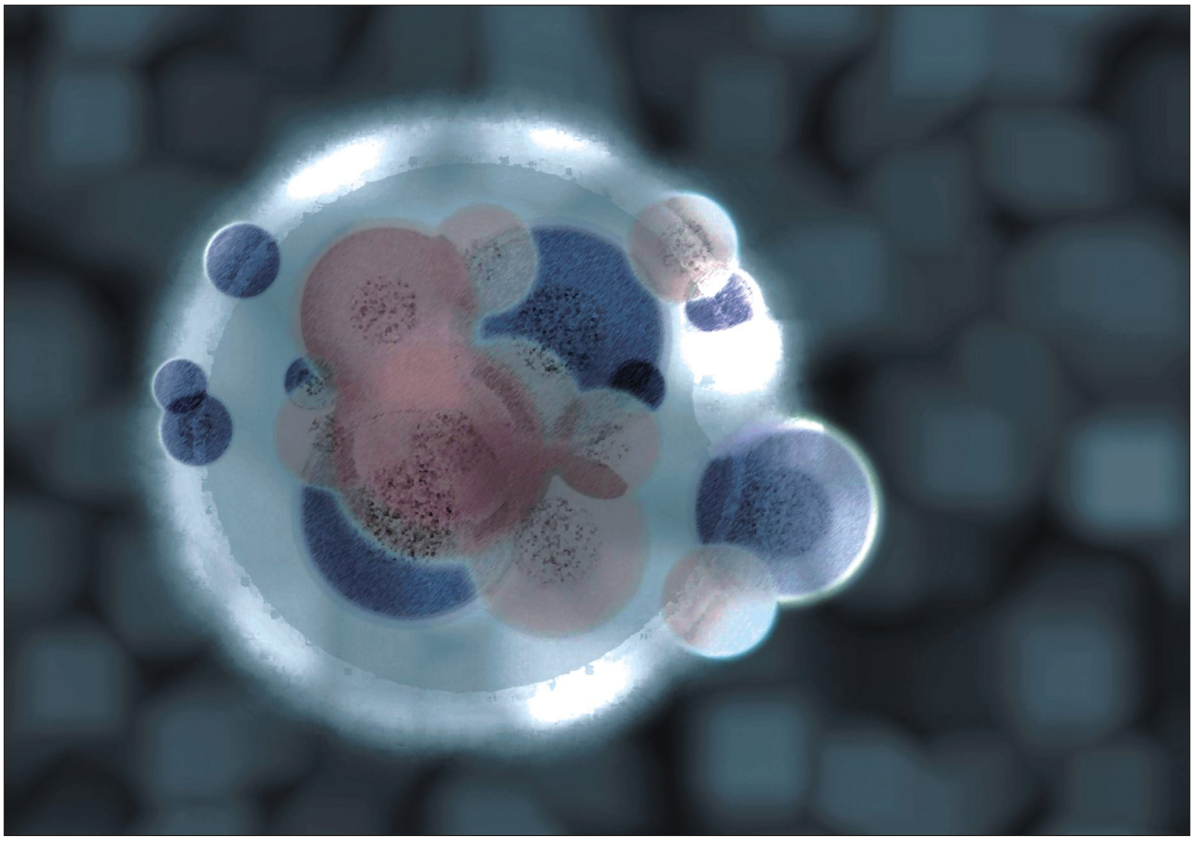

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Research Article
Dietary Fucoidan from *Padina boergesenii* to Enhance Non-specific Immune of Catfish (*Clarias* sp.)

