Total and Differential Cells of Leukocyte Mice (*Mus musculus*) On Evaluation In Vivo Anticancer Extracts Ethanol Marine Sponges *Aaptos suberitoides*

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Abstract. The aim of this research is to know activity of ethanol extract from marine sponges *Aaptos suberitoides* in total number and differentiation leukocyte cells which induce by carcinogenic agent Benzo(a)pyrene. METHODS. Mice (*Mus musculus*) were grouped in 6 group, group I (healthy mice), group II (induce by CMC Na), group III (treatment by cyvlophosamide), group IV (administered by sponges extract 500 mg/kg BW), group V (administered by sponges extract 1000 mg/kg BW) and group VI (administered by sponges extract 1500 mg/kg BW). Benzo(a)pyrene induced for 10 days (5 times). The doses that given is 0.3 gram / 0.2 ml CMC Na. Mice were killed and the blood were taken. The sacrificed and blood sampling were taken. The total number and differentiation of leukocyte cells is made by smear method.

RESULT AND DISCUSSION. The result of this examination is there were average total number of leukocyte cells in group I was 10,800 cells/μl, II and VI group were 3,700-5,500 cells/μl, the groups III, IV, V of 2,100-5,500 cells/μl. The number of neutrophils and lymphocytes showed a significant difference (P <0.05) while the monocyte was no significant difference (P> 0.05). Keywords: *Aaptos suberitoides*, anticancer, total number of leukocytes, leukocyte differentiation

1. Introduction

Indonesia is one of the country who have the most rich of natural resources. Unfortunately, the natural resources in Indonesia was not used properly. One of the natural resources is a sessile benthic organism that has pharmacological activities as anti-microbial, anti-viral, anti-inflammatory and anti-cancer [1]. One of the benthic organism that has high bioactive activity is a sponge. Sponges have the ability to produce high secondary metabolites and synthesize a variety of organic components such as polyketide, alkaloid, peptide and terpene [2].

Sponges also produce toxins and have the prospect of being used in medicine. It has also been reported that some of the compounds isolated from the sponge have high toxic activity to living organisms [3]. *Spongiosorites* sp. is a sponge that lives in tropical waters. Sponges *Spongiosorites* sp. reported to have bioactive potential [4]. Sponges that taken from Pasir Putih of Situbondo have potential bioactive compounds as anti-cancer *A. suberitoides* is the most toxic sponge with an LC50 value of
134.1362 ± 36.611 [5]. This research is a basic development in the field of health and as a pharmaceutical product for anticancer and immune stimulant.

2. Materials and Methods
2.1. Test Animal Preparation
The experimental animals used were male mice (Mus musculus) 3-month-old B albino clone (BALB/c) strain of 24 individuals, grouped randomly in 6 groups. Before the treatment, mice are acclimated in the treated cage for 1 week and were given the same feed and drinking water, namely Par G produced by Comfeed and aquades as drinking water.

2.2. Carcinogenic Induction of Animal Test with Benzo (a) pyrene
The carcinogenic effect treatment is performed by giving a benzo (a) pyrene solution (representing a type of chemical compound that is carcinogenic) to the subcutaneous tissue in the cervical part of the mouse (M. musculus). Benzo (a) 0.3 gram powder dissolved in 0.2 ml CMC Na.

2.3. Test of Sponges A. Suberitoides against Animal Test
After the onset of cancer, at week 15 the mice were given anticancer therapy / treatment with sponge extract A. suberitoides. Treatment is done orally every day for 2 weeks. The concentration of sponge extract of A. suberitoides was 500, 1000, and 1500 mg / kg BW. The dose is based on the dose of drug worthiness in humans. Mice (M. musculus) were grouped randomly in 6 groups. Each group received the following treatment:
- Group I (healthy mice)
- Group II (induce by CMC Na)
- Group III (treatment by cyvlophospamid)
- Group IV (administered by spons extract 500 mg/kg BW)
- Group V (administered by spons extract 1000 mg/kg BW)
- Group VI (administered by spons extract 1500 mg/kg BW)

2.4. White blood cell count (Leucocytes)
Blood sampling was performed at week 17. The number of leukocytes was calculated using the modified Klonz [6]. Calculation of leukocyte amount was performed by using Thoma leukocyte pipette. Blood samples treated with anti-coagulant were smoked with pipettes until the "0.5" mark. The pipette then immersed in the Rees-Ecker solution inhaled until the "11" sign so as to obtain 1:20 dilution. The pipette is flipped for about 3 minutes by forming a quarter of the circle, then the first 2-3 drops of blood are removed. Furthermore, the blood drops on the side of the counting room with an angle of 30 °. Room count is allowed one minute which aims to lyse erythrocytes and give leukocyte chance to occupy count room. The leukocyte amount was performed with a 40x magnification microscope on four large boxes of count chambers. The number of leukocytes per cubic millimeter (mm³) is the number of calculated cells multiplied by 50.

