Chloroform Fraction from Methanol Extract of Starfish *Acanthaster planci* Stimulates Catfish (*Clarias* sp.) Macrophage Immunomodulatory Activity

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Abstract

Catfish (Clarias sp.) is Indonesia's most important and popular freshwater commodity, widely cultured and intensively grown. This study is aimed to find out the effects of the chloroform fraction from a methanol extract of Acanthaster planci on non-specific immunity in Clarias sp. Thin Layer Chromatography analysis of the methanol extract's chloroform fraction yielded three fractions: FrKl3.1, FrKl3.2, and FrKl3.3. The FrKl3.3 fraction of Acanthaster planci had a significant influence on Phagocytic Activity at doses of 0.1 (54.09 ± 8.99 %), 0.3 (48.16 ± 3.34 %), 0.5 (50.39 ± 5.71 %), and 0.7 mg/kg Body Weight (46.58 ± 0.37 %). At 0.5 mg/kg Body Weight (4.03 ± 1.40 %), the Phagocytic Index of FrKl3.2 was significantly higher than the control. At 0.5 mg/kg Body Weight (38.22 ±1.43 cell 10^6/ml) and 0.7 mg/kg BW (40.41 ± 1.92 cell 10^6/ml), the number of leukocytes/white blood cells was significantly higher than the control. Nitroblue Tetrazolium and Total Plasma Protein were not significantly different from the control. Based on the results, the FrKl3.3 was leading on phagocytic capacity and number of leukocytes

Keyword: *Acanthaster planci, Clarias sp., chloroform fraction, non-specific immunity, macrophage*

INTRODUCTION

Catfish (*Clarias* sp.) is Indonesia's most important freshwater commodity (Gustiano *et al.*, 2021). Some cultivators are willing to increase production yield by implementing an intensive cultivation system and rearing fish in a small area with a high stocking density. *Clarias* sp. culture has some deserving perspectives in Indonesia (Diatin *et al.*, 2021; Gustiano *et al.*, 2021; Henriksson *et al.*, 2017). *Clarias* sp. is rich in nutrition, such as protein (16%), lipid (16.92%), fatty acid (24.51%) palmitic acid (29.57%), oleic acid, and 19.07% linoleic acid. Moreover, *Clarias* sp. is also have a valuable amino acids, specifically 13,818% essential amino acids (0.796% Histidine; 1.708% Threonine; 1.719% Valine; 1.205% Methionine; 2.613% Lysine; (Yu *et al.*, 2017). However, high stocking density in an intensive system will lead fish to become stressed, resulting in a decline in fish immunity. As a result, the fish will be susceptible to the disease (Pietrzak *et al.*, 2020; Purbomartono *et al.*, 2019).

Clarias sp. is susceptible to diseases caused by pathogen bacteria such as *Aeromonas hydrophyla* (Sellegounder *et al.*, 2018), *Flavobacterium columnare* (Harikrishnan *et al.*, 2018), and Channel catfish virus (Boon *et al.*, 1988). Chemical substances may effectively control pathogen, but the administration over a long-term period give notice of being harmful (Barman *et al.*, 2013; Chiu *et al.*, 2008; Chopra *et al.*, 2000; Purbomartono *et al.*, 2019; Yang *et al.*, 2018). Pathogenic bacteria can become resistant to chemical substances found in the water environment. Furthermore, the chemical substances' residues can be harmful to human health. By increasing the fish's immunity with an immunomodulator or natural chemical compounds, hazardous substances can be avoided. Furthermore, immunomodulator from natural product is environmentally friendly and does not leave any chemical residues that are harmful to human health (A. Cheng *et al.*, 2008; Jensch-Junior *et al.*, 2006; Magnadottir, 2010; Magnadóttir, 2006).

According to Chiu *et al.* (2008), the use of improper immunomodulators preserves the stressful

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conditions that negatively impact fish physiological conditions. It has been widely reported that natural substances such as polysaccharide polymers, alginates, fucoidan from seaweed, and glucoside from sea cucumbers can boost fish and shrimp immunity (A. Cheng *et al.*, 2008; W. Cheng dan Yu, 2013; Fuchs *et al.*, 2015; Purbomartono *et al.*, 2019; Yudiati *et al.*, 2019). El-Boshy *et al.* (2014) discovered that dietary fucoidan increased non-specific immune response and disease (*A. hydrophila*) resistance in *Clarias gariepinus*. Another study found that *Clarias gariepinus* could withstand a *Flavobacterium columnare* challenge when fed an *Agaricus bisporus*-enriched diet (Harikrishnan *et al.*, 2018).

The primary mechanism of the fish immune response is from aquatic organisms, which includes specific and non-specific responses such as memory, specificity, and recognition of foreign substances (Cheng and Yu, 2013; Mehana *et al.*, 2015; Pietrzak *et al.*, 2020). In terms of macrophage immunomodulatory activity, our previous study demonstrated the effectiveness of *Acanthaster planci* in the chloroform fraction of methanol extract (M. J. Achmad *et al.*, 2018). In fact, in nature, *A. planci* is a dangerous coral destroyer. Pratchett *et al.* (2009) found *A. planci* densities of 162 sea stars per hectare in Papua New Guinea, causing extensive coral mortality. This outbreak killed up to 55% of live corals, reducing overall coral cover from 42.4 % in 2005 to 19.1 % in March 2006. There must be some interesting compound in this particular Sea Star that needs to be explored. As a result, the use of *A. planci* as a potential immunostimulant must be exposed and assessed. This study is aimed to find out the immunomodulatory activities of three active fractions extracted from *A. planci*, namely FrKl3.1, FrKl3.2, and FrKl3.3, and its modulation of *Clarias* sp. non-specific immunity.

