

Immunostimulatory activity of *Holothuria atra* sea cucumber

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Abstract

Immunostimulant is a substance that can stimulate the immune system against pathogenic microbes. Natural products produced by sea cucumbers have the potential to be developed as immunostimulants. This study was aimed to evaluate the immunostimulatory activity of *Holothuria atra* extract using the phagocytocyte assay of macrophage cells and the differentiation of leukocyte in rats. The samples of *H. atra* were taken from Halmahera waters, North Maluku, Indonesia. Extraction was carried out with 96% ethanol. Phagocytocyte activity assay was carried out using macrophage cells isolated from Balb/c mice (*Mus musculus*) using a series of doses 0.5; 1.0; 2.0; 4.0; 8.0; 16.0 and 32.0 mg/kg body weight. Leukocyte differentiation test *in vivo* was conducted using *Rattus norvegicus* rat treated with *H. atra* extract for 90 days with the following series of doses: 0, 25, 50, 100, and 200 mg/kg body weight. The results showed that the highest phagocytosis activity was reached at a concentration of 4.0 µg/ml, but it was insignificantly different from the negative control group ($p < 0.05$). Leukocyte differentiation assay showed that the administration of *H. atra* extract increased the immune system response in the animals which was characterized by the increasing number of lymphocyte cells. *H. atra* extract also decreased the number of monocytes and neutrophils, suggesting the suppression of inflammation in the tested rats. Extract administration for 90 days did not cause a hypersensitivity reaction as indicated by the unchanged number of eosinophil and basophil cells. Based on the results of this study, it is concluded that *H. atra* had a potency to develop as an immunostimulant.

Keywords

Sea cucumber, *Holothuria atra*, immunostimulant, macrophage, leukocyte

Introduction

The immune system of the human body consists of all cells, tissues, and organs that was needed for an immune response. The function of the immune system is to protect the body from pathogens and destroy cells that are not recognized as part of the body cells (James et al. 2008). Immunostimulants are materials that can stimulate the body's immune system through non-specific and specific

immune response mechanisms (Schulz et al. 2004). Acute and chronic infections that are caused by bacteria, fungi, protozoa, and viruses are continuously occurring. This is exacerbated by the decrease of immunological responses due to therapies such as antibiotics, antivirals, and anti-cancer drugs. Therefore, the development of effective new immunostimulants is important (Aminin et al. 2006; Achmad et al. 2018). Immunomodulators are popularly used as complementary therapies and alternative medicines.

Many active ingredients from plants or animals are useful to improve the symptoms of the disease by stimulating the innate and specific immune systems (adaptive immunity) (Wang et al. 2017; Achmad et al. 2014).

Sea cucumbers are marine invertebrates that belong to the phylum Echinoderms and the Holothuridae class. These animals are promising as a source of bioactive ingredients for nutraceutical and pharmacological purposes. Pharmacological properties of sea cucumbers that have been widely reported are anti-angiogenic, anticancer, anticoagulant, anti-hypertensive, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, antitumor and wound healing (Bordbar et al. 2011; Khotimchenko 2018). One species of sea cucumber that is interesting to be studied as a pharmaceutical source is *Holothuria atra* (black sea cucumber).

Black sea cucumbers are edible and widely distributed along the Pacific and Indian Oceans. To date, numerous studies have indicated that *H. atra* extract exhibit a wide range of bioactivities (Nursid et al. 2019; Grauso et al. 2019; Dhinakaran and Lipton 2015). One of the major components of *H. atra* extract is saponin (triterpene glycoside). The bioactivity of saponins is generally related to its role as membranolytic agents (Kalinin 2000; Kalinin et al. 2008).