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Abstract

Background and Objective: Fucoidan is a sulfated polysaccharide with diverse biological activity including immune-potentiating activity, but such activity is little evaluated in fish. This research aimed to evaluate the non-specific immune-stimulating activity of fucoidan from a tropical brown alga, *Padina boergesenii* orally administered in catfish (*Clarias* sp.). **Materials and Methods:** A brown alga of *P. boergesenii* used was collected from Jepara intertidal coastal zone, Central Java, Indonesia and the species was identified base on morphological characteristics. Fucoidan was isolated by acidic methods. $CaCl_2$ was added to separate fucoidan from alginate and then the fucoidan was precipitated by ethanol. Fucoidan was characterized by Fourier Transformed Infra Red (FT-IR) spectrometry and chemical analyses. Catfish was orally administered with fucoidan at 2,000, 4,000 and 6,000 mg kg^{-1} feed. Non-specific immune parameters of phagocytic activity, phagocytic index, superoxide anion (SOA) activity, superoxide dismutase (SOD), serum antibacterial activity, respiration burst and serum bacterial agglutination were evaluated. **Results:** Identification based on morphological characteristics of the brown alga used in this research was *Padina boergesenii* Allender and Kraft. FTIR spectrum of fucoidan extracted from *P. boergesenii* was identical to the bands demonstrated by FTIR spectrum of fucoidan standard from *Fucus vesiculosus*. Chemical analysis showed that fucoidan from *P. boergesenii* composed by sulfate 22.4%, uronic acid 7.5% and sugar content 40.18%. Dietary fucoidan increased SOA activity, serum antibacterial activity, serum agglutination and phagocytic activity ($p<0.05$). However, SOD activity and phagocytic index was not affected by supplemented fucoidan. **Conclusion:** This fucoidan significantly enhanced innate immunity of catfish at 4,000-6,000 mg kg^{-1} feed implying the potential of fucoidan to be used for feed supplement in stimulating non-specific immune response in fish culture.

Key words: Brown seaweed, fucoidan, non-specific immune, *Padina*, catfish, innate immunity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Brown seaweeds contain a large amount of biologically active polysaccharides, mainly alginate, laminarin and fucoidan which account to 40-80% of dry defatted seaweed biomass[1]. Fucoidan is attracted most attention by researcher as this substance has notable wide spectrum bioactivities[2], but low toxicity[3].

Fucoidan is a polysaccharides sulfate and mainly contain fucose in the cell walls and intercellular spaces of brown seaweeds. In addition, fucoidan is also composed by glucose, xylose, mannose, galactose and uronic acid, even acetyl and protein group. Bioactivity of fucoidan may vary depending on the source of seaweed, chemical structure and composition[4]. According to Sinurat and Marraskuranto[5], fucoidan is the most widely distributed and relatively cheap compared to other sulfate polysaccharides.

Fucoidan has extensively studied because of its multi bioactivity such as anticoagulant, antitumor, immunostimulant, antivirus and anti-inflammatory activities. Isnansetyo *et al.*[6] reported that injection of fucoidan isolated from *S. cristaefolium* increases phagocytic activity, total plasma protein, leukocrit and leukocyte count significantly, but does not affect the differentiation of leucocytes and phagocytic index. Futhermore, fucoidan activates and enhances the immune systems to protect against invading pathogens[3]. Yang *et al.*[7] proved that dietary fucoidan from *S. horneri* significantly influences the non-specific immune response parameters such as superoxide dismutase (SOD), phagocytic index (PI) and respiratory burst (RB) in juvenile yellow catfish, but does not significantly decrease in mortality when the fish is challenged with *Aeromonas hydrophila*. According to El-Boshy *et al.*[8], dietary fucoidan improves its resistance to immunosuppressive stressful of heavy metal pollution and could be used by African catfish farmer of *C. gariepinus* as immunostimulant. Although several researchers have been studied fucoidan, a lack information is found regarding to fucoidan from *Padina*, its chemical characters and immunostimulating activity in freshwater fish. Almost researcher studied fucoidan from *Sargassum* or commercial fucoidan from *Fucus vesiculosus*[9].

The aim of this research were to isolate fucoidan from *Padina boergesenii* Allender and Kraft and to know the yield and non-specific immune response activity of isolated fucoidan in catfish. This is first report on the evaluation of fucoidan of *P. boergesenii* to enhance non-specific immune response in catfish.