MATERIAL AND METHODS

A. planci was collected on Ternate Island, North Maluku, Indonesia. Ethyl acetate 99.5% (Sigma, USA), Chloroform 99% (Merck, USA), Methanol 95% (Merck, USA), NaOH 99% (Merck, USA), Silica gel 60 F254 plates (Merck, USA), preparative silica gel GF254 (Merck, USA), C18 (Reversed-phase, Pyrex USA), and KLT (E. Merck) plates were used as solvents and purification materials. HCl 37 % (Merck, USA), diethyl ether 99.7% (Merck, USA), acetone 99% (Merck, USA), ethanol 96% (Merck, USA), Calcium Chloride 99% (Sigma, USA), 100 mg/ml Bovine Serum Albumin/BSA 99% (Sigma, USA), test kit protein, Biorad methods (Sigma, USA), and fish blood samples were used in the haematology test. For the fish diet, commercial fish feed (Pokphand 781-2-a) was used.

Isolation and Purification of Bioactive Compound *A. planci*

Extraction and partition were carried out following the method used by M. Achmad *et al.* (2020); M. J. Achmad *et al.* (2018); M. J. Achmad *et al.* (2014). Methanol was used to extract and filter *A. planci* samples. To obtain the methanol extract, a rotative evaporator (Rotavapor® R-100, Buchi) was used at a temperature of 35°C. By separatory funnel, the methanol extract was partitioned to chloroform, hexane, and water in a 1:1:1 ratio. Partitioning was performed to obtain three fractions: hexane, chloroform, and water. After that, the fractions were evaporated. Since the water and hexane fractions were done in our previous study, in this present study we continue with the chloroform fraction (M. J. Achmad *et al.*, 2018).The chloroform fraction was then fractionated using eluent Chloroform: Methanol. This fractionation was then chemically profiled on a 5 cm column using Choloform: Methanol = 8:2 (10 ml) as eluent. FrKl3 fractions were chosen for hematological testing based on the results of the chemical profiling process.

Administration of Fraction FrKl3 *A. planci*

During fish cultivation, Pokphand 781-2-a commercial feed was administered. Catfish were raised in a fiber tank and feeding was done twice a day on an ad libitum basis. The average size of the fish was 40 cm long and 30 cm wide. The injection method was used to administer FrKl3 fraction to fish, and it was repeated three times. Three fish were injected with FrKl3 fractions at 0.1 mg/kg BW, 0.3 mg/kg BW, 0.5 mg/kg BW, and 0.7 mg/kg BW in each treatment. Three days after the first injection, the injections were repeated. The blood draw occurred after the second injection and on the fifth day following the second injection.

Hematology Test

Immunomodulatory activity of fish injected with FrKl3 fractions to non-specific defenses was assessed using hematological parameters such as macrophage phagocytosis, number of leukocytes, nitroblue tetrazolium (NBT), total plasma protein, and leukocyte differentiation (monocytes, neutrophils, and lymphocytes), as well as physiological parameters (hematocrit and leucocyte). The hematological parameters were observed using the techniques followed by M. J. Achmad *et al.* (2014); A. Cheng *et al.* (2008).

Phagocytosis Test

Blood fish sample was carried out using a syringe which was previously coated with EDTA solution and the sample was then transferred into a microtube, immediately. The blood was then drawn using a capillary tube and centrifuged at 1500 rpm within 5 minutes. Afterward, the capillary tube was cut to take the white blood cells to be placed into a microtube. A total of 50 µl of white blood was inserted into the well of microtiter plate 96. Furthermore, the antigen *Staphylococcus* sp. with a volume of 50 µl was mixed into a microtiter plate containing white blood and mixed. Subsequently, it was incubated for 30 minutes and stained with 10% Giemsa solution in ethanol. 5 µl of incubated white blood was taken out using a micropipette and then dropped on a glass preparation to be examined. Before this, absolute methanol was used for fixation, and then dried, and followed by staining with 10% Giemsa solution for 15 minutes. The observation of macrophages was conducted using a microscope with 1000 times magnification, and the results of the observations were analyzed to measure the phagocytic activity (PA) and phagocytic index (PI).

White Blood Cell (WBC) Test

The calculation of white blood cells (leukocytes) was done by taking ± 50 µl of fish blood. Blood samples were taken using 0.5 of a hemocytometer tube and added with 1N HCl. It was then mixed before being observed with a hemocytometer under a microscope to calculate the number of white blood cells.

Total Plasma Protein Test

The measurement of total plasma protein was conducted by filling the microtube with 798 µl aqua bides according to the number of samples. Each microtube was added with 2 µl serum and a 200 µl protein test kit (Biorad) before being mixed using the pipette, and followed 15 minutes incubation. The absorbance was measured using a spectrophotometer at a wavelength of 595-610 nm, and the concentration of total plasma protein was then determined using a standard curve.

Nitrobluetetrazolium (NBT)

100 µl of fish blood was put into a microtube and added with 0.2% NBT solution at a similar volume. The mix was incubated for 30 minutes at 30^{o}C. After the incubation, a 50 µl sample was then put into 1 ml N-N dimethylformamide solution and then centrifuged for 5 minutes at 3000 rpm. The supernatant was observed, and its absorbance was measured using a spectrophotometer at a wavelength of 595-610 nm.

Statistical Analysis

The obtained data were tested for variance using analysis of variance (ANOVA), using R Studio application. If the analysis of the variance test revealed a significant difference, it was further tested using Tukey HSD multiple comparisons of means with a 95 % family-wise confidence level ($P<0.05$). NBT, PA, PI, total plasma protein, hematocrit, leucocrit, number of SDPs, and % of leukocyte types among the variables were examined.