Monosulfated glycosides of sea cucumbers *Cucumaria japonica* (Aminin et al. 2006) and *C. frondosa* (Aminin et al. 2008) showed immunostimulatory activity, while exhibiting non-toxicity to the immune cells (Aminin et al. 2009). Mucopolysaccharide acid of *Stichopus japonicus* increased the index function of the spleen and thymus glands and can increase the phagocytosis index of macrophages and NK cells. The mucopolysaccharide acid effectively inhibited hepatocellular carcinoma (HCC) through immune system stimulation and tissue proliferation thereby increasing cellular immunity in mice (Song et al. 2013). Glycosaminoglycan isolated from *Apostichopus japonicus* can significantly increase immunoregulation activity. It was also shown to play an important role in the immune enhancement and protection against cyclophosphamide-induced immunosuppression and oxidative damage (Wang et al. 2017). Based on this research, glycosaminoglycan from *Apostichopus japonicus* has the potential to be applied as an immunomodulatory and adjuvant agent in cancer patients.

To the best of our knowledge, studies on the immunostimulatory activity of *H. atra* extract are still rare. Therefore, this study aimed to determine the activity of macrophage cell phagocytosis in Balb/c (*Mus musculus*) mice and the differentiation of white blood cells in *Rattus norvegicus* rats that were treated by the ethanolic extract of *H. atra*.

Materials and methods

Sea cucumber collection and extraction procedure

Holothuria atra sea cucumber was collected from Halmahera waters, North Maluku, Indonesia on April, 2016.

Samples were kept in cold conditions (-20 °C). The identification was carried out at the Research Center for Oceanography, Indonesian Institute of Science, Jakarta. A specimen voucher (no. HA-01) was deposited in the Biotechnology Laboratory, Research Center for Marine and Fisheries Product Processing and Biotechnology, Jakarta Indonesia. Extraction was conducted by maceration method (12 hours, three times) using 96% ethanol with a ratio of 1 : 2 (w/v) according to Nursid et al. (2019).

Preparation of macrophage cells

Macrophage activity assay was carried out according to the methods of Aminin et al. (2010) and Achmad et al. (2014). Macrophage cells were isolated from Balb/c mice (male, ± 20 g). One ml of extract with various concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 mg/kg body weight) was injected via intraperitoneal. The isolation procedure was carried out by the following method. Mice were euthanized with neck dislocation. Ten ml of RPMI medium was injected into the cavity in the peritoneum. A mixture of peritoneum and RPMI were taken from the stomach using a syringe then centrifuged at 1200 rpm at 4 °C for 10 minutes. The supernatant was removed and the pellet was re-suspended with complete RPMI medium (containing 10% fetal bovine serum/FBS, penicillin-streptomycin 2%, and fungizon 0.5%). Cells were diluted up to 20 times using a complete RPMI medium and then the number of cells was calculated using a hemocytometer. The cell suspension (200 μ l/well, density 5×10^5 /well) was then cultured in a 24-well microplate with coverslips and subsequently incubated for 24 hours in a CO₂ incubator.

Macrophages activity test

The cells were washed with RPMI medium and added with 20 μ l/well latex beads (2 μ m in diameter) suspension and incubated for 60 min in a 5% CO₂ incubator at 37 °C. The cells suspension was washed three times with PBS to remove unphagocytosed latex. The cells suspension was then dried at room temperature and fixed with methanol. Coverslips were dyed with Giemsa 20% for 20 minutes, rinsed with aquadest and dried at room temperature. The macrophages activity was calculated as the number (%) of consumed latex (substrates), visualized by a light microscope (magnified 400 $\times$). Phagocytosis capacity (PC) was indicated by the percentage of active macrophages in 100 macrophages, and the phagocytosis index (PI) was indicated by the number of latex be consumed by active macrophages. These data were compared to the negative controls.

$$PC = \frac{\text{Number of macropages phagocytozing}}{\text{Total of macropages counted}}$$

$$PI = \frac{\text{Number of latex inside macrophages}}{\text{Number of macrophages phagocytozing}}$$

