The Nitric Oxide-Induced Immune Effect After Temulawak (Curcuma Xanthorrhizae Roxb.) Starch Treatment on Cyclophosphamide-Induced Rats

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Abstract. Temulawak (Curcuma xanthorrhizae RoxB.) is known as an immunostimulant medicinal herb, however its starch has not been extensively investigated. Therefore, this study aimed to assess the immunostimulant effects of temulawak starch (TS) in cyclophosphamide (CYD)-induced Sprague-Dawley male rats. Temulawak starch (TS) were prepared with simple method using alpha-amylase enzyme to obtain the polysaccharide fraction. The quality of TS were evaluated using dextrose equivalent value and moisture content, which were 23.79 and 7.63%, respectively. The animals were divided into six groups, each five rats a group, namely, carrier, untreated, and positive control (levamisole), and TS at doses of 200, 400, and 800 mg/kg BW orally. Meanwhile, all animals except the carrier control group received CYD induction at a dose of 100 mg/kg BW on days 16 and 18. The investigated parameters, such as leukocytes and thrombocyte value, body weight, and nitric oxide (NO) secretion of the peritoneal macrophage were examined after 56 days of treatment. The results showed that there were no significant differences in the parameters of leukocyte and and thrombocyte value between the groups ($p > 0.05$). However, there was a significant increase in nitric oxide (NO) secretion at the dose of 200 mg/kg BW compared to the other groups ($p < 0.05$). This increase in NO production could be associated to a rise in the non-specific immune response. Therefore, it can be concluded that TS treatment at a dose of 200 mg/kg BW showed an immunostimulant effect on the CYD-induced rat model via nitric oxide production pathway. Nevertheless, further studies are needed to confirm the immunostimulant mechanism on other pathways.

Keywords: Immunostimulant activity · cyclophosphamide · temulawak (Curcuma xanthorrhizae RoxB.) starch · nitric oxide

1 Introduction

The immune system is the body's defense mechanism that responds to the invading external agents in the form of living things such as whole micro-organisms and small

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compounds, such as free radicals and toxins [1, 2]. In general, there are two types of immune systems, namely, the non-specific and specific. The non-specific response is innate immunity that naturally body gained, while the specific is acquired, or often called an adaptive response that arises against certain antigens after a previous exposure [3]. Macrophages are the immune cells that provide the first response to the presence of foreign body invasion. The modern genetic insight stated that this cell in vertebrata was generated from erythro-myeloid progenitors (EMPs) of the yolk sac and matured in the fetal liver, and subsequently colonize organs throughout the body, giving rise to local macrophage populations such as the microglia of the brain, Langerhans cells of the skin, Kupffer cells of the liver, and resident macrophages of the lung. The presence of inflammation or damage has a significant impact on the mechanism of replenishment of tissue-resident macrophages [4, 5]. The defense mechanisms of macrophage against invading pathogens are by producing proinflammatory cytokines and releasing several inflammatory molecules such as tumor necrosis factor (TNF), interleukin (IL) 1β, IL6, or nitric oxide (NO). The contributing of NO as host defense compound toward microorganism and tumor cells was previously reported both in vitro and in vivo [6, 7]. The other study reported that the immunostimulant activity of a tested sample can be determined from its ability to level up the hematological parameters on an animal model with suppressing body's immune system, such as gallic acid that was gavaged on the CYD-induced mice increase white blood cells, red blood cells, platelet counts and hemoglobin levels [8].

Temulawak (Curcuma xanthorrhizae RoxB.), also known as Java Turmeric and belonging to the Zingiberaceae family is a medicinal plant widely used in Indonesian herbal medicine (Jamu) [9]. Among temulawak's secondary metabolites, curcuminoids, terpenoids and xanthorrhizol are the most abundant compound in temulawak and reported demonstrating important biological effects (10). Some studies reported, both such kind of crude extracts and chemical entities of temulawak demonstrated pharmacological activities such as antioxidant, anti-inflammatory, antibacterial, immunostimulant, and antifungal [10, 11]. The mode of action of temulawak as immunostimulant agent is probably multi-targeted such as 18.2 μg xanthorrhizol/ml inhibitied PAF-receptor binding and it was also reported that methanolic extract inhibited the release of reactive oxygen species (ROS) and chemotactic migration of phagocytes in vitro (IC50 0.7 μg/ml) [11].