RESULT AND DISCUSSION

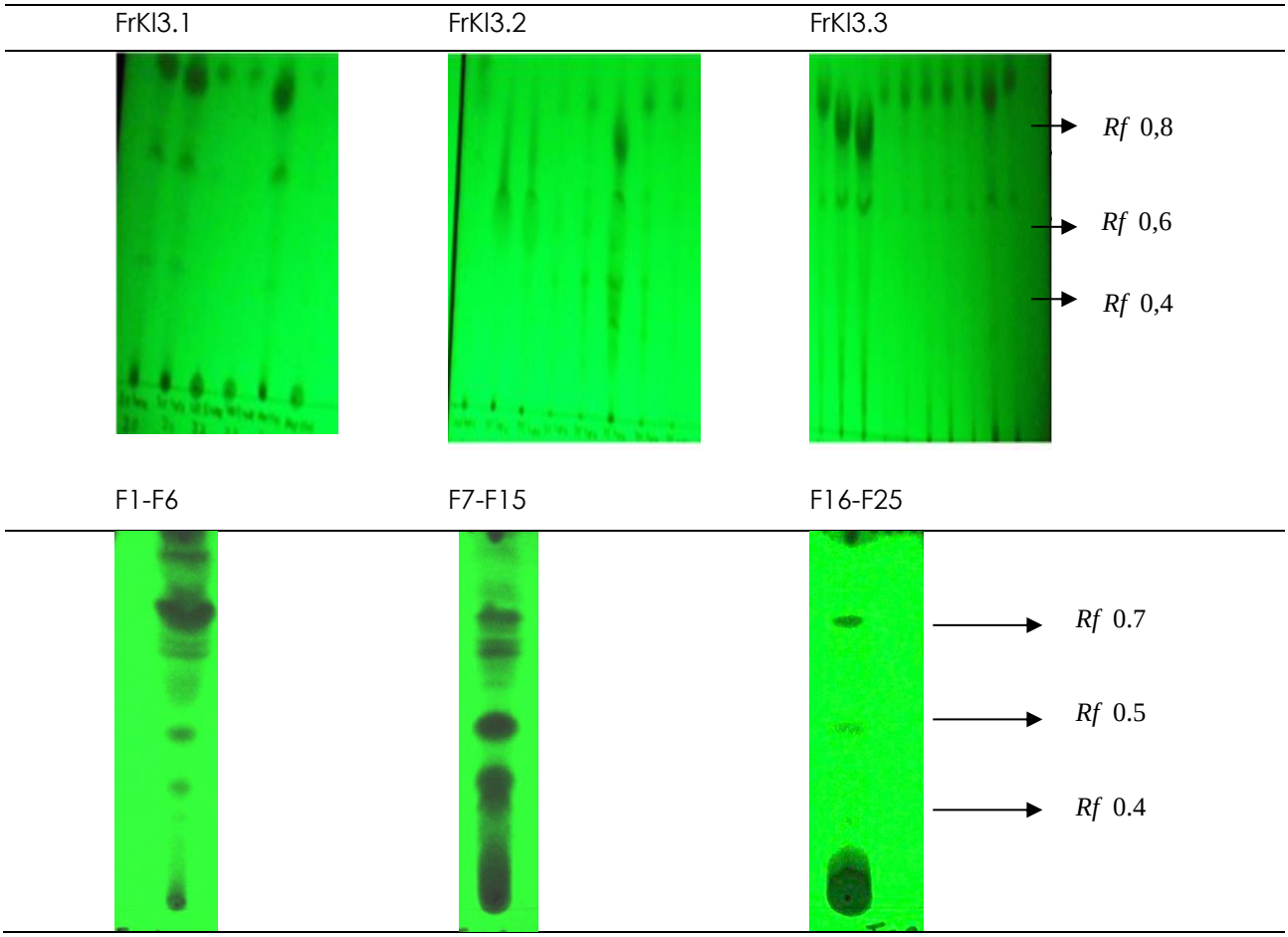
Chemical Profiling

Table 1 shows the results of fractionation with four elusions: CHCl3: MeOH = 2:1, CHCl3: MeOH = 2:2, CHCl3: MeOH = 1:2, and MeOH elution. FrKl3.1; FrKl3.2; and FrKl3.3, as a combination of several

fractions (F1-F6, F7-F15, and F16-F25), respectively, comprised the fractionation in 25 fractions. This classification is based on the similarity of retention factors (RF), and the combination of these fractions results in chemical profiling from the TLC plate. The first fraction (FrKl3.1) was developed from six fractions namely F1, F2, F3, F4, F5, and F6. The second fraction (FrKl3.2) was developed from nine fractions, namely F7, F8, F9, F10, F11, F12, F13, F14, and F15. The last third fraction (FrKl3.3) was developed from 10 fractions F16, F17, F18, F19, F20, F21, F22, F23, F24, and F25. FrKl3.1; FrKl3.2, and FrKl3.3 were then continued with chemical profiling with static phase SiO_2 60 F $_{254}$: mobile phase n-Hx: EtOAc = 9 :1 v/v. The spot developed was detected under UV light at 254 nm. The Rf value from major to minor of FrKl3.1; FrKl3.2 and FrKl3.3 were 0.7; 0.5; and 0.4, respectively. The major spot developed from FrKl3.1, followed by FrKl3.2., and the FrKl3.3 developed spot was minimum.

The differences in Rf value are based on the distance of the spot developed in the TLC plate. The Rf value of each compound is related to the polarity. In comparison with the polar compound, the nonpolar compound will move more distance on a plate, so, therefore leads to a higher Rf value. In contrast to the lower compound polarity, the higher compound polarity has a stronger interaction with silica gel (static phase) rather than the lower ones (Fair & Kormos, 2008; Reich & Schibli, 2007; Stoddard *et al.*, 2007). The chemical profiling in this present study is to monitor the fraction movement, identify the existing compound in the crude extract, and to define the compound purity (Reich & Schibli, 2007; Stoddard *et al.*, 2007). Hematological tests were then performed on the three fractions to get the best fraction.

