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Fittrie Meyllianawaty Pratiwy
Department of Fisheries, Faculty of Fisheries and Marine Science, Universitas Padjadjaran, Jl. Raya Sumedang KM 21 Sumedang, Indonesia

Dian Yuni Pratiwi
Department of Fisheries, Faculty of Fisheries and Marine Science, Universitas Padjadjaran, Jl. Raya Sumedang KM 21 Sumedang, Indonesia

Corresponding Author:
Fittrie Meyllianawaty Pratiwy
Department of Fisheries, Faculty of Fisheries and Marine Science, Universitas Padjadjaran, Jl. Raya Sumedang KM 21 Sumedang, Indonesia

The effects of bioactive compound (Antioxidant) from *Sargassum* extract on the erythrocytes and differential leucocytes of catfish (*Clarias* sp.)

Fittrie Meyllianawaty Pratiwy and Dian Yuni Pratiwi

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Abstract
This study aims to confirm the antioxidant activities of brown seaweed, Sargasum as a potential additive for catfish feed. The research conducted with various solvents (n-hexane, ethyl acetate and ethanol) to determine phytochemical screening and antioxidant activities. The bioactive compound from Sargassum extract was used in Catfish diets which is expected to optimize the number of erythrocytes and differential leukocytes (the number of neutrophils monocytes and lymphocytes).
The result of showed that the total phenol content of the ethyl acetate extract of Sargassum was 127.5 mg GAE/g extract, while the total flavonoid content was 107.66 mg QE/g extract. The ethanol extract showed the highest antioxidant activity, with an IC50 of 29.84 ppm.
Meanwhile, in the observation of fish blood test, the use of extract Sargassum in diets with doses 5, 10 and 15 g/Kg during 21 days gave an impact on the number of erythrocytes and percentage of lymphocytes. The highest value of erythrocytes was found in treatment with dose 15 g/Kg on 21st day, 16.57 ± 1.43 x 105 cells/mm^3. In case of differential leucocytes test, there was not indicated the influence of the Sargassum extract in diets to the percentage of neutrophils and monocytes of the blood fish. It can be concluded that 15 g/Kg additional extract of Sargassum in diets, which contains the bioactive compound, give the effectiveness of the optimal response immune system in catfish.

Keywords: Catfish, sargassum, antioxidant, number of erythrocytes, differential leucocytes

1. Introduction
Catfish is one of the most important freshwater fish in term of production. However, the problem in catfish farming is dealing with the bad condition of water quality, so diseases can easily attack. One of the ways to prevent this problem is using the bioactive compounds in fish feed as a supplement. The bioactive compounds, such as antioxidant, in fish are pointers to the health benefits of fish ingestion.
Antioxidants are both nutritional and non-nutritional substances contained in food, which can prevent or slow down the oxidation process [1]. Very antioxidant beneficial to health and cosmetics and plays a role important in maintaining the quality of food products [1, 2]. The most commonly used antioxidants are synthetic antioxidants, such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tertbutylhydroquinone (TBHQ) and propyl gallate (PG) [2,3]. Synthetic antioxidants are carcinogenic and might be caused a liver damage [2]. As a result, the demand for natural antioxidants continues to increase.
There are various sources of natural antioxidants from the sea, such as seaweed [2, 3, 4], seagrass [5], microalgae [6], and so on. Seaweed, especially from class *Phaeophyceae* such as Sargassum, is grows widely in coastal sea areas all over the world from temperate to tropical regions [7].
Sargassum, which are known in Indonesia there are about 12 species, has been used as an anti-cholesterol [8], biofuel [9], antibacterial [10], anti-tumor [11], anticancer [12], and antivirals [13]. Extract of *Sargassum* sp. also potential as an antioxidant. Therefore, the present work has been carried out to investigate the effects of bioactive compound (antioxidant) from *Sargassum* sp. extract as supplements in diets on erythrocytes and differential leucocytes of catfish (*Clarias* sp.).

2. Materials and Methods

The research consisted of three stages. The first stage are sample collection, identification, preparation, extraction and phytochemical analysis. The second stage is the total phenol and flavonoid test and continue with antioxidant activity test by the1,1-diphenyl-2-picrylhydrazil (DPPH) method. The third stage is application of bioactive compound from sargassum extract as a fish supplement in diets.

2.1 Seaweed collection and extraction

Sargassum sp. was collected manually from Tidung Island, Kepulauan Seribu, Indonesia. Epiphytes were thoroughly removed from fresh thalli, rinsed, and dried. The dried sample was placed away from sun light directly to avoid damage of bioactive compound before grinded.

The sample was weighted as much as 250 grams and put into the Erlenmeyer, then the solvent was added up to 1000 ml with a ratio of 1: 4 (w/v). Extraction procedure carried out by immersing the sample with n-hexane, ethyl acetate and ethanol sequentially. The saturation results was filtered by filter paper Whatman 42, until resulting filtrate and residue. The soaking procedure was done in 3 times until the filtrate was clear enough. The filtrate obtained from the previous step then concentrated with a vacuum rotary evaporator at a temperature of 40 ^{0}C until a crude extract was attained in the form of paste. The phytochemical analysis was done to identify the bioactive compounds contained in each solvent from the Sargassum extract. The phytochemical analysis carried out included the alkaloid, triterpenoid and steroid, saponin, phenol, flavonoid and quinone test.