It had been reported that mono, oligo and polysaccharide compounds isolated from many plants demonstrated pharmacological activities and also involved in modulating immune system [12–14]. Kim and team (2007) claimed that polysaccharide fraction of temulawak induce NO production on RAW 264.7 cell in vitro. This polysaccharide also enhances the macrophage phagocytic index of fluorescence-labeled Escherichia coli on RAW cells [6].

However, polysaccharide isolation developed by Kim and team required sophisticated and complex equipment. It meant that the application of this process on an industrial scale would require a large investment cost. In addition, the studies carried out previously were still limited to on RAW cells. Further studies to evaluate the immunostimulant activity of TS on high-level taxonomy animals (in vivo) are still needed because the in vivo study was a mimic of the human body rather than on a cell system. As a result, the goal of these studies was to refine the TS isolation using a simple method on laboratory scale,

followed by assessing the immunostimulant activity on CYD-induced rats using the NO level of peritoneal macrophages as a parameter tested. Hopefully, this TS production can be optimized in a low-scale industrial process by local enterprises.

2 Materials and Methods

The dried rhizome of temulawak was purchased from the local market in South Tangerang, Banten, Indonesia. An alpha-amylase enzyme was purchase from Novozyme (Liquozyme®Supra, China) and 3,5-dinitrosalycil acid was from Merck, Germany. RPMI 1640-medium was from Gibco, Fisher Scientific, English. Griess reagent (modified), sodium nitrite, and lipopolysaccharides were all from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethanol, cyclophosmaphide (CPY), levamisole was from the local market. Sprague-Dawley rats (30 males, 14 weeks old) were obtained from The Indonesian FDA (National Agency of Drug and Food Control (BPOM). The animals were housed in a controlled ambient temperature (22 ± 20C) and 12 h dark/light cycles. Also, standard fed laboratory (21% protein level, PT. Indofeed, Indonesia) and tap water were given ad libitum. Animal experiment protocol had been approved by the Ethics Committee of Medicine Faculty, University of Indonesia (1170/UN2.F1/ETIK/2018).

2.1 Preparation of Temulawak Starch

Temulawak starch preparation was conducted based on the previous research [15] with modification. Briefly, the dried-powdered-rhizome of temulawak (100 g) was soaked in water (1000 mL) and heated until 80 °C for 1 h. Alpha-amylase enzyme (diluted with distilled water 1:1) was added to the heated rhizome, followed heating for 2 h later and then the mixture was cooled in an ice bath for 1 h. After being filtrated using a double molisch cloth filter and a Whatman paper, ethanol was poured into the filtrate (1:1). Furthermore, the sediment polysaccharide formed was collected and dried using an oven at a temperature less than 50 °C.

2.2 Determination of Dextrose Equivalent (DE)

DE measurement was based on reduction of 3,5-dinitrosalicylic acid (DNS) with the reducing moiety of the sample forming 3-amino-5-nitrosalicylic, as explained on the previous study [15]. Sample solution was prepared by dissolving 50 mg sample in 2 mL distilled water, sonicated in the sonicator chamber, and filtered using a membrane cellulose acetate syringe filter with a pore size of 0,45 μm. The filtrate was collected for DE determination.

2.3 Animal Experiment

After being acclimatized in the laboratory environment for 7 days, 30 male rats were randomly divided into six groups (n = five rats per group). The first group assigned as carrier control (normal) (carboxymethylcellulose, CMC 0.5%), the second was model or untreated (CMC 0.5%), the third served as the positive control (Levamisole), while four, five, and sixth received TS at the dose of 200, 400 and 800 mg/kg BW, respectively. The treatment received by each group was summarized in Table 1.

