The Activity Test of Ethanol Extract Tea Parasite Herb (Scurrulla Artopurpurea) as an Immunostimulator on Wistar Strain Rat Sensitized with Sheep Red Blood Cell

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Abstract. Tea parasite (Scurrulla artopurpurea) is one of the hemiparasite plants suspected as an immunostimulator agent. The aims of this study was to prove the activity of tea parasite herb extract as immunostimulator in Wistar strain rats that are sensitized by sheep red blood cell suspension (SRBC). Twenty eight Wistar rats were divided into 4 groups. The control group was given CMC 1%, the treatment group was given the tea parasite herb extract at doses of 750 mg, 1.5g and 3 g/kgBW/day, each group was sensitized by SRBC 10% as much 0.5 ml intraperitoneally on the 0th and 9th days and 0.1 ml intraplantar on 12th days. The total number of leukocyte and lymphocyte cells was performed using blood smear and analyzed by GLMRM test. The macrophage cell activity and the capacity of the peritoneal fluid preparation on day 14 and analyzed by ANOVA test. The histopathological features are demonstrated after 48 hours of intraplantar injection, quantitatively perceived perivascular and periadnexal infiltrates. The results showed that there were average differences on leukocyte cell counts (F = 46.249) and total lymphocyte cells (F = 58.144) on each calculation (day), but there were no average differences between each treatment group. The highest activity of phagocytosis is 78% and continues to increase along with the additional dose of extract, without the increased capacity of macrophage phagocytosis. Histopathological features of slow-type IV reaction shows mild severity. It can be concluded that the active compound of the extract tea parasite herb are able to improve immune system.

1. Introduction
Indonesia is a part of a tropical country. Areas that have a tropical climate are perfect for the growth of various disease agents such as bacteria, viruses and so on. This is what facilitates the immune system weakened, resulting in various diseases [1]. Humans since birth have been equipped with a specific and non-specific body defense system. The immune system works to counteract various bacteria, viruses, fungi, and other foreign substances that can cause various diseases [2]. If interference occurs by the antigen (foreign body), then the body’s defense system will be activated to maintain the body against infection of microorganisms, homeostasis against the elimination of old components of the body and supervision against the destruction of cells that mutate, especially the malignant [3].
Immunostimulator is a substance capable of improving the function and activity of the immune system, so as to restore the imbalance of the impaired immune system by stimulating and improving the function of it. Both immune systems work simultaneously in eliminating the antigens that enter the body. Materials that can stimulate immune cells can be from biological or synthetic groups [4]. Immunostimulators relate to the presence of antigens that enter our body and are able to strengthen the immune system naturally by inducing immune responses through complementary phagocytosis, IgA antibody secretion, release of α and β interferons, increasing T and B lymphocytes, synthesizing specific antibodies [5].

Tea parasite (Scurrulla artropurpurea) is one of the hemiparasite plants suspected as an immunostimulator agent that has 16 bioactive compounds such as polysaccharide compounds, steroids / triterpenoids, alkaloids, saponins, tannins, quinones and flavonoids [6,7]. Benalu has long been used as a cure for various diseases, according to Winarno et al. (2000) that tea parasites have. Infusion of tea parasite is suspected to have immunostimulator activity in cancer cells in C3H mice by increasing the concentration of immunoglobulin G (IgG). The immunostimulator effect is due to the content of quercetin contained in the parasitic extract of tea. Quercetin is thought to induce the proliferation and maturation of macrophages and is able to induce T lymphocytes or lymphoid maturation [7]. In addition, the Lectin content contained in tea parasite is cytotoxic and able to boost the immune system [8]. Polysaccharides contained in the parasite can increase the secretion of antibodies and cytokines, either by increasing the function of Natural Killer cells and T and B lymphocytes [9].

In this research, immunostimulator activity of ethanol extract of parasite (Scurrulla artropurpurea) in white male Wistar strain is first induced by sheep red blood cells as antigen.

2. Research Methods

The design of this study used a preposttest experimental method with a control group, conducted in the laboratory using tea parasite test (Scurrulla atropurpurea) materials and white male rat (Rattus norvegicus) strain of male Wistar strain previously induced with sheep red blood cells, then blood sampling for the total number of lymphocytes, leucocyte cells, activity and capacity of phagocytosis and histopathologic features as a quantitative type of slow hypersensitivity immune response.

The ethanol extract of tea parasite was obtained from BALITRO, Bogor using the maceration method and the phytochemical simplicia has been tested that includes examination of secondary metabolites consisting of alkaloid compounds, flavonoids, saponins, tannins, steroids / triterpenoids.