2.2 Antioxidant activity test

The antioxidant activity test for the Sargassum extract was used the free radical 1,1-diphenyl-2-picrylhydrazil (DPPH) method as conducted by Molyneux (2004) and Vijayabaskar and Shiyamala (2012).

2.3 The experimental animal and trial conditions

The experimental fish, Catfish (*Clarias* sp.) were obtained from a local farmer. They were acclimatized for two weeks in the concrete tank and fed by commercial fish diets. After being kept in the concrete tank, they were moved and maintained in experimental tanks. They were divided into four types of experimental rearing condition.

This research was conducted for 21 days and the method used in this research is experimental with a completely randomized design (CRD). The treatments used were 4 treatment and 5 replications. The treatments were fed by supplemental diets contained bioactive compound of sargassum extract on days 0, 7, 14, and 21. The number of erythrocytes and differences leukocytes (neutrophils, lymphocytes and monocytes) were measured during the experimental period. The treatment divided by the difference in dosage extract of *Sargassum* sp. namely, SE0 (Control/without Sargassum extract), SE1 (5 gr extract/ kg of feed), SE2 (10 g of extract / kg of feed) and SE3 (15 gr extract/kg feed). The amount of feed given as much as 5% of the biomass.

2.4 Analysis of number of erythrocytes and differential leucocytes

The number of erythrocytes were counted in a certain volume by using a conversion factor. The count begun with filling the erythrocytes pipette, namely blood sucked up to the mark 0.5 and diluent solution (hayem solution) up to line 101, removed the aspirator tube then homogenized for 5-10 minutes to make a good mixing. Then, put in the counting booth and analyzed under a microscope in magnification forty times.

Erythrocytes = (A / N) x (1 / V) x Df

A = Σ calculated cells

V = volume of haemocytometer box

N = Σ observed haemocytometer box

Df = dilution factor

The differential leucocytes were examined through the blood smear preparation, stained with 10% Giemsa for 10-15 minutes. Blood samples were mixed homogeneously before taking it with a capillary pipette, then a small drop of blood was placed close by the end of the object glass position flat surface. The second object glass was placed with the tip touched the surface of the object glass. The blood samples were fixed with methyl alcohol for 3-5 minutes. Then the preparations soaked with dye Giemsa new for 10-15 minutes and washed with water in many times. The percentage calculation of lymphocytes were analyze under microscope with the magnification.

$$\text{Lymphocyte percentage} = \frac{L}{100}100\%$$

$$\text{Monocyte percentage} = \frac{M}{100}100\%$$

$$\text{Percentage of Neutrophils} = \frac{N}{100}100\%$$

3. Result and Discussion

3.1 Phytochemical analysis results

The Sargassum extract contains alkaloids, triterpenoids, steroids, saponins, phenols, flavonoids and quinones. The ethyl acetate extract showed the highest total phenol and flavonoid content. The total phenol content of the ethyl acetate extract of Sargassum was 127.5 mg GAE /g extract, while the total flavonoid content was 107.66 mg QE / g extract. The ethanol extract showed the highest antioxidant activity, with an IC50 of 29.84 ppm.

3.2 The number of Erythrocytes

The highest value was found in treatment SE3 on 21st day, 16.57 ± 1.43 x 105 cells/mm^3. Meanwhile, the lowest value was in treatment C on the 7th day, with the result 13.43± 2.33x105 cells/mm^3. From the statistical analysis of variance (ANOVA) and continued with Duncan test at a 95% confidence interval (p<0.05) the results obtained on the 21st day of control was significantly different (p<0.05) with treatment A, B and C indicated by the number of erythrocytes is lower than all three treatments (Table 1).

This results range within the normal range means fish are in healthy condition. This is appropriate with the statement of Robert (1978) in Mulyani (2006) [14] which stated that at teleost fish, the normal number of erythrocytes is equal to 1.05 - 3.00 x 106 cell/mm^3.

Table 1: The Average Percentage of Erythrocytess for Each Treatment (%) during the Experimental Period

Treatment	Day 0	Day 7	Day 14	Day 21
C	14.24±1.66	13.43 ± 2.33	15.18 ± 1.11	14.08 ± 1.52^a
SE 1	13.52 ± 2.55	13.74 ± 1.45	15.10 ± 2.65	16.10 ± 1.50^b
SE 2	13.70 ± 1.34	13.85 ± 2.12	15.50 ± 1.80	16.06 ± 1.22^b
SE 3	13.56 ± 1.48	13.98 ± 1.05	15.34 ± 2.78	16.57 ± 1.43^b

Different letters in the same column show significantly different effects (p <0.05)

The number of erythrocytes will make the fish unable take in large amounts of oxygen despite the availability of oxygen in the waters is sufficient. As a result, the fish will experience anoxia (lack of oxygen) [15].