MATERIALS AND METHODS

Sampling and identification: The raw material used in this work was a brown alga of *Padina* sp. collected from Jepara intertidal coastal zone, Central Java, Indonesia. The sample was washed with freshwater and air-dried without direct sunlight kept and packaged into plastic bags. Identification of this *Padina* sp. was conducted base on morphological characteristics. The research was carried out at the Laboratory of Hidrobiologi, Universitas Gadjah Mada and Laboratory of Biological Pharmacy, Universitas Muhammadiyah Purwokerto during February, 2016-February, 2017.

Extraction of fucoidan: Extraction was done by using acid methods[10]. Maceration was conducted for 100 g of dried alga in 1 L of 0.1 N HCl for 24 h at room temperature, then filtration with nylon cloth was carried out. The retentate was remacerated in 1 L of 0.2 N HCl for 2 h at 70°C and refiltration. The obtained filtrates were combined, filtered through Whatman paper No. 40 and evaporated to obtain a final volume of 150 mL using rotary evaporator.

Ethanol precipitation was conducted by adding 3 times volume of 95% ethanol while stirred, then allowed for 2 h. Centrifugation was conducted at 8000 g, 4°C for 5 min and the obtained pellet was dissolved in distilled water at pH 2. $CaCl_2$ was poured to obtain the final concentration of 2 M to separate fucoidan from alginate. Ethanol was used to precipitate fucoidan from the supernatant.

Spectrometry of fucoidan by FT-IR: The analysis of fucoidan was done by FT-IR spectrophotometer (Spectrum One) in potassium bromida and the spectrum was compared to that of standard fucoidan from *Fucus vesiculosus* (Sigma). Wave number between 500 and 4,000 cm^{-1} was used to record the fucoidan sample.

Chemical analysis of fucoidan: Total sugar content of fucoidan was determined by the Masuko *et al.*[11] method using phenol-sulfuric acid and the absorbance was measurement at 490 nm using Elisa Reader (Heales type MB 580). The sulfate content was quantified based on the $BaCl_2$-gelatin method using Na_2SO_4 (Merck) as the standard[12], while uronic acid content used the glucuronat acid standard[13]. Absorbance measurements were recorded by Elisa Reader (Heales type MB 580) at 360 nm for sulfate and 450 nm for uronic acid.

Dietary fucoidan and blood sampling: Catfish (*Clarias* sp.) (100±6 g) were reared in rounded plastic tank (0.5 m^3). Four tanks were used to reare 4 fishes each thank as individual replication. The fish were fed with a commercial feed (P.T. Central Proteina Prima, Sidoardjo, Indonesia) at feeding rate of 3% of biomass/day. After acclimatization for 1 week, the fish were fed with the commercial feed containing fucoidan at 2,000, 4,000 and 6,000 mg kg^{-1} and control. Feeding was conducted twice a day at 8:00 am and 5:00 pm.

Phagocytic activity (PA) and phagocytic index (PI): The preparation of leucocyte was conducted according to the method previously published by Isnansetyo *et al*.[6]. Fifty microliter of leucocytes was poured into a 96-well microplate and filled in the same volume of 10^8 cells mL^{-1} formalin-killed *Staphylococcus aureus*. After incubation at 30°C for 30 min, each sample was smeared on object glass, fixation with ethanol 96%, air-dried and then stained with 0.15% safranin. The PA and PI was observed under microscope at 1,000x magnification from 100 phagocytes each slide.

Nitroblue tetrazolium (NBT) activity test: Heparinized 15 µL of blood was put in microtube and poured with 15 µL of 0.2% NBT in 0.85% NaCl steril, then incubation for 30 min in room temperature. N-N Dimethylformamide (Merck) at a volume of 600 µL was added and then centrifuged at 3,000 rpm for 5 min. The absorbance of the supernatant was determined by a spectrophotometer[14] (UVmini 1240, Shimadzu) at 540 nm.