Table 1. Chemical profiling of the FrKl3 fraction resulted in three (3) subfractions (FrKl3.1; FrKl3.2; and FrKl3.1)



Remarks: stationary phase of SiO_2 60 F $_{254}$: mobile phase of n-Hx: EtOAc = 9:1 v/v, detected under UV 254 nm.

Phagocytic Activity (PA) and Phagocytic Index (PI)

Figure 1 depicts the results of the phagocytic activity in three fractions of FrKl3. FrKl3.1 (Figure 1A) significantly exceeded control at doses of 0.3 (51.11±5.47 %) and 0.5 (50.96±0.64 %) mg/kg BW, whereas FrKl3.2 exceeded control (36.3±7.47 %) at a dose of 0.7 mg/kg BW. Furthermore, the PA value of FrKl3.3 significantly exceeded the control (30.99±4.51 %) at all doses, i.e., 46.58-54.09 % with a standard deviation of 0.37-8.99 %. Only a dose of 0.5 mg/kg BW FrKl3.2 (4.03±1.40 %) significantly outperformed the control (1.69±0.32 %) in the PI value (Figure 1B).

The chloroform fraction from the methanol extract of *A. planci* stimulated the nonspecific fish immune system in terms of phagocytic capacity (Phagocytic Activity and Index) and the number of leukocytes/white blood cells (WBC). At 0.3-0.5; 0.7 mg/kg BW and all doses, these three fractions of FrKl3.1, FrKl3.2, and FrKl3.3 had a significant effect on Phagocytic Activity. PA indicates an increase in cellular response, which eventually leads to an increase in humoral response (M. J. Achmad *et al.*, 2018; M. J. Achmad *et al.*, 2014; Jensch-Junior *et al.*, 2006; Yudiati *et al.*, 2019). The PI (Phagocyte Index) value was significantly higher than the control, FrKl3.2 at a dose of 0.5 mg/kg BW (4.031.40).

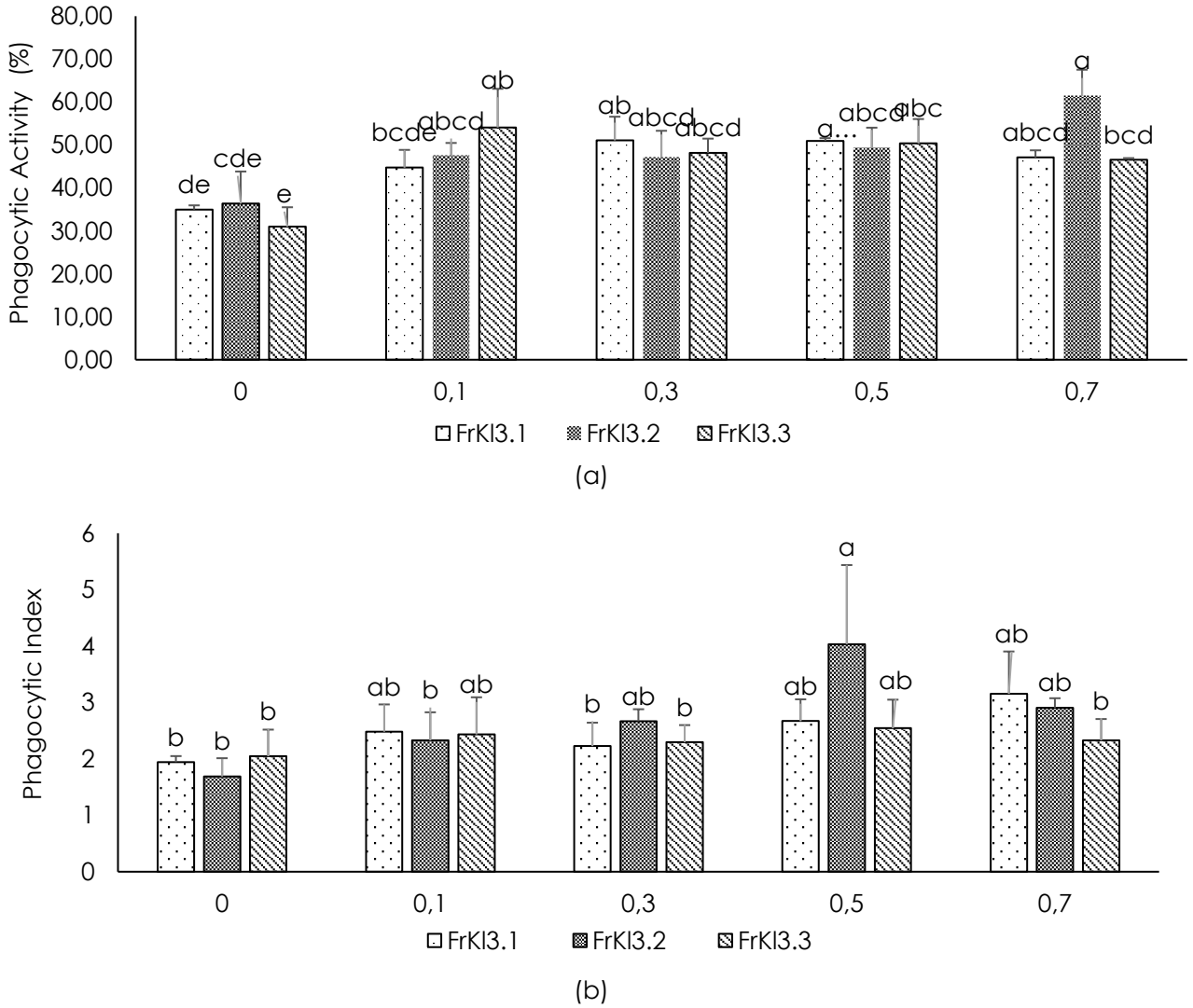


Figure 1. Phagocytic Activity (a) and Phagocytic index (b) of FrKl3.1; FrKl3.2; and FrKl3.3 on *Clarias* sp., analyzed with two-way ANOVA (R Studio Application). The significant differences were indicated by different notations. Phagocytic activity and index were significant in the dose and not significant in fractions. Only phagocyte activity had an interaction between dose and fraction.