The ethanol extract of tea parasite was suspended with 1% Na CMC 2 ml, then administered to the orally daily rats for 14 days with dose of 750 mg / kgBW / day, 1.5g / kgBW / day and 3g / kgBW / day.

Animal for testing is done by ethical approval by Medical Ethics Committee of Faculty of Medicine UPN Veteran Jakarta with approval number B / 1156 / VII / 2017 / KEPK. Prior to the experiment, Wistar strain rat were adapted for one week and each group of rats was kept in a cage, fed standard feeding ad libitum. Then each group was sensitized with 10% of red blood cell sheep (SRBC) suspension intraperitoneally on days 0 and 9 and 0.1 ml intraplantar on day 12 [10]. The samples were taken from animal experiments with a minimum sample size of 28 tails calculated on the Faraday formula. Then divided into 4 groups namely 1 control group given 2 ml CMC 1% and 3 treatment groups.

According to Nurmaya Effendi (2014), 1 mL of fresh sheep blood is accommodated in a tube containing 1 mg EDTA as an anticoagulant. Then centrifuged 3000 rpm for 10 minutes. After forming two layers of plasma and red cell deposits, then the plasma fluid is removed by using a micropipette. Then the precipitate was washed with PBS pH 7.2 in the tube three times. This procedure is repeated until the top layer is completely clear and colorless. The clear top layer is removed and the bottom layer is a 100% SRBC suspension. Take 0.5 mL of 100% SRBC suspension, add PBS with same volume, so get 50% SRBC suspension. Prepare the antigen to be used by diluting 0.4 mL of 50% SRBC suspension with 1.6 mL PBS to obtain 2 mL of antigen suspension (SRBC 10%) [11].
The white rat was taken by the ventral part of the vein by the tail section compressing warm water for 5 minutes in advance to dilate the vein, then the rat tail was cut. Blood that came out immediately taken and accommodated on a 1.8 ml effendorf tube which drops with EDTA 4% 2 drops. The pipette is then shaken for three minutes until homogeneous. Blood sampling was performed on days 0, 7 and 14.

Take the blood from the tube by inhalation using a pipette until the mark 0.5 and the tip of the pipette is cleaned with a tissue. Then suction the Turk solution with the same pipette until it reaches the limit of the number 11. Before the solution is put into the count chamber, remove the first two or three drops. After the solution is put into the counting chamber awaited for one minute, the next leukosit is calculated using magnification 10 times or 40 times on the objective lens. The leukocyte count formula is number of cells x 50.

Total lymphocyte counts were performed by using the multiplication of leukocyte count and lymphocyte count [3, 12]. The calculation of the absolute number of each leukocyte type is calculated by the formula is the number of cell of the leukocytes type :100) x total number of leukocytes.

On the 14th day, the euthanized rat was then dissected in the stomach by using surgical scissors and sterile tweezers. Intraperitoneal fluid is taken using a micropipette. Then the intraperitoneal fluid is applied to the object glass and fixed with methanol for 5 minutes with Giemsa staining. Then let stand for 20 minutes and then flowed with running water. After dry preparation of the dosage under a microscope and with the added emergent oil with 10x-100x enlargement is calculated the activity and capacity of macrophage phagocytosis. Phagocyte activity is determined by percentage of the number of phagocyte cells that will perform phagocytosis process in 100 phagocyte cells. The phagocyte capacity is based on the amount of SRBC to be in phagocytosis by 50 active phagocyte cells [13].

Sensitized rats with intraperitoneal SRBC suspension were injected with an intradermal 0.1 ml SRBC10% on the sole of their right foot on day 12. Injecting effects were seen after 48 hours of exposure to sheep red blood cells, then rats in anesthesia with later ether rats were killed by means of cervical dislocations, then taken the soles of their feet to be processed into histologic preparations after paraffin blocks were made. Values for inflammatory severity are seen separately for periadnexal and perivascular inflammation for each section. Each part of the skin was given an overall score based on the number of points for periadnexal and perivascular inflammation [14].

### 3. Research and Discussion Result

The result of phytochemical simplicia test from qualitatively tea extract by BALITRO laboratory, Bogor was found to contain positive alkaloid, saponin, tannin, phenolic, flavonoid, triterpenoid, steroid and glycoside compounds. This is consistent with a study by Simanjuntak (2004), that tea parasites in the genus Scurrula contain 16 important compounds, six unsaturated fatty acid compounds, two xantins, two flavonol glycosides, one monoterpene glycoside, one lignin glycoside and four flavones [15].