Superoxide dismutase (SOD) activity test: Measurement of SOD activty was conducted using nitroblue tetrazolium (NBT) by addition of riboflavin. About 100 µL of heparinized whole blood was added with 0.5 mL of buffer phosphate (50 mM, pH 7.8), homogenized and centrifuged at 6,000 g, 4°C for 5 min. The supernatant was removed, heated at 65°C for 5 min and centrifugated to obtain supernatant.

One hundred microliter of supernatant was added with 20 µL mixture of NBT (0.1 mM EDTA, 13 µM methionine, 0.75 mM NBT and 20 µM riboflavin in 50 mM phosphate buffer, pH 7.8) were placed under fluorescent light for 2 min, then the absorbance of the sample was measured[15] at 560 nm. The results were expressed as a relative enzyme activity.

Serum antibacterial activity test: *A. hydrophila* was cultured in Tryptic Soy Broth (TSB) medium (Merck) at 30°C for 24 h. The bacterial density was estimated using Mc. Farland standard and diluted to obtain a density of 10^6 cells mL^{-1}. About 100 µL bacterial suspension was transferred to a 96-microwell plate, added with 100 µL of serum sample and incubated at 30°C for 30 min. After incubation, 20 µL mixture was taken and added into 180 µL sterile 0.8% NaCl and serial diluted with sterile 0.8% NaCl. Finally, 100 µL of serially diluted samples were poured on a selective medium of Glutamat Starch Phenile (GSP) (Merck). The plate was incubated at 30°C for 24 h and colony was counted[16].

Serum bacterial agglutination test: Bacterial density of *A. hydrophila* was estimated by using Mc. Farland standard to obtain a bacterial density of 10^8 cells mL^{-1}. Serum sample with a volume of 20 µL was aliquoted in a 96-microwell and serially diluted with the same volume of sterile phosphat buffer saline (PBS). *A. hydrophila* suspension with a volume of 20 µL was added to each well and incubated[16] at 25°C for 1 h.

Data analysis: The data were analyzed by one way analysis of variance (ANOVA) with *post hoc* Duncan multiple comparison test using SPSS 21.0 software (IBM). The significant differences among treatment groups, p-values smaller than 0.05 ($p<0.05$) were considered as statistically significant.

RESULTS

The brown alga sampel used in this research was morphologically identified to be *Padina boergesenii* Allender and Kraft. The fucoidan yield was 4.26±0.67% of dry matter of the seaweed. Chemical analyses indicated that the fucoidan contained 22.4% sulfate, 7.5% uronic acid and 40.18% total carbohydrate. FT-IR spectrum of this fucoidan showed the bands at 3,443.87, 2,933.46, 2,115.99, 1,667.50, 1,496.80, 1,390.76, 1,255.38, 1,101.36, 1,062.99 and 662.63 cm^{-1} and agree with bands showed by spectrum of fucoidan standard (Fig. 1).

Dietary fucoidan was able to stimulate phagocytic activity (PA) at day 4 in all treatments and showed significantly different compared to control ($p<0.05$) (Fig. 2). However, the effect was not noticeable at the further blood sampling periods ($p>0.05$) (Fig. 2). On the other hand, dietary fucoidan did not affect ($p>0.05$) the phagocytic index (PI) (Table 1) and SOD of catfish (Table 2). These results indicated that the PA was one of non-specific immune responses of catfish that was improved by the oral administration of fucoidan, but PI and SOD were two parameters that was not improved by such treatments.