According to Magnadóttir (2006); Purbomartono *et al.* (2019), if the PI value in the treatment group is higher than the control group, it indicates a stimulatory effect on increasing PI due to the active compound from the material test. In line with previous research, the hexane fraction of the methanol extract had a significant effect on Phagocyte Activity and Index (M. J. Achmad *et al.*, 2018).

Number of Leukocytes/White Blood Cells (WBC)

The results of total leukocytes in three fractions of FrKl3 are presented in Figure **2**. The FrKl3.1 fraction did not differ significantly from the control ($p>0.05$). FrKl3.2 at 0.5 mg/kg BW (38.22 ± 1.43 cell.10^6/ml) and FrKl3.3 at 0.7 mg/kg BW (40.42 ± 1.92 cell.10^6/ml) were significantly different from the FrKl3.2 control (21.34 ± 2.25 cell.10^6/ml) and FrKl3.3 control (22.63 ± 3.00 cell.10^6/ml, respectively. The number of leukocytes/white blood cells (WBC) significantly exceeded the control.

Fish health, environmental conditions, and fish species all influence the number of leukocytes (M. J. Achmad *et al.*, 2018; Chiu *et al.*, 2008; Jensch-Junior *et al.*, 2006; Purbomartono *et al.*, 2019; Yudiati *et al.*, 2019). Le *et al.* (2016) discovered two new pyrrole oligoglycosides, plancipyrrosides A and B (1 and 2), in methanol extract from *A. planci* Macrophages. Macrophages in RAW264.7 was able to suppress Lipopolysaccharide-Induced Nitric Oxide Production. NO is a signaling molecule that regulates physiological processes (such as vasodilation and neurotransmission) as well as the inflammatory response (Pacher *et al.*, 2007). It has cytotoxic properties against microbial pathogens but can also be harmful to host tissues (Korhonen *et al.*, 2005). Prevention of NO overproduction in living organisms via regulatory pathway control can aid in the treatment of high NO-mediated disorders without changing the physiological level of the NO radical point (Aktan, 2004).

Nitroblue Tetrazolium (NBT)

The results of NBT in three fractions of FrKl3 are presented in Figure 3. All FrKl3 fractions were not significantly different from the control ($p>0.05$). The respiratory level of macrophage cells was tested during the phagocytosis process using nitro-blue tetrazolium (NBT) solution, which was reduced into formazan when the reaction occurred with the radical oxygen produced by macrophage (Fuchs *et al.*, 2015; Nieves-Rodríguez *et al.*, 2018; Wang *et al.*, 2017). During phagocytosis, the macrophage frequently performed a respiratory burst, resulting in reactive oxygen species (ROS), which play an important role in pathogen defense. NADPH oxidase was a key enzyme in this process (Fuchs *et al.*, 2015; Nieves-Rodríguez *et al.*, 2018; Vetvicka *et al.*, 2013; Wang *et al.*, 2017).

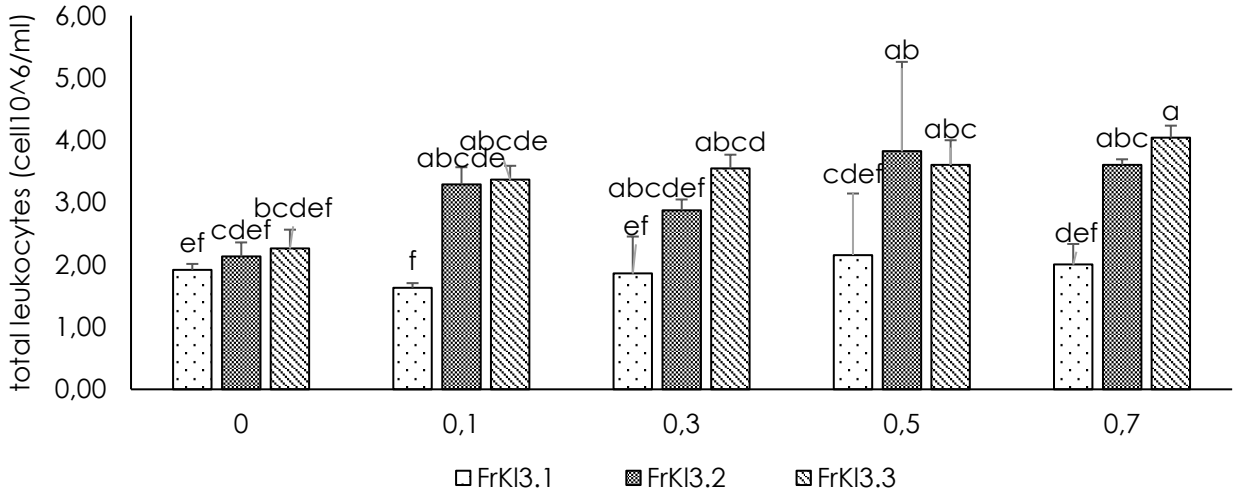


Figure 2. Total leukocytes of FrKl3.1; FrKl3.2; and FrKl3.3 on *Clarias* sp., analyzed with two-way ANOVA (R Studio Application). The significant differences were indicated by different notations. Leucocyte was significant in dose and fractions. Leucocytes have no interaction between dose and fraction.