Cytotoxicity test

This test was carried out to evaluate the cytotoxicity of *H. atra* extract against normal cells. Cytotoxicity test against normal Vero cell was performed according to Nursid et al. (2011) and Ebada et al. (2008) with some modifications. Vero cells (isolated from African green monkey kidney) were obtained from the Laboratory of Parasitology, Faculty of Medicine, Gadjah Mada University, Yogyakarta Indonesia. Cells were cultured in M199 medium supplemented with FBS 10%, penicillin-streptomycin 2%, and fungizon 0.5% and it was incubated in a CO₂ incubator at 37 °C with a flow rate of 5 ml/min. A total of 1 × 10⁴ cells/wells were cultured in 96-well microplate and incubated for 12 hours in a CO₂ incubator. Furthermore, 100 µl of *H. atra* extract with concentrations of 2.5, 5.0, 10.0, 20.0 and 40.0 µg/ml were put into the cells-containing microplate and incubated at 37 °C in a CO₂ incubator. After 24 hours of incubation, 100 µl of MTT solution (concentration of 0.5 mg/ml) was added into the microplate and the cells were further incubated for 4 hours in a CO₂ incubator, prior to the addition of 100 µl sodium dodecyl sulfate (SDS) 10%. After 12 hours of incubation time, the absorbance of the samples was measured with a microplate reader (Thermo Scientific) at a wavelength of 570 nm. The absorbance data was subsequently used to calculate cell mortality (%). The percent of mortality cells were calculated with formula:

$$(A - D) - (B - C) / (A - D) \times 100\%$$

where A: control cell absorbance, B: extracts absorbance, C: control extracts absorbance, and D: control media absorbance.

Differentiation of leukocyte assay

Animals used

Adult *Rattus norvegicus* male rats (220–250 g) were obtained from The National Agency of Drug and Food Control Republic of Indonesia. The experiment was conducted at the Faculty of Veterinary Medicine, Bogor Agricultural University. The rats were housed in cages measuring 39 × 30 × 11 cm (two animals/cage). Animals were acclimatized for two weeks before the experiment and fed with standard rodent pellets diet, whereas drinking water is given filtered water with Pureit ad libitum. The investigational procedures adopted in this experiment were in accordance with the requirements of the Experimentation Ethics Committee on Animal Use of the Faculty of Veterinary Medicine, Bogor Agricultural University, Indonesia.

Animal treatment

Before the administration of sea cucumber extract, rats were weighed to determine the proper dose. The experimental animals were divided into five groups, each group consisting of seven to eight rats. The extract doses used in this study were 25 mg/kg body weight (bw) (group B),

50 mg/kg bw (group C), 100 mg/kg bw (group D), 200 mg/kg bw (group E), and 400 mg/kg bw (group F). The control groups (group A) were treated with the same volume of distilled water. The extract was administered by oral gavage at 1 mL/kg body weight on daily basis for 90 days.

Analysis of leukocyte differentiation

The animal blood was taken from the coxigea vein using a 1 ml syringe and was smeared on the slide. Dried smears were then soaked in methanol for 5–6 minutes. A 10% Giemsa stain (Merck) was added on to the fixed blood smear and left for 15 minutes, prior to washing with running water. After drying, the DPX Mountant (Merck) was dripped on the blood smear, then covered with a glass cover and allowed to stand dry. The blood smear was observed under a light microscope (100× magnification) and documented, the cell count was carried out to reach 100 leukocyte cells. The parameter analyzed was the response of the animal immune system to *H. atra* extract by calculating the differentiation of rat leukocytes, namely the number of lymphocytes, neutrophils, monocytes, macrophages, eosinophils and basophils in the number of 100 leukocyte cells.

Data analysis

Data on phagocytosis capacity, phagocytosis index, differentiation of leukocytes and viability of Vero cells were obtained in triplicates. The difference in each treatment was analyzed by one way ANOVA followed by the Tukey test. The IC₅₀ value of *H. atra* extract against Vero cells was calculated by probit analysis. The ANOVA and probit analyzes were performed using the MINITAB version 16.0 software.

Results

Phagocytosis activity

Phagocytosis activity of *H. atra* extract showed that the phagocytosis capacity (PC) values ranged from 2.5 to 101.7. The lowest mean PC value was found at a dose of 32.0 µg/mL and the highest at a dose of 4.0 µg/mL. Even though the 4.0 µg/mL dose had the highest PC value, statistically it was not significantly different ($p > 0.05$) to the control (without treatment), 0.5 µg/mL, 1.0 µg/mL, and 2.0 µg/mL. The PC values in the dose group were only significantly different from the PC values of doses 8.0 µg/mL, 16.0 µg/mL and 32.0 µg/mL ($p < 0.05$) (Fig. 1A).