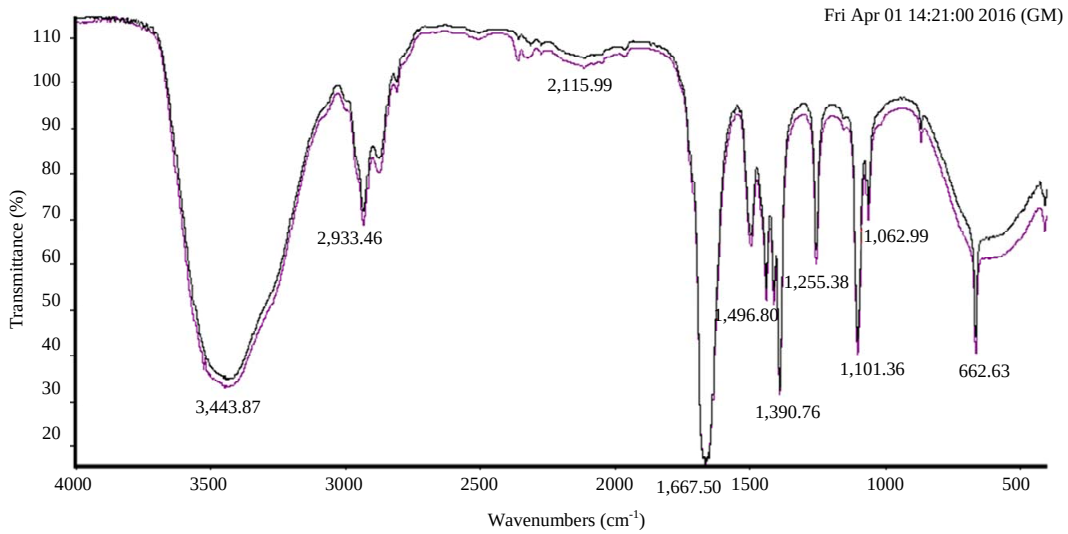

Fig. 1: FT-IR spectra of fucoidan from *P. boergesenii* and standard fucoidan (Sigma). Red Line (standard fucoidan); black line (*Padina* fucoidan)

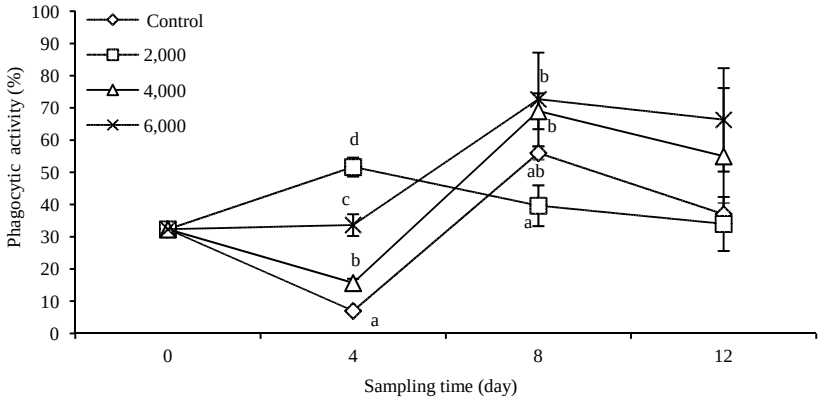

Fig. 2: Phagocytic activity of phagocytes of catfish orally administered with fucoidan from *P. boergesenii*
Same letters at the same sampling time indicates insignificant difference (p>0.05), T0: Control, T1: 2,000 mg kg^{-1} PF, T2: 4,000 mg kg^{-1} PF, T3: 6,000 mg kg^{-1} PF

Table 1: Phagocytic index of phagocytes of catfish orally administered with fucoidan from *P. boergesenii*

	Days			
Dose (mg kg^{-1})	0	4	8	12
Control	1.97	2.13±0.78^{a}	1.71±0.15^{a}	2.06±0.27^{a}
2000	1.97	1.99±0.38^{a}	1.76±0.22^{a}	1.88±0.54^{a}
4000	1.97	2.51±0.62^{a}	2.05±0.07^{a}	2.36±0.55^{a}
6000	1.97	1.75±0.37^{a}	1.67±0.28^{a}	2.11±0.47^{a}

Same superscript letter in one column indicates insignificant difference (p>0.05)

Table 2: SOD activity of whole blood of catfish orally administered with fucoidan from *P. boergesenii*