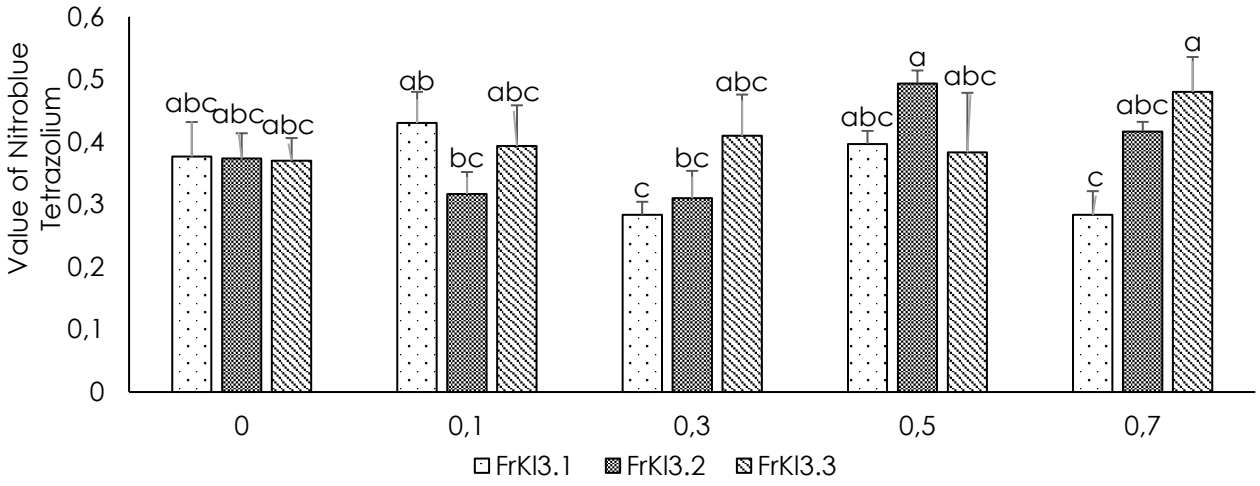


Figure 3. Values of nitroblue tetrazolium (NBT) of FrKl3.1; FrKl3.2; and FrKl3.3 on *Clarias* sp. were analyzed with two-way ANOVA (R Studio Application). The significant differences were indicated by different notations.

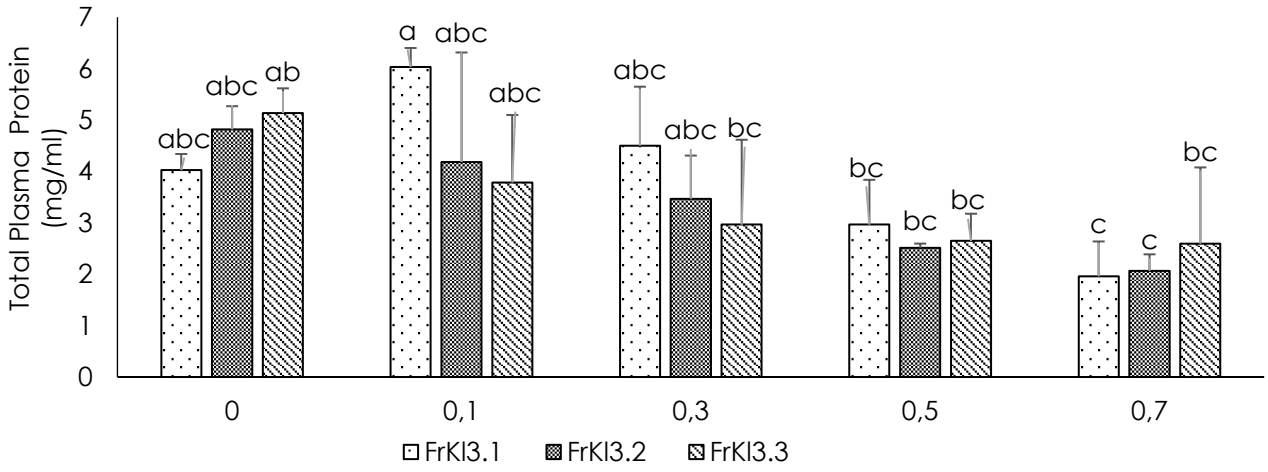


Figure 4. TPP of FrKl3.1; FrKl3.2; and FrKl3.3 on *Clarias* sp. were analyzed with two-way ANOVA (R Studio Application). The significant differences were indicated by different notations.

The best dose of NBT on *Clarias batrachus* and rainbow trout fish was 0.2-0.4 mg/kg body weight. Fish had a killing mechanism by phagocytosis cells through the production of radical faculae lysosome that could increase the permeability of bacterial cells, allowing fluid to enter the cells and cause plasmolysis (Fuchs *et al.*, 2015; Guzmán-Villanueva *et al.*, 2014).

Total Protein Plasma (TPP)

Results of TPP in three fractions of FrKl3 are presented in Figure 4. All FrKl3 fractions were not significantly different from the control ($p>0.05$). Total protein plasma may also contribute to an increase in fish immunity to disease. Furthermore, total protein plasma may indicate the amount of nitrogen in amino acids (Fuchs *et al.*, 2015; Wang *et al.*, 2017).

CONCLUSION

The methanol extract's chloroform fraction yielded three fractions: FrKl3.1, FrKl3.2, and FrKl3.3. The best PA was gained in FrKl3.3 of *Acanthaster planci* fraction at the dose at doses of 0.1 mg/kg

BW. The PI, and number of leukocytes/white blood cells, the FrKl3.2 shows the best results. Eventhough, the Nitroblue Tetrazolium and Total Plasma Protein were not significantly different from the control. This present research shows that fractionation can stimulate Macrophage Immunomodulatory Activity even at low doses.