A similar pattern occurred in the phagocytosis index (PI) value where the highest value was found at a dose of 4.0 µg/mL at 7.1, and the lowest at a dose of 32.0 µg/mL at 1.0. The best PI value at the 4.0 µg/mL dose was insignificantly different from the control, 0.5 µg/mL, 1.0 µg/mL, and 2.0 µg/mL doses. At higher doses, 8.0 µg/mL, 16.0 µg/mL, and 32.0 µg/mL, PI values decreased dramatically (Fig. 1B). The low PC and PI values at doses higher than 8.0 µg/mL were most likely caused by the toxicity of *H. atra* extract.

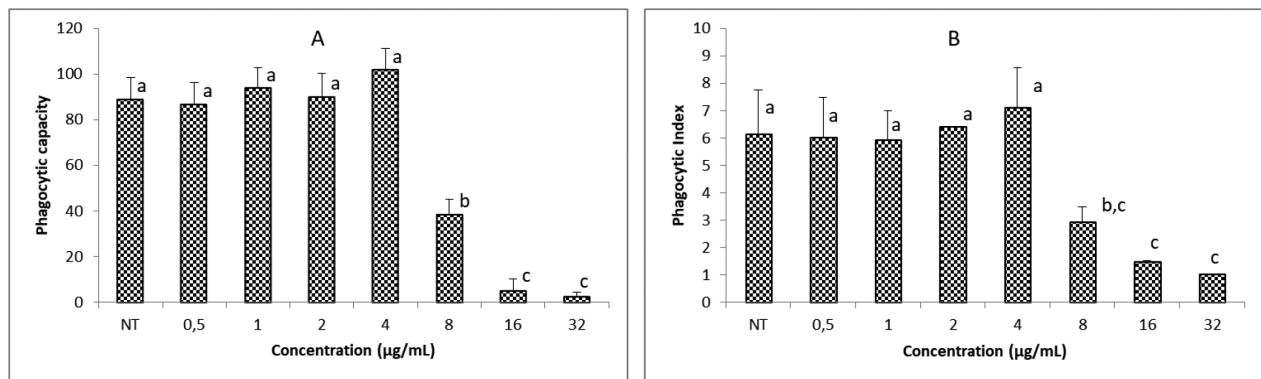


Figure 1. Phagocytosis activity of *H. atra* extract, phagocytosis capacity (A) and phagocytosis index (B). **Note:** NT (not treated); different letters indicated statistical differences at $p < 0.05$.