Table 1. The Summary of animal treatment

Day of experiments	Activity
Day 0	Blood withdrawal for hematological evaluation
Day 1–56	Administration of test sample/levamisole/carrier p.o. Bodyweight evaluation (weekly)
Day 16 and 18	Immunosuppressant agent Induction (CYD) i.p.
Day 21 and 29	Blood withdrawal for hematological evaluation
Day 57	Animal sacrifice Intraperitoneal macrophage isolation
	Evaluation of nitric oxide release

2.4 Hematological Evaluation

After being fasted overnight, about 500 μL of blood was drawn from retro-orbital and collected in a microtube with heparin. The leukocyte and thrombocyte were determined using Hematology Analyzer Sysmaz KX-21 at day 0 (before), 21, and 29 after CYD injection.

2.5 Statistical Analysis

Data of body weight, leukocyte, thrombocyte, and nitric oxide level was stated as mean ± standard deviation. One-Way ANOVA test followed by the LSD test with SPSS 25 was used for statistically analysis. The level of statistical significance was set at $p < 0.05$.

3 Results

3.1 Determination of DE

The DE value determination was carried out based on the reduction reaction of a yellow 3,5-dinitrosalicylic acid to be the orange red colorant 3-amino-5-nitro-salicylic acid in alkali condition and the oxidation of the aldehyde group of reducing sugars to the carboxylic acid (16, 18). The calibration curve of dextrose standard was obtained a linear in the range concentration of 100–1300 ppm with following equation $y = 0.0008x\text{-}0.0303$ ($R2 = 0.9985$). The DE value of TS powder was 23.79, while the water content was 7.63%.

3.2 Animal Experiment

The average body weight of each group was depicted in Fig. 1. It was seen that there was a small weight loss between weeks 2 and 3 in the group who received cyclophosphamide. After three weeks, the body weight of cyclophosphamide-induced groups showed gradual increase, and it became comparable to those of the normal group.