REFERENCES

Achmad, M., Isnansetyo, A., Andriani, R., Samman, A., & Marus, I. (2020). The analysis of challenges test of catfish (*Clarias* sp.) with fatty acid compounds from starfish (*Acantaster planci*). *IOP Conference Series: Earth and Environmental Science,* 584, p.012014. doi:10.1088/1755-1315/584/1/012014

Achmad, M.J., Isnansetyo, A., & Kasanah, N. (2018). Macrophage Immunomodulatory Activity of Unsaturated Fatty Acid Isolated from the Crown-of-thorns Star Fish (Acanthaster planci). *Pharmacognosy Journal,* 10(5), 951-957. doi: 10.5530/pj.2018.5.161

Achmad, M. J., Isnansetyo, A., Kasanah, N., Ustadi, U., & Kamiso, K. (2014). Immunostimulatory effect of fatty acid from star fish (acanthaster planci) on lymphocyte proliferation in-vitro. *Squalen Bulletin of Marine Fisheries Postharvest Biotechnology and Bioprocess Engineering,* 9(3), 107-114. doi: 10.15578/squalen.v9i3.109

Aktan, F. (2004). iNOS-mediated nitric oxide production and its regulation. *Life Sciences,* 75(6), 639-653. doi: 10.1016/j.lfs.2003.10.042

Barman, D., Nen, P., Mandal, S., & Kumar, V. (2013). Immunostimulants for Aquaculture Health Management. *Journal of Marine Science: Research & Development,* 3(3), 1-11. doi: 10.4172/2155-9910.1000134

Boon, J.H., McDowell, T., & Hedrick, R.P. (1988). Resistance of the African (*Clarias gariepinus*) and the Asian catfish (*Clarias batrachus*) to channel catfish virus. *Aquaculture,* 74(3), 191-194. doi: 10.1016/0044-8486(88)90362-6

Cheng, A., Wan, F., Wang, J., Jin, Z., & Xu, X. (2008). Macrophage immunomodulatory activity of polysaccharides isolated from Glycyrrhiza uralensis Fish. *International Immunopharmacology, 8*(1), 43-50. doi: 10.1016/j.intimp.2007.10.006

Cheng, W., & Yu, J.S. (2013). Effects of the dietary administration of sodium alginate on the immune responses and disease resistance of Taiwan abalone, Haliotis diversicolor supertexta. *Fish & Shellfish Immunology,* 34(3), 902-908. doi: 10.1016/j.fsi.2012.12.020

Chiu, S.T., Tsai, R.T., Hsu, J.P., Liu, C.H., & Cheng, W. (2008). Dietary sodium alginate administration to enhance the non-specific immune responses, and disease resistance of the juvenile grouper Epinephelus fuscoguttatus. *Aquaculture,* 277(1), 66-72. doi: 10.1016/j.aquaculture.2008.01.032

Chopra, A.K., Xu, X., Ribardo, D., Gonzalez, M., Kuhl, K., Peterson, J.W., & Houston, C.W. (2000). The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. *Infection and Immunity,* 68(5), 2808-2818. doi: 10.1128/iai.68.5.2808-2818.2000

Diatin, I., Shafruddin, D., Hude, N., Sholihah, M. a., & Mutsmir, I. (2021). Production performance and financial feasibility analysis of farming catfish (*Clarias gariepinus*) utilizing water exchange system, aquaponic, and biofloc technology. *Journal of the Saudi Society of Agricultural Sciences,* 20(5), 344-351. doi: 10.1016/j.jssas.2021.04.001

El-Boshy, M., El-Ashram, A., Risha, E., Abdelhamid, F., Zahran, E., & Gab-Alla, A. (2014). Dietary fucoidan enhance the non-specific immune response and disease resistance in African catfish, *Clarias gariepinus*, immunosuppressed by cadmium chloride. *Veterinary Immunology and Immunopathology,* 162(3), 168-173. doi: 10.1016/j.vetimm.2014.10.001

Fair, J.D., & Kormos, C.M. (2008). Flash column chromatograms estimated from thin-layer chromatography data. *Journal of Chromatography A,* 1211(1-2), 49-54. doi: 10.1016/j.chroma.2008.09.085

Fuchs, V.I., Schmidt, J., Slater, M.J., Zentek, J., Buck, B.H., & Steinhagen, D. (2015). The effect of supplementation with polysaccharides, nucleotides, acidifiers and Bacillus strains in fish meal and soy bean based diets on growth performance in juvenile turbot (*Scophthalmus maximus*). *Aquaculture,* 437, 243-251. doi: 10.1016/j.aquaculture.2014.12.007

Gustiano, R., Prakoso, V., Radona, D., Dewi, R., Saputra, A., & Nurhidayat, N. (2021). A sustainable aquaculture model in Indonesia: multi-biotechnical approach in *Clarias* farming. *IOP Conference Series: Earth and Environmental Science,* 718, p.012039. doi: 10.1088/1755-1315/718/1/012039

Guzmán-Villanueva, L.T., Ascencio-Valle, F., Macías-Rodríguez, M.E., & Tovar-Ramírez, D. (2014). Effects of dietary β-1,3/1,6-glucan on the antioxidant and digestive enzyme activities of Pacific red snapper (*Lutjanus peru*) after exposure to lipopolysaccharides. *Fish Physiology and Biochemistry,* 40(3), 827-837. doi: 10.1007/s10695-013-9889-0

Harikrishnan, R., Naafar, A., Musthafa, M.S., Ahamed, A., Arif, I.A., & Balasundaram, C. (2018). Effect of *Agaricus bisporus* enriched diet on growth, hematology, and immune protection in *Clarias gariepinus* against *Flavobacterium columnare*. *Fish and Shellfish Immunology,* 73, 245-251. doi: 10.1016/j.fsi.2017.12.024

Henriksson, P.J.G., Tran, N., Mohan, C.V., Chan, C.Y., Rodriguez, U.P., Suri, S., Mateos, L.D., Utomo, N.B.P., Hall, S. & Phillips, M.J., (2017). Indonesian aquaculture futures – Evaluating environmental and socioeconomic potentials and limitations. *Journal of Cleaner Production,* 162, 1482-1490. doi: 10.1016/j.jclepro.2017.06.133