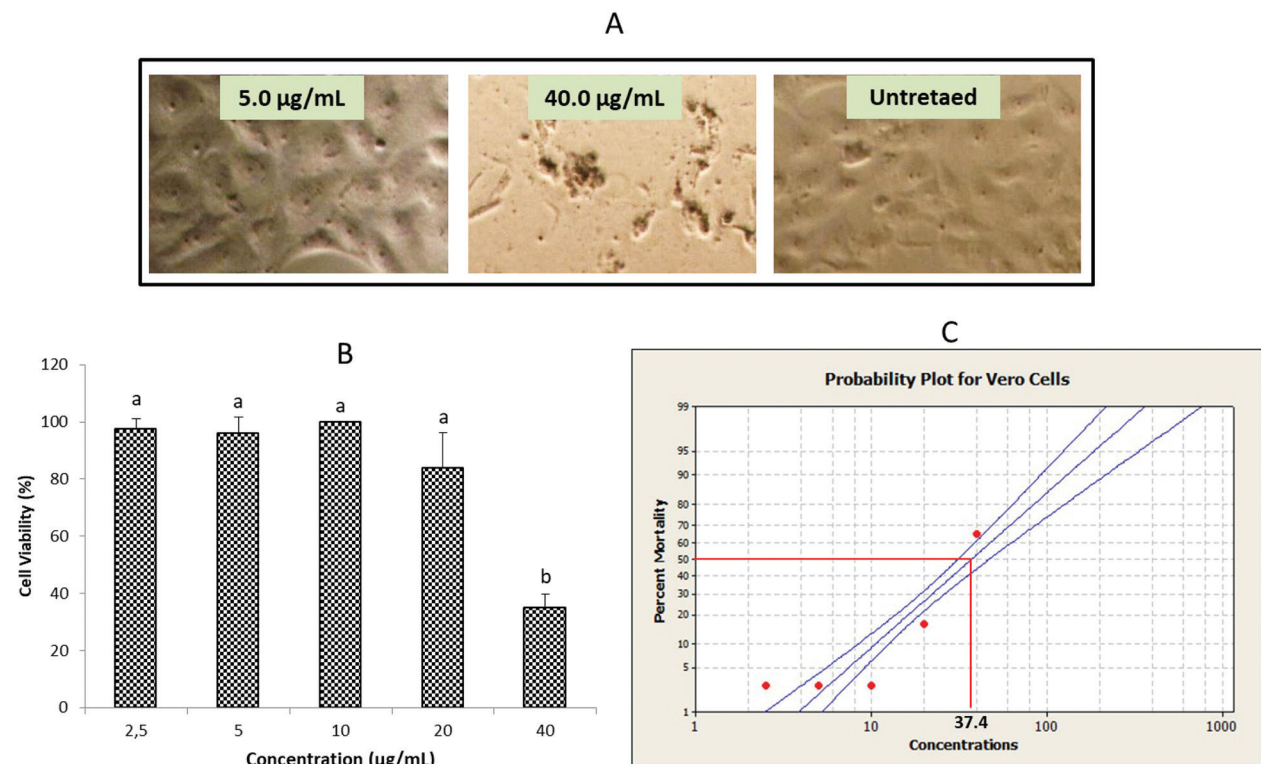


Figure 2. Morphology (A), viability (B) and probit analysis (C) of normal Vero cells after being treated with *H. atra* extract for 24 hours. **Note:** different letters in the graph indicated statistical differences at $p < 0.05$.

Cytotoxicity against Vero Cells

Cytotoxicity tests showed that the morphology of the Vero cells were unaltered at 5.0 µg/mL and were disintegrated when treated with 40.0 µg/mL of extract (Fig. 2A). The Vero cells viability after exposure to *H. atra* extract was maintained up to 20 µg/mL, then decreased sharply at 40 µg/mL (Fig. 2B). Probit analysis showed that the IC₅₀ value of *H. atra* extract against Vero cell was 37.4 µg/mL (Fig. 2C).

Leukocyte differentiation

Morphology of lymphocyte, monocyte, eosinophil and neutrophils isolated from *R. norvegicus* was presented in

Fig. 3. The number of rat lymphocytes in each treatment was found to be dose-dependent. The number of lymphocytes was significantly different ($p < 0.05$) compared to that of controls. The highest lymphocytes were exhibited at 400 mg/kg bw (Table 1). The number of monocytes was inversely correlated to the dose of extract ($p < 0.05$). The mean numbers of eosinophils and neutrophils were unaffected in all treatments ($p > 0.05$).

Discussion

In this study, it was found that the ethanol extract of *H. atra* did not affect the activity of macrophage cells of Balb/c mice. However, the administration of extract for 90 days increased

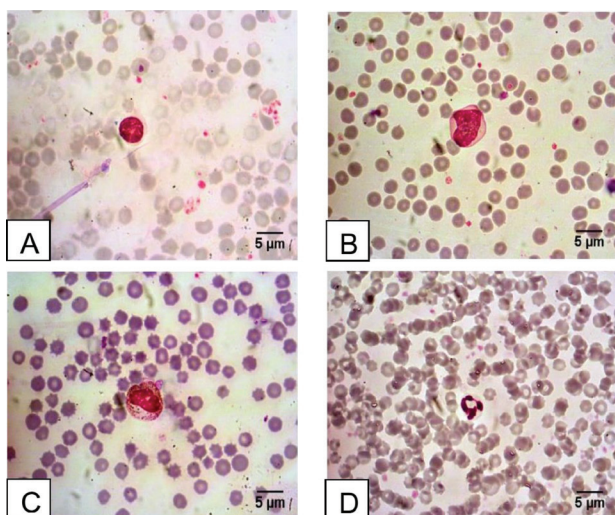


Figure 3. Morphology of leukocytes (100 x). A Lymphocytes; B Monocytes; C Eosinophils; D Neutrophils.

the leukocyte cell differentiation in *R. norvegicus* rat, indicating the immunostimulant effect of *H. atra* extract.