The test animals used were male rats aged 3-4 months, according to the adult age in humans, so that the expected hormone levels in the body have been stabilized and the immune system in male rats is less affected by the reproductive hormone. Basically the human’s and rats’ immune system are almost the same [16].

| Control Group | Treatment Group 1 | Treatment Group 2 | Treatment Group 3 |
|---------------|-------------------|-------------------|-------------------|
| Leukosit      | Limfosit total    | Leukosit          | Limfosit total    | Leukosit          | Limfosit total |
| day-0         | 12.76             | 7.79              | 15.36             | 10.51             | 13.86           | 9.38           | 12.19           | 7.75 |
| day-7         | 23.22             | 16.68             | 24.11             | 18.27             | 23.07           | 17.88           | 18.46           | 13.87 |
| day-14        | 6.69              | 4.22              | 8.69              | 5.18              | 7.58            | 5.60            | 7.72            | 4.67 |

Table 1. Result of Calculating Average Number of Leukocyte Cells and Total Lymphocyte Cells in Wistar Wistar Tissue Between Treatment Group Compared to Control Group on Day 0, 7, and 14 (Thousand / mm3)
The first intraperitoneal sheep red cell sensitization will stimulate non-specific immune systems, i.e leukocyte cells to eliminate pathogens, although the body has not been exposed before, whereas the specific immune system will be triggered after the body gets the same previous antigen exposure through memory immune cells. Specific immune cells play primarily lymphocyte cells, because they have the main characteristics of specificity, diversity, memory, specialization and self-limiting [17].

The result showed that the average number of leukocyte cell and total lymphocyte cell of Wistar strain on each treatment on days 0, 7, and 14 as shown in Table 1. The average difference of the total lymphocyte cells number in Wistar rats on day 0, 7th and 14th day can be known by the Within subjects contrasts test as shown in Table 3 and differences between treatment groups can be obtained by testing the Between subjects effects as listed in Table 4 based on analysis general linear test model repeated measures (GLM-RM) test.

### Table 2. Within-Subjects Contrasts Test Results

| Source  | Day               | df | F    | Sig. |
|---------|-------------------|----|------|------|
| Day     | Level 1 vs. Level 2 | 1  | 58.144 | .000 |
|         | Level 2 vs. Level 3 | 1  | 225.137 | .000 |
| Day * treatment | Level 1 vs. Level 2 | 3  | 0.355  | .786 |
|         | Level 2 vs. Level 3 | 3  | 1.236  | .319 |
| Error(Day) | Level 1 vs. Level 2 | 24 |       |      |
|         | Level 2 vs. Level 3 | 24 |       |      |

Information:
- Level 1 = day 0, Level 2 = Day 7, Level 3 = Day 14
- Treatment = control group (placebo CMC 1%), treatment (tea parasite extract 750 mg, 1.5g, 3g / kgBB / day)

The average difference of the leukocyte cells number in Wistar rats on day 0, 7th and 14th day can be known by the Within subjects contrasts test as shown in Table 3 and differences between treatment groups can be obtained by testing the Between subjects effects as listed in Table 4 based on analysis general linear test model repeated measures (GLM-RM) test.

### Table 3. Between subject effects Test Results

| Source  | df | F    | Sig. |
|---------|----|------|------|
| Intercept | 1  | 527.078 | .000 |
| Perkalian | 3  | 1.825 | .17  |
| Error    | 24 |       |      |

### Table 4. Within-Subjects Contrasts Test Results

| Source  | Day               | df | F    | Sig. |
|---------|-------------------|----|------|------|
| Day     | Level 1 vs. Level 2 | 1  | 46.249 | .000 |
|         | Level 2 vs. Level 3 | 1  | 313.686 | .000 |
| Day * treatment | Level 1 vs. Level 2 | 3  | 0.474  | .703 |
|         | Level 2 vs. Level 3 | 3  | 2.485  | .085 |
| Error(Hari) | Level 1 vs. Level 2 | 24 |       |      |
|         | Level 2 vs. Level 3 | 24 |       |      |

Information:
- Level 1 = day 0, Level 2 = Day 7, Level 3 = Day 14
- Treatment = control group (placebo CMC 1%), treatment (tea parasite extract 750 mg, 1.5g, 3g / kgBB / day)