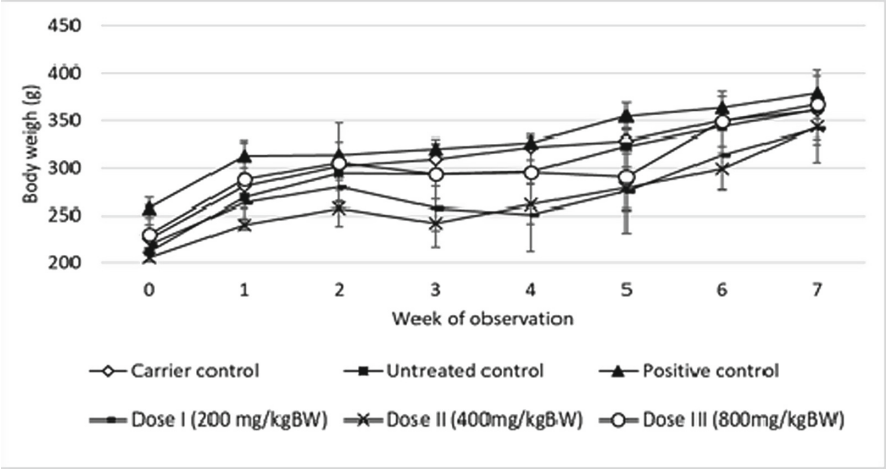

Fig. 1. Bodyweight variation of each group

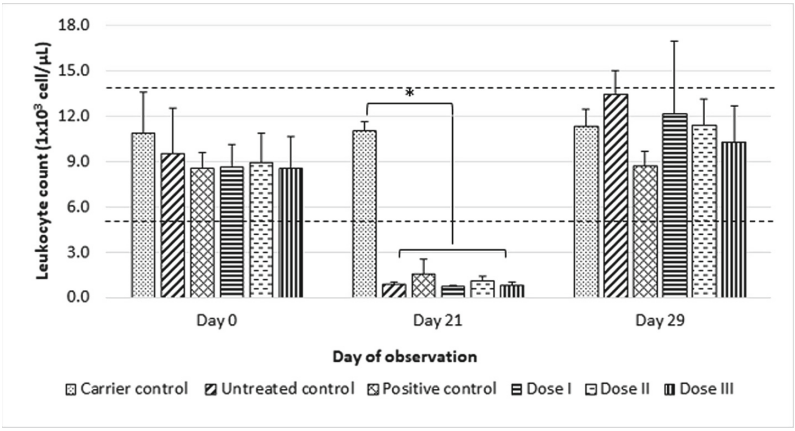

Fig. 2. The average leukocyte value of all groups at the point of measurements.* = Significant difference ($p < 0.05$). The range value between dot lines indicated a normal value level. Value was an average of 5 rats

The leukocyte and thrombocyte count on day 21 and 29 after treatment was depicted in Fig. 2 and 3, respectively. The level of leukocyte and thrombocyte on day 0 was considered as a baseline. On the 16th and 18th day, cyclophosphamide was administered to all groups except to the carrier control group. Three days after the last treatment (day 21), the number of leukocytes and thrombocytes was decreased in all groups. On the 29th day, leukocytes value back to normal in the treatment group but there was an increase in thrombocytes exceeding the normal value (shown with stripped line).

The average nitrite oxide concentration excreted from the intraperitoneal macrophages were depicted in Fig. 4. LPS was shown to stimulate the production of

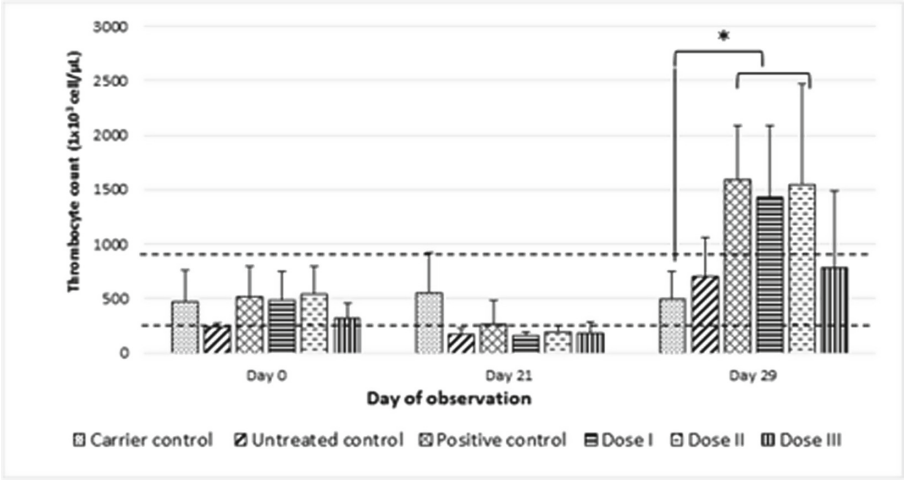

Fig. 3. The average thrombocyte value of all groups at the point of measurements.* = Significant difference ($p<0.05$). The range value between dot lines indicated a normal value level. Value was an average of 5 rats