The toxicity of *H. atra* extract is correlated to the major component of its secondary metabolite, saponins. The holothuroid saponins have a strong membranolytic action against cells by inhibiting chemokine receptor subtype 5 (CCR 5) that is responsible for toxicity. Membranolytic is the capability to induce disturbances in cellular membrane permeability up to lysis (Kalinin et al. 2008).

Leukocytes are formed in the spinal cord and consist of monocytes, lymphocytes, neutrophils, eosinophils and basophils which play a role in phagocytosis foreign objects and pathogens that cause allergic reactions. Immune reactions can be determined by analyzing the differentiation of leukocytes (Klein 2012; Vegad 2007). Lymphocyte cells are a key component in the immune system, mainly responsible for adaptive immunity. Based on the function and surface characteristics, lymphocytes divided into B cells (play a role in humoral immunity), T cells (play a role in cellular immunity) and natural killer cells (NK cells). Lymphocytes are made in the spinal cord and mature in primary lymphoid organs (thymus for T cells and lymph nodes, spleen, or other lymphoid organs for B cells) (Hoffman et al. 2017). The lymphocyte population contained in blood samples shows that many lymphocytes circulate in the peripheral blood.

The number of monocytes decreased significantly with increasing extract dose. This showed that *H. atra* extract might suppress the population of monocytes circulating in the peripheral blood. Monocytes will come out of blood vessels and circulate to the tissue when there are foreign objects, and will turn into macrophages in the tissue for phagocytosis. Normal monocyte number in white rat range from 3–11% of total leukocytes (Reddy 2014). The normal amount of basophils ranges from $0.0\text{--}0.3 \times 10^3/\mu\text{l}$ (Freeman and Klenner 2015). This result showed that the administration of sea cucumber extract to rats did not cause hypersensitivity reactions. Basophils play a role in hypersensitivity reactions and defenses against parasites, as well as an

Table 1. Differential leukocytes of white rats treated with *H. atra* extract for 90 days.

Groups	Leucocyte cells (%)				
	Lymphocyte	Monocyte	Basophil	Eosinophil	Neutrophil
A	70.70 $\pm$ 2.40 ^d	12.60 $\pm$ 2.36 ^a	0.00 $\pm$ 0.00	0.50 $\pm$ 0.52 ^a	15.70 $\pm$ 1.56 ^a
B	72.30 $\pm$ 2.35 ^{cd}	10.50 $\pm$ 1.90 ^{ab}	0.00 $\pm$ 0.00	0.40 $\pm$ 0.51 ^a	16.40 $\pm$ 2.06 ^a
C	73.10 $\pm$ 1.52 ^{bc}	9.40 $\pm$ 1.57 ^b	0.00 $\pm$ 0.00	0.50 $\pm$ 0.52 ^a	16.80 $\pm$ 1.22 ^a
D	73.70 $\pm$ 1.25 ^{bc}	8.60 $\pm$ 0.51 ^{bc}	0.00 $\pm$ 0.00	0.30 $\pm$ 0.48 ^a	17.00 $\pm$ 1.56 ^a
E	74.90 $\pm$ 0.73 ^b	7.00 $\pm$ 1.76 ^c	0.00 $\pm$ 0.00	0.30 $\pm$ 0.48 ^a	17.30 $\pm$ 1.76 ^a
F	80.20 $\pm$ 1.11 ^a	3.10 $\pm$ 0.99 ^d	0.00 $\pm$ 0.00	0.20 $\pm$ 0.42 ^a	15.80 $\pm$ 1.47 ^a

Note: Different superscript letters in the same column showed significant differences ($p < 0.05$) Group A = Control (no treatment), B = dose 25 mg/kg bw, C = dose 50 mg/kg bw, D = dose 100 mg/kg bw, E = dose 200 mg/kg bw, F = dose 400 mg/kg bw.

important role in fat metabolism and hemostasis (Latimer 2011). Basophils also have a function as a stimulator of inflammation and allergic reactions (MacPherson et al. 2012).