Jensch-Junior, B.E., Pressinotti, L. N., Borges, J.C.S., & da Silva, J.R.M.C. (2006). Characterization of macrophage phagocytosis of the tropical fish Prochilodus scrofa (Steindachner, 1881). *Aquaculture*, 251(2), 509-515. doi: 10.1016/j.aquaculture.2005.05.042

Korhonen, R., Lahti, A., Kankaanranta, H., & Moilanen, E. (2005). Nitric Oxide Production and Signaling in Inflammation. *Current Drug Targets - Inflammation & Allergy,* 4(4), 471-479. doi: 10.2174/15680 10054526359

Le, T.V., Hanh, T.T.H., Huong, P.T.T., Dang, N.H., Van Thanh, N., Lyakhova, E., Cuong, N.X., Nam, N.H., Van Kiem, P., Kicha, A. and Van Minh, C., 2016. Pyrrole oligoglycosides from the starfish Acanthaster planci suppress lipopolysaccharide-induced nitric oxide production in RAW264. 7 macrophages. *Chemical and Pharmaceutical Bulletin*, *64*(11), 1654-1657. doi: 10.1248/cpb.c16-00585

Magnadottir, B. (2010). Immunological control of fish diseases. *Marine biotechnology,* 12(4), 361-379. doi: 10.1007/s10126-010-9279-x

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish and Shellfish Immunology,* 20(2), 137-151. doi: 10.1016/j.fsi.2004.09.006

Mehana, E.S., Rahmani, A., & Aly, S. (2015). Immunostimulants and Fish Culture: An Overview. *Annual Research and Review in Biology,* 5, 477-489. doi: 10.9734/ARRB/2015/9558

Nieves-Rodríguez, K.N., Álvarez-González, C.A., Peña-Marín, E.S., Vega-Villasante, F., Martínez-García, R., Camarillo-Coop, S., Tovar-Ramírez, D., Guzmán-Villanueva, L.T., Andree, K.B. & Gisbert, E. (2018). Effect of β-Glucans in Diets on Growth, Survival, Digestive Enzyme Activity, and Immune System and Intestinal Barrier Gene Expression for Tropical Gar (*Atractosteus tropicus*) Juveniles. *Fishes, 3*(3), p.27. doi: 10.3390/fishes3030027

Pacher, P., Beckman, J.S., & Liaudet, L. (2007). Nitric Oxide and Peroxynitrite in Health and Disease. *Physiological Reviews,* 87(1), 315-424. doi: 10.1152/physrev.00029.2006

Pietrzak, E., Mazurkiewicz, J., & Slawinska, A. (2020). Innate Immune Responses of Skin Mucosa in Common Carp (*Cyprinus Carpio*) Fed a Diet Supplemented with Galactooligosaccharides. *Animals,* 10(3), p.438. doi: 10.3390/ani10030438

Pratchett, M.S., Schenk, T.J., Baine, M., Syms, C., & Baird, A.H. (2009). Selective coral mortality associated with outbreaks of *Acanthaster planci* L. in Bootless Bay, Papua New Guinea. *Marine Environmental Research, 67*(4-5), 230-236. doi: 10.1016/j.marenvres.2009.03.001

Purbomartono, C., Isnansetyo, A., Murwantoko, & Triyanto. (2019). Dietary Fucoidan from *Padina boergesenii* to Enhance Non-specificImmune of Catfish (*Clarias* sp.). *Journal of Biological Sciences,* doi: 10.3923/jbs.201

Reich, E., & Schibli, A. (2007). High-performance thin-layer chromatography for the analysis of medicinal plants. Thieme. FAO

Sellegounder, D., Gupta, Y.R., Murugananthkumar, R., & Senthilkumaran, B. (2018). Enterotoxic effects of *Aeromonas hydrophila* infection in the catfish, *Clarias gariepinus*: Biochemical, histological

and proteome analyses. *Veterinary Immunology and Immunopathology,* 204, 1-10. doi: 10.1016/j.vetimm.2018.08.008

Stoddard, J.M., Nguyen, L., Mata-Chavez, H., & Nguyen, K. (2007). TLC plates as a convenient platform for solvent-free reactions. *Chemical Communications,* (12), 1240-1241. doi: 10.1039/b616311d

Vetvicka, V., Vannucci, L., & Sima, P. (2013). The Effects of β - Glucan on Fish Immunity. *North American Journal of Medical Sciences, 5*(10), 580-588. doi: 10.4103/1947-2714.120792

Wang, W., Sun, J., Liu, C., & Xue, Z. (2017). Application of immunostimulants in aquaculture: current knowledge and future perspectives. *Aquaculture Research,* 48(1), 1-23. doi: 10.1111/are.13161

Yang, P., Hu, H., Liu, Y., Li, Y., Ai, Q., Xu, W., Zhang, W., Zhang, Y., Zhang, Y. & Mai, K., (2018). Dietary stachyose altered the intestinal microbiota profile and improved the intestinal mucosal barrier function of juvenile turbot, *Scophthalmus maximus* L. *Aquaculture,* 486, 98-106. doi: 10.1016/j.aquaculture.2017.12.014

Yu, L., Zzaman, W., Akanda, M. J., Yang, T., & Easa, A. (2017). Influence of superheated steam cooking on proximate, fatty acid profile, and amino acid composition of catfish (*Clarias batrachus*) fillets. *Turkish Journal of Fisheries and Aquatic Sciences,* 17, 935-943. doi: 10.4194/1303-2712-v17_5_09

Yudiati, E., Isnansetyo, A., Murwantoko, Triyanto, & Handayani, C.R. (2019). Alginate from *Sargassum siliquosum* simultaneously stimulates innate immunity, upregulates immune genes, and enhances resistance of Pacific white shrimp (*Litopenaeus vannamei*) against white spot syndrome virus (WSSV). *Marine Biotechnology,* 21(4), 503-514. doi: 10.1007/s10126-019-09898-7