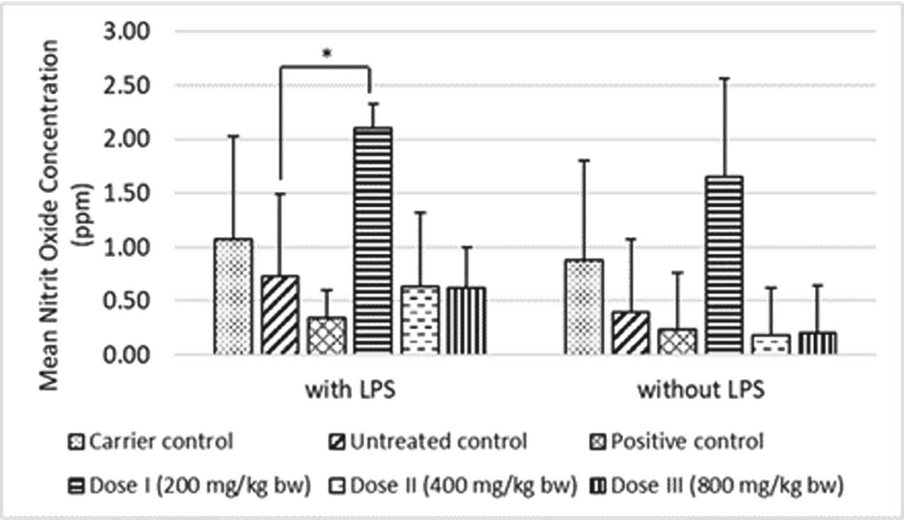

Fig. 4. The average NO of all groups after treatment. * = Significantly difference ($p < 0.05$). Value was an average of 3 rats

nitric oxide in all groups. Furthermore, there was a statistically significant difference between untreated group and the group who received TS on dose 200 mg/kg BW.

4 Discussion

In this study, we investigated whether the temulawak starch (TS) have a potential as an immunostimulant agent on the cyclophosphamide-induced rats. We prepared the TS using local source with simple method, as opposed to more time-consuming method found in previous references [6]. For evaluation of the TS quality, we use DE (dextrose equivalent) parameter and water content. Dextrose equivalent (DE) expressed the degree of conversion of starch to dextrose [18]. Previous studies showed that monomer or short chain products of hydrolyzed-starch exhibited some benefits such as a source of soluble dietary fiber, stimulating bacterial growth in the human colon, and also as an important material in industrial bioprocess [6, 19]. The overall water content was evaluated because it is a critical factor for bacterial growth [20]. To avoid microbiological growth and achieve product stability, the Indonesian FDA specifies that the water content of the dried-solid natural product be less than 10% [21]. Our result was 7.63%, indicating that this product meets the requirement established by authorities.

Cyclophosphamide (CYD) has a known side effects such as weight loss [22], and this effect also observed in this study as depicted in Fig. 2. There was a slight weight loss between weeks 2 and 3 in the group who received cyclophospamide. We deduced that this weight loss probably due to the induction of cyclophosphamide on days 16 and 18.

Another side effects of CYD is leukopenia and thrombocytopenia. The initial leukocyte value shown in Fig. 3 corresponded to the data from a study by Nadzirah and Oduola [23] that the baseline leukocyte number was between 5.00–14.45 x10^3 cells/μL. The reduction of leucocyte on 21th day was consistent with a previous work which reporting the effect of CYD administration on leukocyte, namely leukopenia [24]. The leukocyte number of carrier control group, on the other hand, could be maintained at the normal value. Previous studies reported that levamisole prevented CYD immunosuppression effect in vivo only when rats were pre-treated for minimum 30 days before induction [25]. In addition, a week after the 2nd blood measurement (day 29), leukocyte count increased back to the normal acceptable value. According to a previous study, rats' blood counts (leukocytes, neutrophils, and lymphocytes) decreased after CYD induction for 7 days, then gradually restored to normal from day 10 to day 21. [26].

The other parameter to be assessed was thrombocyte count, and the average number of each group at the point of measurements was depicted in Fig. 4. Thrombocyte value on day 0 (before any treatment) was considered as a baseline. This level also corresponded with previous literature which was between 253.50–963.50 x 10^3 cells/μL [23]. On days 16th and 18th, CYD was administered into all groups except carrier control. Three days after administration (day 21), the thrombocyte count decreased significantly in all the groups. This result confirmed the side effect of administration as stated in the literature, which is thrombocytopenia (28, 29). Treatment of TS cannot ameliorate the decrease of thrombocyte level, and this same result was also observed in the levamisole group. However, approximately a week after 2nd blood measurement (day 29), the thrombocyte count increased significantly even above normal value (thrombocytosis) in all groups that received CYD injection compared to carrier control. This phenomenon is known as rebound thrombocyte count and is also discovered in a previous report in the human clinical trial [29]. The previous study suggested that increasing thrombocyte counts