Eosinophils of the observed rats showed similarity across different treatments, suggesting that there was no hypersensitivity reaction in rats upon administration of the extracts. Eosinophils will increase if there is an infection due to parasites, by playing a role in healing tissue damaged by parasitic infections (Aspinall and Capello 2015). In this study, rats were pre-treated with anthelmintic and antiprotozoal to reduce internal parasites. Increasing the number of eosinophils can also occur in conditions of metabolic disorders, humoral immunodeficiency, autoimmune diseases, and eosinophilic leukemia. Normal eosinophil counts of white mice range from 0.1 to 4.3% of total leukocytes (Montgomery et al. 2013).

The average numbers of neutrophils were similar in all treatments ($p > 0.05$). This showed that the administration of *H. atra* extract might protect the cells from free radicals or infiltrating microorganisms. Increase in neutrophils can be caused by physical or emotional stress, suppurative acute infections, myelocytic leukemia, and trauma (Zachary 2012). Neutrophils will migrate to the tissue that is inflamed. Neutrophil migration from blood vessels to tissue occurs due to changes in the surface of the endothelium. These changes are stimulated by inflammatory mediators (histamine, cysteinyl leukotrienes, and cytokines), produced when leukocytes come in contact with pathogens (Kalaczewska and Kubes 2013). Also, neutrophil granules contain macrophages such as azurocidin, defensins, and cathelicidin (Tizard 2013). This study showed that the administration of sea cucumber extract did not cause inflammation in experimental mice, so that cell integrity can be maintained. This also parallels to the decrease of the number of monocytes in the blood circulation.

In *Cucumaria japonica*, the saponin cucumarioside A₂-2 has been found to increase macrophage activity (Pislyagin et al. 2017). Three saponin compounds isolated from *Cucumaria okhotensis* namely frondoside A₁, frondoside A, and cucumarioside A₂-5 stimulated spreading and lysosomal activity of mouse macrophages (Aminin et al. 2010). Some glycosides possess immunostimulatory action in subtoxic doses. The most effective immunostimulants are monosulfated glycosides. On the other hand,

di- and trisulfated glycosides are immunosuppressors (Kalinin et al. 2008). Additionally, glycosaminoglycan from *Apostichopus japonicus* has also been reported to increase immune enhancement in mice (Wang et al. 2017).

The saponin content in *H. atra* is very diverse, many of which have not been identified. Holothurins A, B, D, echinosides A and B, calcigeroside B are saponins currently identified in *H. atra*. These compounds have remarkable cytotoxicity against several cancer cell lines (Shahinoz-zaman et al. 2018; Grauso et al. 2019). In recent years, there has been an increasing interest in the use of natural immunostimulants as supplements in combination with general therapeutic modalities in cancer treatment. The anticancer activity of this immunomodulator is caused by anti-inflammatory, antioxidant, and induction effects of apoptosis, anti-angiogenesis, and anti-metastasis (Mohamed et al. 2017). Previous studies (Putram et al. 2017; Halimatushadyah et al. 2018) also revealed that *H. atra* extract exhibited cytotoxicity to T47D, HeLa, MCF7, and WiDr cells. The anticancer mechanism of saponin compounds found in *H. atra* in T47D cells occurs through the induction of apoptosis and cell cycle arrest (Nursid et al. 2019). The results of this study may lead to further studies of immunostimulant and anticancer activity in *H. atra*. Considering the height and diversity of saponins in sea cucumbers, we proposed that the saponin contained in

ethanol extract was responsible for immunostimulatory activity in *H. atra*.

Conclusion

The ethanolic extract of *H. atra* increased macrophage activity despite it was insignificantly different from the control group. The administration of *H. atra* extract may increase total lymphocytes, which indicated an increase in the body's immune system response. Decreased monocytes and neutrophils suggested that there was no inflammation or cell damages occurred in the body of rats. Also, the extract used did not cause a hypersensitivity reaction, indicated by unchanged number of eosinophils and basophils.

Acknowledgements

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