2.5. Type/Differential Types of White Blood Cells (leukocytes)
Blood samples without EDTA were dripped on the object glass and made smear using right hand placed another object in front of the blood drops at an angle of 30-40 °C. The second object glass was pushed forward to form a thin smear. After drying the smear is fixed with methanol for 3-5 minutes, allowed to dry in the air. The preparations are then stained with giemza solution with 1: 9 dilution for 30 minutes (phosphate buffer pH 6.8-7.2). The preparations were then washed with aquades and allowed to dry on the shelf. After dry preparations are examined under a microscope with 100x magnification calculated each type of leukocytes by battlement method, using a blood counter tabulator. Interrupted count of at least 100 cells and calculated the percentage of leukocyte types. The number obtained is the relative amount of each type of leukocyte from all types of leukocytes.
3. Results and Discussion

3.1. Number of white blood cells (Leukocytes) Mice (M. musculus) on In Vivo Evaluation Anticancer from Sea Sponges Aaptos aubertoides

The total number of leukocyte cells in the Group I treatment remained within the total number of normal leukocyte cells of 5,100 - 11,600 cells / μl [18]. The total number of leukocyte cells control (Group I) differed significantly (P <0.05) by treatment of Group I, II, III, IV and VI. In control, leukocyte cell amount was greater than other treatments. The total number of leukocyte cells at treatment Group II and K6 ie 3,700-5,500 cells / μl did not differ significantly (Table 1).

Table 1. Total Number of Leukocyte Mice Cells (Mus musculus) on In Vivo Evaluation of Anticancer from Sea Sponges A. aubertoides.

| Repetition | Group (cell/μl) |
|------------|----------------|
|            | I   | II  | III | IV  | V   | VI  |
| 1          | 11.950 | 4.400 | 2100 | 2.250 | 3.150 | 3.800 |
| 2          | 10.700 | 5.500 | 2000 | 3.700 | 3.250 | 4.150 |
| 3          | 9.750 | 3.800 | 4650 | 3.100 | 2.750 | 3.700 |
| Average    | 10.800cd±1.103 | 4.566,67ab±862 | 2916,67±0.502 | 3.016,67±29 | 3.050±265 | 3.883,33±2.23 |

(Description: different superscripts in the same column show significantly different (P <0.05)

3.2. Differentiation White blood cells (Leukocytes) Mice (M. musculus) on In Vivo Evaluation Anticancer from Sea Sponges A. aubertoides

In leukocyte cell differentiation, the writer only found neutrophils, lymphocytes and monocytes, whereas eosinophils and basophils were not found (Table 2). The average percentage of neutrophil cells in Group I is 59.67%, still close to the normal range of 60-70% [7]. But statistically, Group I did not differ significantly with Group III and VI (59-73%) (Table 2).

Table 2. Percentage of Mice Neutrophils (M. musculus) on In Vivo Evaluation Anticancer from Sea Sponges Aaptos Suberitoides.

| Repetition | Group (%) |
|------------|-----------|
|            | I   | II  | III | IV  | V   | VI  |
| 1          | 55  | 36  | 73  | 51  | 50  | 59  |
| 2          | 64  | 42  | 59  | 42  | 33  | 66  |
| 3          | 60  | 48  | 65  | 47  | 41  | 71  |
| Average    | 59.67 cd±4.509 | 42±6.00 | 65.67 ed±7.024 | 46.67 ab±4.509 | 41.33 ab±8.505 | 65.33cd±6.028 |

(Description: different superscripts in the same column show significantly different (P <0.05
The average percentage of K1 lymphocyte cells was 27.67%, and the value still in the normal lymphocyte cell percentage range of 25-33%. The percentage of lymphocyte cell amount in Group III and VI were 7-17% did not differ significantly. Group II and V do not differ significantly (Table 3).

Table 3. Presentation of Mice Lymphocytes Mice (M. musculus) in Differentiation Leucocyte Evaluation In Vivo Anticancer from Sea Sponges A. suberitoides.

| Repetition | Group (%) |
|------------|-----------|
|            | I         | II        | III      | IV        | V         | VI        |
| 1          | 34        | 56        | 9        | 42        | 44        | 15        |
| 2          | 28        | 49        | 17       | 42        | 55        | 7         |
| 3          | 21        | 45        | 8        | 42        | 55        | 11        |
| Average    | 27.67b    | 50d,e     | 11.33a   | 42cd      | 51.33d,e  | 11a       |
|            | ±6.506    | ±5.568    | ±4.933   | ±0.00     | ±6.351    | ±4.00     |

(Note: different superscripts in the same column show significantly different (P <0.05)

The percentage of lymphocyte cell amount in the Group III and VI of 7-17% did not differ significantly. Group III and VI have decreased lymphocyte amount (Table 3). On average the percentage of monocyte cells did not differ significantly between treatments (Table 4).