	Days			
Dose (mg kg^{-1})	0	4	8	12
Control	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{a}
2000	1.00±0.00^{a}	0.78±0.39^{a}	1.17±0.76^{a}	0.80±0.35^{a}
4000	1.00±0.00^{a}	0.72±0.25^{a}	1.50±0.50^{a}	0.66±0.15^{a}
6000	1.00±0.00^{a}	1.11±0.19^{a}	1.00±0.00^{a}	0.90±0.47^{a}

Same superscript letter in one column indicates insignificant difference (p>0.05)

Significant increase in NBT activity by oral administration of fucoidan was found in day 8 and 12 ($p<0.05$) (Fig. 3). More noticeable increase in the NBT was found in the longer period of oral administration of fucoidan. The

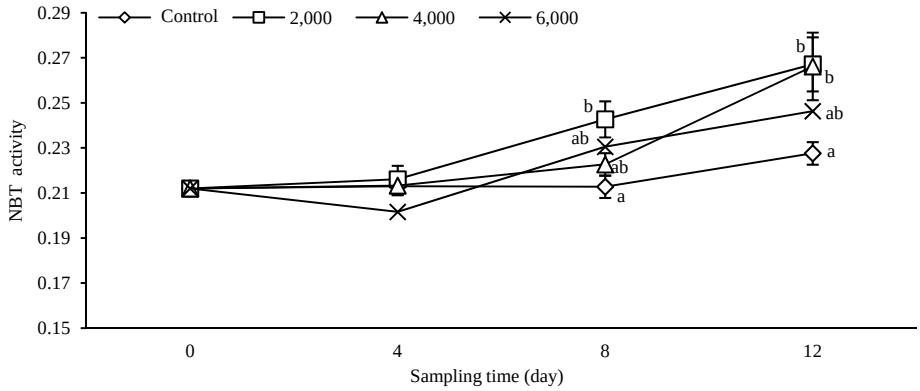

Fig. 3: NBT activity of whole blood of catfish orally administered with fucoidan from *P. boergesenii*
Same letters at the same sampling time indicates insignificant difference (p>0.05), T0: Control, T1: 2,000 mg kg^{-1} PF, T2: 4,000 mg kg^{-1} PF, T3: 6,000 mg kg^{-1} PF

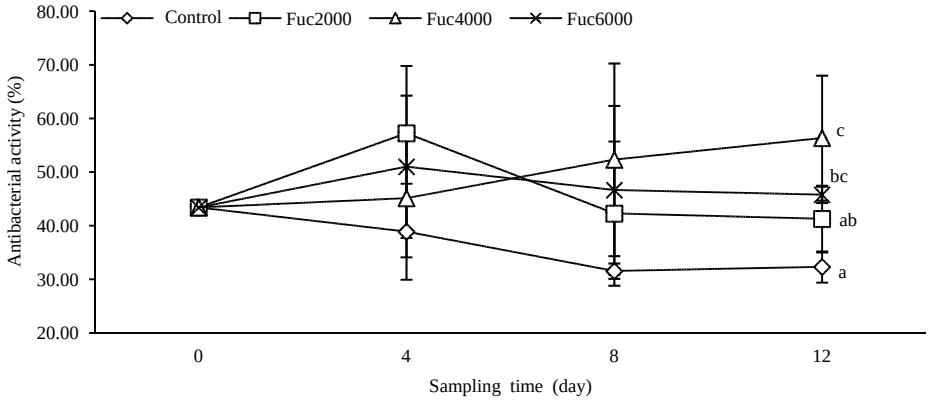

Fig. 4: Antibacterial activity of serum of catfish orally administered with fucoidan from *P. boergesenii*
Same letters at the same sampling time indicates insignificant difference (p>0.05), T0: Control, T1: 2,000 mg kg^{-1} PF, T2: 4,000 mg kg^{-1} PF, T3: 6,000 mg kg^{-1} PF

stimulating activity of fucoidan to NBT activity might be correspond to the stimulating activity of this substance to PA.