3.3 The differential leucocytes

The total percentage of lymphocytes of all treatments were increased from day 0 to 21 days (table 2). The increasing occurred in lymphocyte percentage at treatments 5, 10 and 15 g extract/kg feed on day 21st, which indicate that the extract Sargassum gave the influence to the percentage of lymphocytes in catfish blood. This is due to the chemical content naturally extracted from Sargassum such as vitamin C, while the high doses of vitamin C can increases the immunity [16] and polysaccharides has a function as boosters of the immune system in fish and increase protection against bacteria [17]. Besides that, other ingredients in the extract of Sargassum such as fucoidan compounds could increase the immune system by stimulating production of immune cells [18].

Table 2: The Average Percentage of Lymphocytes for Each Treatment (%) during the Experimental Period

Treatment	Day 0	Day 7	Day 14	Day 21
C	78.80 ± 4.32	81.60 ± 2.41	81.40 ± 1.67	78.44 ± 1.33^{a}
SE 1	80.40 ± 1.14	82.45 ± 3.39	83.72 ± 3.56	85.92 ± 3.12^{b}
SE 2	79.60 ± 4.93	81.60 ± 2.19	84.00 ± 3.54	86.33 ± 3.45^{b}
SE 3	78.20 ± 2.86	80.90 ± 2.92	85.40 ± 4.04	86.43 ± 2.43^{b}

Different letters in the same column show significantly different effects (p <0.05)

The increasing of lymphocyte percentages is a reflection of the success of the system fish immunity in developing a non-specific response as a trigger for the immune response. The activated lymphocytes will be differentiable from that cognitive cell recognize antigens to be effector cells serves to get rid of anti-genes into cells effectors that remove antigens [19]. The observation of mean percentage neutrophils in the blood of catfish indicates that the neutrophil count has a tendency to decreases every week. In control, 5, 10 and 15 g extract / kg of feed the percentage of neutrophils has the same decreasing patterns, but result of control group decrease rapidly and had a significant difference with other groups (Table 3). According to the Tizard (1988), this is related to the main function of neutrophils such as the destruction of external material through the process of phagocytosis, namely chemotaxis where the cell will migrate towards the particles, laying of particles on cells, particle ingestion by cells, and the destruction of particles by enzymes lysozymes in phago-lysosomes. Therefore, without the stimulation of external materials such bacteria, viruses, or pathogens, neutrophil will not increase.

Table 3: The Average Percentage of Neutrophils for Each Treatment (%) during the Experimental Period

Treatment	Day 0	Day 7	Day 14	Day 21
C	13.00 ± 4.95	10.60 ± 2.07	10.60 ± 1.82	11.20 ± 0.93^{a}
SE1	11.40 ± 1.82	9.00 ± 2.83	8.20 ± 3.96	7.80 ± 1.23^{b}
SE2	13.40 ± 4.51	10.40 ± 3.05	7.20 ± 1.44	8.40 ± 1.98^{b}
SE3	11.40 ± 2.82	11.00 ± 3.32	8.20 ± 2.28	8.20 ± 1.09^{b}

Different letters in the same column show significantly different effects (p <0.05)

The monocytes of the treatment fed by Sargassum extract were decreased in each week 191 (Table 4) related to function monocytes such as macrophages, where monocytes were not needed 192 to phagocyte, because there is no incoming infection into the body which stimulates the production monocytes. This is in line with previous study by Witeska (2005) which stated that lower lymphocyte and higher neutrophil numbers in peripheral blood as typical stress response, vice versa.

Table 4: The Average Percentage of Monocytes for Each Treatment (%) during the Experimental Period

Treatment	Day 0	Day 7	Day 14	Day 21
C	8.20 ± 1.02	7.80 ± 0.90	8.00 ± 0.28	8.60 ± 1.05^{a}
SE1	8.20 ± 0.99	8.00 ± 1.06	7.70 ± 0.44	7.40 ± 1.23^{b}
SE2	9.00 ± 0.89	8.45 ± 1.43	8.07 ± 0.60	7.54 ± 0.99^{b}
SE3	8.40 ± 0.76	8.20 ± 1.58	7.78 ± 0.39	7.20 ± 1.67^{bs}

Different letters in the same column show significantly different effects (p <0.05)

In addition, Sargassum which used in this study were used the extract which contained the bioactive compounds. Nevertheless, other study, such as in Nile tilapia also revealed that sargassum meal in fish feed could increase the growth performance [15, 21].

4. Conclusion

In conclusion, the present study demonstrated that the extract Sargassum which contains bioactive compounds (alkaloids, triterpenoids, steroids, saponins, phenols, flavonoids and quinones) could be an effective supplement for Catfish. The extract give an effect on increasing the number erythrocytes and the percentage of catfish blood lymphocytes but had no significant effect on the percentage of monocytes and neutrophils. The dose of extract as much as 15 g of extract/kg feed was able to provide an optimal erythrocytes and lymphocytes. Further research is needed also regarding to the optimum feeding time to make its use appropriate for practical culture.

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