Table 4. Presentation of Monocyte Cells Mice (M. musculus) on Differentiation Leucocyte Evaluation In Vivo Anticancer from Sea Sponges A. suberitoides.

| Repetition | Group (%) |
|------------|-----------|
|            | I         | II        | III      | IV        | V         | VI        |
| 1          | 11        | 8         | 9        | 7         | 6         | 15        |
| 2          | 8         | 9         | 17       | 16        | 12        | 7         |
| 3          | 17        | 7         | 8        | 11        | 4         | 11        |
| Average    | 12        | 8         | 11.333   | 11.333    | 7.333     | 11        |

In cancer-infected mice (Group II) had the same total number of leukocyte cells as mice treated with A. suberitoides sponge extract at the highest dose of 1500 mg / kg BW (Group VI). This means that sponge A. suberitoides extract with the highest dose has not been effective in the treatment of cancer. While the mice treated with A. suberitoides sponge extract at doses of 500 mg / kg and 1000 mg / kg body weight have the total number of leukocyte cells that are relatively the same as the mice induced by cyclophosphamidc cancer drugs so that it can be said that the extract of sea sponges A. suberitoides At doses of 500 mg / kg and 1000 mg / kg body weight has the same effectiveness as anti-cancer drugs. It is suspected that the extract of A. suberitoides sponge is a more effective and useful type of traditional medicine when used at appropriate doses and appropriate use times [8].

In cancer-infected mice occurred damage to the blood-producing system that is bone marrow. All blood cells are formed in the bone marrow so that if the bone marrow is damaged then the production of blood cells will decrease including leucocyte cells. In addition, there are other factors that cause low levels of leukocytes, namely nutrition and stress. Stress over a long period of time will cause weight decreased so bad impact on the immune system. This is because the immune system requires sufficient leptin levels to function properly. Thus a deficiency in fatty deposits can lead to immune deficiency. Poor nutrition can lead to decreased leukopenia and phagocytosis [9].

Sponge activity of A. suberitoides at low doses (500 mg/Kg BW and 1000 mg / Kg BW) as anticancer drugs is suspected to contain bioactive compounds. The most commonly found compound in the marine
sponge *A. suberitoides* is the alkaloid bioactive compound [10]. There are three alkaloids that can be isolated from sponges *A. suberitoides* are aaptamine, 9-dimetylaaptamine and isoaaptamine [11]. The aaptamine compound can induce the expression of the p21 protein that serves to withstand the cell cycle in G2 / M phase. Protein p21 is a member of the family of kinase inhibitor proteins (Cyclin Dependent Inhibitor Kinase / CDIK) which inhibits cell cycle and its expression is controlled by p53-independent [12]. Protein p53 acts as a regulator of cell proliferation and mediators in apoptosis [13], so that uncontrolled cancer cells may experience apoptosis. If cancer cells experience cell death (apoptosis) then the number of cancer cells will decrease so that the number of leukocytes that respond to the presence of cancer cells will be lower.

In leukocyte cell differentiation, only neutrophils, lymphocytes and monocytes are discovered, whereas eosinophils and basophils are not found. There are no infection or inflammation caused by bacteria. Neutrophils play a major role in the early defense of non-specific immunity against bacterial infections [9]. Similarly, eosinophil cells were not observed. And mice is expected not experience allergies or parasitic diseases. Eosinophils cells play a role against parasitic and allergic diseases [14]. While the absence of basophile cells because the number of basophile cells in the blood circulation is relatively small (0.07%). Basophil cells are precursors to mast cells. In the basophile cell contained heparin (anticoagulant). This heparin is released in an inflammatory area to prevent freezing and static blood and lymphs [15].

Lymphocytes play a role in the immune response, responsible for the presence of antigens or foreign bodies by forming circulating antibodies in the blood or in the cellular immune system [15]. In mice infected with cancer (K2) and treated with *A. suberitoides* sponge extract at dose 1000 mg / Kg BW (K5) showed no significant difference (44-56%). In cancer-infected mice, an increase in the number of lymphocyte cells is caused by a specific immune response to cancer cells. Cancer cells are not familiar with the cell death program known as apoptosis. Apoptosis (programmed cell death) is a process that runs physiologically in the cell's life through specific molecular signals that play a role to regulate and determine the process of cell death itself [16] [17]. Protein p53 serves as a regulator of cell proliferation and mediators in apoptosis. Loss of p53 gene function or mutation of the gene makes the cell protected from DNA damage, uncontrolled cell growth and death, cancer cell division occurs continuously without apoptosis [13]. So it can encourage the production of lymphocytes to respond to cancer cells.

On average the percentage of monocyte cells did not differ significantly between treatments. It is expected there is no bacterial infection in mice, so there is no phagocytosis. Monocytes are the largest leukocytes that are 15 to 20 μm in diameter and account for 3 to 9% of all white blood cells. Monocytes are formed in the bone marrow, entering the circulation in immature form and undergoing the maturation process into macrophages after entering the tissues [7]. In addition, there is also difficulty in the identification of monocytes in the presence of transitional forms between small and large lymphocytes.

4. Conclusion
The conclusions of this paper are sponge ethanol extract *A. suberitoides* did not significantly affect the number of leukocyte cells and the percentage of basophil cells, eosinophils and monocytes. Extract ethanol sponge *A. suberitoides* effect on lymphocyte and neutrophil cells.

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