Figure 4 indicates that the oral administration of fucoidan was not able to increase the serum antibacterial activity promptly, but significantly improved the activity after 12 days of administration. This result indicated by statistically insignificant different ($p>0.05$) of serum antibacterial activity at day 4 and 8 of blood sampling period, but significant different in day 12 of the blood sampling ($p<0.05$). These present study results suggested that the serum antibacterial activity was one of the non-specific immune parameters that might be improved after 12 days of oral administration of fucoidan.

The agglutination test demonstrated that serum natural agglutination of catfish was effectively improved by the oral administration of fucoidan. Consistence significant differences ($p<0.05$) of the serum natural agglutination were observed at the beginning of blood sampling (days 4) until the end of the blood sampling periods (day 12) after oral administration of fucoidan (Fig. 5). Although in the end of blood sampling period, the agglutination activities of all treatments including control group decreased in various levels, the statistical test indicated that the agglutination activity of all treatment groups were significantly higher than that of control groups.

DISCUSSION

Extraction of fucoidan from *P. boergesenii* used acid methods yielded 4.26±0.67% of dry matter the seaweed. For comparison, previous study[17] has demonstrated that fucoidan yield from *P. tetrastromatica*, *T. ornata* and *S. wightii* are

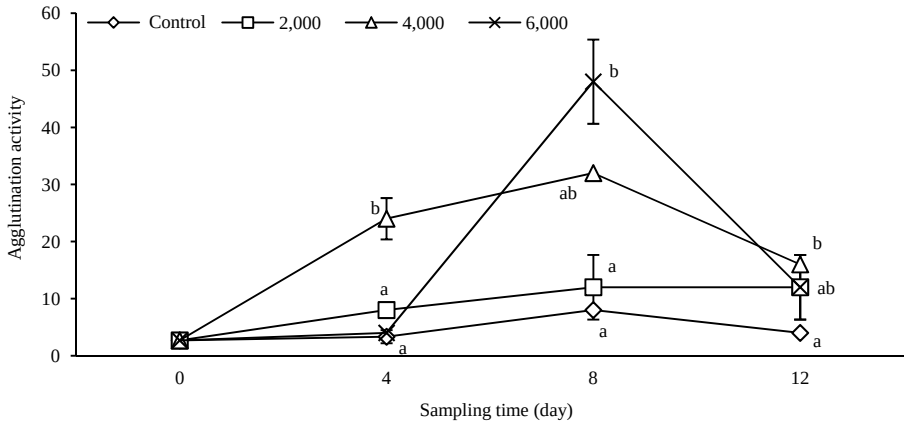

Fig. 5: Serum agglutination activity of catfish orally administered with fucoidan from *P. boergesenii*
Same letters at the same sampling time indicates insignificant difference (p>0.05), T0: Control, T1: 2,000 mg kg^{-1} PF, T2: 4,000 mg kg^{-1} PF, T3: 6,000 mg kg^{-1} PF

9.45±2.7, 5.53±2.5 and 3.91±1.04%, respectively. Yield of fucoidan varies depending on species, geographic location and season[17].

FTIR spectra showed the same typical bands of polysaccharide both for fucoidan extracted from *P. boergesenii* and standard fucoidan. Wave number of 3,444 cm^{-1} is assigned to hydrogen bonded O-H stretching vibration derived from a hydroxyl group[18-20]. Futhermore wave number of 2,933 cm^{-1} indicates the ring of C-H pyrenoid and C6 of fucose and/or galactose[20,21]. Wave number of 1,668 cm^{-1} indicates the presence of vibrations of asymmetric carboxylates O-C-O vibration[19]. The sulfate ester group which is the main component of the fucoidan appeared in the wave number of 1,255 cm^{-1} which indicates the presence of the S = O bond[19,21], close to the presence of O=S=O stretching vibration of sulfate esters at band[22] 1,258 cm^{-1}.