after CYD injection is directly linked to increasing megakaryocyte colony-forming cells both in bone marrow and spleen, which is responsible for producing blood cells, such as thrombocytes. Also, it is speculated that this phenomenon is feedback mechanism which controls megakaryocytopoiesis, with a resultant simulation of the megakaryocyte progenitor compartment [30]. Both groups that received TS at the dose of 200 and 400 mg/kg BW demonstrated a higher level of thrombocyte count than the last dose of 800 mg/kg BW. Statistical analysis of both groups showed significant differences compared to the carrier control group ($p < 0.05$).

The usage of CYD in developing immunosuppressive animal models had been previously demonstrated due to CYD can break the structure of DNA, alter macrophage proliferation and differentiation, destroy immune cells, and weaken the immune system, [31]. NO is a highly reactive molecule produced by activated macrophages as a response of the innate immune system's reaction [32]. It also plays a key role in host defense against germs, protozoa, and cancer cells [33]. As a result, a rise in NO levels could signal a stronger immunological response in the host. The results of peritoneal macrophage NO production after sample treatment were illustrated in Fig. 5. The animal model (untreated control group) induced by CYD indicated that production activity of NO on peritoneum macrophage decreased compared to the carrier control group. Treatment of animals with TS could increase NO production. NO release was marked on the dose I group than others. In this group, the NO level was significantly different compared to the untreated control group ($p < 0.05$). Furthermore, as compared to its counterpart, NO released in the medium with LPS was greater (the one without LPS). This was consistent with the fact that LPS is among the most potent activators of macrophages to produce inflammatory mediators such as nitric oxide [34]. These results supported previous studies that claimed that crude polysaccharide extract from C. xanthorriza increased phagocytic fluorescence-labeled E. coli on RAW 264.7 in vitro [6]. In addition, this polysaccharide increased the release of NO, H2O2, TNF-alpha, and PEG-2 in a dose-dependent manner in vitro [6]. When macrophages are stimulated by an invading pathogen or antigen, they can release a high amount of nitrite that can destroy foreign microorganisms, parasites, and tumor cells. The other mechanism of NO in preventing the invading was by inducing inflammatory reactions [35]. Previous studies described that polysaccharide isolated from plants can modulate the immune system in multiple ways [1, 35]. It can support immune system cells like T-cell and B-cell lymphocytes, macrophages, and NK cells, as well as trigger and activate the complement system that produces cytokines [35]. These results also showed that levamisole as the positive control did not exhibit an immunostimulant effect via the nitric oxide pathway. It is speculated that this compound is involved in reestablishing the immune system through other pathways rather than stimulating response to above-normal levels. Also, it was previously reported that the levamisole treatment on cyclophosphamide-induced rats increased gamma globulin level, leucocyte, neutrophil, and monocyte counts as well as significantly increase phagocyte activity compared to normal [36, 37]. The immunomodulatory mechanism of levamisole is by modulating the interaction between its imidazole ring and the sulfur moiety of T-cell [38].

In summary, it could be concluded that temulawak starch (TS) isolated with this simpler method had the potential as an immunostimulant agent on the cyclophosphamide-induced rats at a dose of 200 mg/kg BW in vivo by attenuating the release of nitric oxide compound. Nevertheless, further studies are needed to confirm the immunostimulant mechanism on other pathways.

5 Conclusion

In summary, it could be concluded that temulawak starch (TS) isolated with this simpler method compared to the previous one had the potential as an immunostimulant agent on the cyclophosphamide-induced rats at a dose of 200 mg/kg BW in vivo by attenuating the release of nitric oxide compound. Nevertheless, further studies are needed to confirm the immunostimulant mechanism on other pathways.

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