On day 0, the amount of leukocyte cell is within the normal range that is 4.5x103-11x103 cell/µL. On day 7, after a desensitize of SDMD antigen, a respond of non specific immune increases the amount of leukocyte cells. This indicate that antigen diffused to the vascular, continues to be phagocytosised by leukocyte cells. Limfocyte cells appear on day 5 and reach the maximum amount on day 7. According to theory, the increasing amount of limfocyte cells are caused because of
proliferation dan the differences are triggered by new antigens that is shown by APC. Later on day 14, there is a tendency of the decreasing amount of leukocyte cells and limfocyte cells because the extract of Benalu tea intensify the immune response on a limited time, with a limited effect. This happened because it depends on the amount of dose tested, if it is high it will cause imunosupression and if it is low it will cause imunostimulation. Furthermore, the technique of dose tested will affect the strength of immune response that appears. This imunostimulation ability seems to be related to polysaccharide content in parasite tea extract which can increase the secretion of antibodies and cytokines by stimulating the function of natural killer cells and also T and B lymphocytes.

Macrophages have a very important role as phagocytes in the innate immune system and as antigen presenting cells (APC) that initiate an adaptive immune response. TLR can activate innate immune responses and stimulate the production of various proteins that play a role in the important function of macrophages. From the results of the research, the calculation of activity and capacity macrophage phosphocytic of Wistar strain rats on each treatment as shown in Table 6 and 7.

| Table 6. Results of Fagocytosis Macrophage Rats Wistar Triangle Tests | Table 7. Results Calculation of Meaning of Intergroup Macrofag Fagocytosis Capacity |
| --- | --- |
| **Group** | **Macrophage Activity** | **Group** | **Kapasitas Fagositosis** |
| Control | 70% | Control | 567 |
| Treatment 1 | 71% | Treatment 1 | 310 |
| Treatment 2 | 69% | Treatment 2 | 216 |
| Treatment 3 | 78% | Treatment 3 | 371 |

Group 1 : inflammatory cells are located in small numbers around glandular and follicular structures, some inflammatory cells may be present in the superficial dermis. No infiltrate perivascular inflammatory

Group 2 : inflammatory cells cuffs some adnexal structures, small numbers inflammatory cells scattered in the superficial dermis. Inflammatory cells are located around vessels and scatters around these areas, they may be present in small numbers around adipocytes in the hypodermis, present around the majority of vessels

Group 3 : inflammatory cells cuffs some adnexal structures, small numbers inflammatory cells scattered in the superficial dermis. Inflammatory cells are located around vessels and scatters around these areas, they may be present in small numbers around adipocytes in the hypodermis, present around the majority of vessels

Group 4 : inflammatory cells are located in small numbers around glandular and follicular structures, some inflammatory cells may be present in the superficial dermis. No infiltrate perivascular inflammatory

**Figure 1.** Histopathology of the skin as a response to slow type hypersensitivity
Statistical test results using One-Way ANOVA and showed significance value ($p<0.05$) showed that there was a significant difference in macrophage phagocytosis activity between groups and no significant ($p>0.05$) difference in macrophage phagocytosis activity between groups. Further statistical test results with Post Hoc test to see comparison between groups showed similar results with the Anova test.

According to the results of macrophage measurements, Benalu tea extract is not high enough to increase the capacity of macrophage phagocytosis, even though with the maximum additional dose. The differences taken of the activity of macrophage fagocytosis in this research is allegedly caused by presence of flavonoid and saponin contained in the Benalu tea extract. Francis et al [18] stated that saponin increases the macrophage fagocytosis. Flavonoid potentially works on limfokin that is produced by T cell, so it will stimulate the phagocyte cells that responds to phagocytosis [19]. As an imunostimulan, flavonoid and saponin compound are used to improve the immune system by increasing the phagocytosis by granulocyte and macrophageand stimulate the production of sitokin by Th2 cells so the production of antibodies will increase [20].

Delayed Type hypersensitivity (DTH) response is a reaction of increased reactivity and sensitivity to antigens previously exposed and used to determine the activation of T cells involved in cell-specific immune responses. Histopathologically can be seen the existence of inflammatory response, existing cell types, inflammatory severity and location of infiltration is by assessing periadneksal and perivaskular infiltrates (Figure 1).

Overall, histologic features do not show a significant effect of DTH response. This is possible because flavonoid compounds can affect complex immune responses in complex and biphasic action, ie low doses can stimulate lymphocyte proliferation but otherwise high doses preclude it.

4. Conclusions
Overall, test animals treated with tea extracts at various doses did not show any significant difference, so further studies of other immunity parameters were necessary.

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