Phagocytic activities (PA) of phagocytes in catfish increased significantly in day 4 post-treatment ($p<0.05$) at 2,000; 4,000 and 6,000 mg kg^{-1} (Fig. 2). The increase in phagocytic activity at the beginning of this treatment was also accompanied by an increase in the percentage of monocytes and neutrophils. Monocytes or macrophages and neutrophils are required to phagocyte a foreign materials. Activation of monocytes and neutrophils in this study might be due to sulfate content in fucoidan. Sulfate content in this experiment reaching 22.4%, is higher than *S. glaucescens* (12.37%) and *S. horneri* (12.09)[23]. In general, sulfate content level directly coresponded to the higher biological activity of fucoidan[4]. Sulfate group in fucoidan is essential to bind macrophage cell surface receptors and plays a key role in the immune-regulating activity of macrophages[24]. Higher sulfate contents are reported in fucoidan extracted from *F. evanescens* (36.3%)[25] and *Turbinaria* conoides (38%)[19], but the non-specific immune-stimulating activity have not been reported yet.

Besides sulfate, uronic acid content also affects the bioactivity of fucoidan. The uronic acid levels in fucoidan may be a factor that determines the immune-activating capacity, at least the capacity of activating dendritic cells (DCs) and T cells[26]. In this present study, the content of uronic acid in fucoidan was 7.46%; quite lower than that of *S. stenophyllum*[27] 9.17%.

Dietary fucoidan in this research did not affect SOD activity. Ussually, increase in NBT activity is followed by increase in SOD activity in various level as the NBT activity level may determine the SOD activity to neutralize the damage. Additionally, the amount of free radicals affects the work of endogenous antioxidants. The work of endogenous antioxidants will be alleviated by a small amount of free radicals[28].

Significant difference on serum antibacterial activity was demonstrated in day 12 at 4,000 and 6,000 mg kg^{-1} (Fig. 4). This result proved that fucoidan from *P. boergesenii* is able to increase non-specific immune of catfish. The similar finding have been reported in fish and shellfish after treating with *S. wightii*[15]. Bactericidal activities in this experiment was assumed by higher production of O_2.(SOA)-free radical. This antibacterial activity corresponded to the NBT assay that increased on day 8-12 at 2,000-4,000 mg kg^{-1} (Fig. 3), as phagocytosis process produces ROS known as respiratory burst[29].

Besides SOA, serum antibacterial activity can be performed by neutrophils that secrete antimicrobial peptides (AMPs). According to Mulero *et al.*[30], AMPs which are secreted by granula from granulocytes, plays a role as bactericidal substances. This might be assumed that fucoidan modulates the function of various immune cells such as granulocytes including neutrophils. This also correspond to the fact that in day 12 dietary fucoidan at 4,000 mg kg^{-1} was in accordance with the increase in the percentage of neutrophil.

Diet of fucoidan exhibited a significant effect on the increase in agglutination of serum in day 4-12 at 4,000-6,000 mg kg^{-1} (Fig. 5). *In vitro* fucoidan enhances lymphocytes proliferation[31]. Lymphocytes produce antibody which bind with antigen to stimulate complement activity. Activated complement induces agglutination through the complement bind to carbohydrate moieties of microbe[32].

CONCLUSION

Dietary fucoidan from *P. boergesenii* stimulated non- specific immune response in catfish by increasing NBT, serum antibacterial, serum agglutination and phagocytic activites, but did not affect SOD activity and phagocytic index. Dietary fucoidan effectively increased non-specific immune at 4,000-6,000 mg kg^{-1} feed, suggesting that this fucoidan is potential to be used for feed supplement to stimulate non-specific immune in fish.

SIGNIFICANCE STATEMENT

This study discover the role and function of a brown alga of *P. boergesenii* Allender and Kraft that can be beneficial for increasing immunity and preventing diseases. This study will help the researcher to uncover the critical areas of biological source of seaweed that many researchers were not able to explore. Thus a new theory on immune-enhancing mechanism of fucoidan may be arrived at present for future.

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