The Effects of Red Betel (*Piper crocatum*) Leaf Extract to Decreasing Profile Population of $CD4^+$ and $CD8^+$ Cells in Rheumatoid Arthritis-Induced Mice

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Abstract. Rheumatoid Arthritis (RA) is one of the autoimmune diseases resulted by oxidative stress. Red betel leaves (*Piper crocatum*) with a high content of flavonoid are expected to function as antioxidant and to reduce inflammation. This research aimed to identify the effects of red betel leaf extract on the amount of the $CD4^+$ and $CD8^+$ cells. 24 male Swiss mice, aged eight 8 weeks, with the weight of 27±3 g. 20 mice induced-RA were made by injecting 0.01 mL Complete Freund's Adjuvant (CFA) by intraperitoneal; after seven days, they were then boostered by injecting 0.03 mL of Incomplete Freund's Adjuvant (IFA) into the forelimbs. The animals were divided into six groups: normal, RA (C-), RA+aspirin (C+), RA+red betel leaf extract 100 mg/kg BW (T1), RA+red betel leaf extract 200 mg/kg BW (T2), and RA+leaf extract red betel nut 400 mg/kg BW (T3). The animals were given treatment for 21 days. At the end of the treatment, the mice were sacrificed, and the removed lymphocyte cells were isolated to measure the number of the $CD4^+$ and $CD8^+$ using Flow-cytometry. The results of this research showed that red betel leaf extract was significantly different to decreasing the amount of the $CD4^+$ cells, but it was not significantly different to reducing the amount of $CD8^+$ cells. The effective dosage in this research was 200 mg/kg BW.

Keywords: $CD4^+$ cell, $CD8^+$ cell, Rheumatoid Arthritis, red betel, *Piper crocatum*

1. Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease caused by chronic inflammation and connective tissue degeneration in the synovial joint membrane [1,2]. RA disease causes inflammation in the joint synovium; it creates long-term joint damage, prolonged pain, loss of function and disability [3]. Based on the data from the World Health Organization (WHO) (2015), the prevalence of RA disease in the world was approximately between 0.8-1% across the world population and affecting 2-4 times more women than men [4]. RA prevalence reached up to 0.4% among the populations in South East Asian [5].

The inflammation in RA was influenced by free radicals such as *Reactive Oxygen Species* (ROS). $CD4^+$ and $CD8^+$ also have been characterized in RA disease [2]. Chronic inflammation in RA is characterized by high levels of proinflammatory cytokines in the blood circulatory, including Tumour Necrosis Factor α (TNF-α) and Interleukin 6 (IL-6) secreted by $CD4^+$ cells [1]. $CD8^+$ cell also can

produce high amounts of cytokine which contributes to joint inflammation in RA. $CD8^+$cell has several subtypes of cell, i.e. Tc1 and Tc2. Tc1 can produce Interferon γ (IFNγ), while Tc2 secrets Interleukin 4 (IL-4) and IL-5. Tc1 and Tc2 also can induce inflammation by activating $CD4^+$ effector T cells (Th1 in cellular and Th2 in humoral immune response) rather than recruiting those effector cells into the inflammatory site [6].

RA disease also can be characterized by oxidative stress. Oxidative stress is a condition with the characteristics of imbalance between free radicals and antioxidants, where the number of free radicals is higher than antioxidants. Proinflammatory cytokines TNF-α and IL-6 can initiate other tissue damage pathways caused by the activation of ROS as free radicals. Immune cells are located in inflamed joints such as macrophages, neutrophils, and lymphocytes that can produce free radicals [7]. The increase in free radicals can cause the decrease in endogenous antioxidants such as glutathione reductase and superoxide dismutase [7,8].

The treatment for RA disease widely uses Non-Steroid Anti-Inflammation Drug (NSAID). The NSAID can inhibit the activity of cyclooxygenase enzyme which has a role in the biosynthesis of prostaglandins (PG) for COX-1 and the inflammation pathway for COX-2. However, the NSAID which can inhibit COX-1 also cause gastrointestinal ulceration and bleeding [9]. Therefore, an alternative treatment is vital to reduce the side effect of the treatment. Herbal medicine was widely used for any kinds of diseases that has a low adverse effect as long as it was consumed appropriately [10].

Indonesia is one of the countries which uses the plant as a traditional and herbal medicine [11]. Red betel (*Piper crocatum*) is one of the herbal medicines widely used as alternative medicine as anti-inflammatory and immunomodulatory activity [12–14]. Red betel contains bioactive compound including polyphenols, flavonoids, tannins, and alkaloids. Flavonoid has antioxidant activity that can be scavenging free radicals. Alfarabi *et al.* reported that flavonoid can reduce malondialdehyde (MDA) level where MDA is one type of free radicals [15]. The whole plant of betel also can be used in the treatment of inflammation [16]. This research aimed to identify the effects of red betel leaf extract with the indicator of $CD4^+$ and $CD8^+$ cells amount, and the most efficient dosage of the red betel leaf extract to reduce the amount of $CD4^+$ in mice induced-RA.

2. Methods

2.1. Piper crocatum leaf extraction

The extraction of *Piper crocatum* leaves used percolation method. 100 g of red betel leaves were added into 500 mL of 70% ethanol, subsequently put into a percolator. The extract was rained by using a rotary evaporator until obtaining a semi-solid extract. 0.5 g of extract was taken. The results of this extraction were reputed as stock. The doses used in this experiment were 100, 200, and 400 mg/kg BW. The determining of the doses was based on previous research [17].

2.2. Animal model and experimental design

This research used 24 male Swiss mice (*Mus musculus*) ± eight weeks old (28±2 g). The animals were obtained from Gajah Mada University. The animals were housed in standard cages and given free access to water and food. The animals were acclimatized for two weeks and fed with a normal diet. After two weeks, the animals were injected with 0.01 mL Complete Freud's Adjuvant (CFA) by intraperitoneal injection. One week later, the animals were injected with Incomplete Freud's Adjuvant (IFA) into the forelimbs. This injection was given to make animals RA models. This study was approved by the institutional ethics committee of Brawijaya University No. 983-KEP-UB. The animals were divided into six groups, i.e.:

N : normal
C- : RA (negative control)
C+ : RA + aspirin (positive control)
T1 : RA + red betel leaf extract at 100 mg/kg BW

T2 : RA + red betel leaf extract at 200 mg/kg BW
T3 : RA + red betel leaf extract at 400 mg/kg BW

The animals were given treatment for 21 days. The animals were sacrificed by dislocation. The spleen was removed to be analyzed using flow-cytometry.

2.3. Flow-cytometry analysis

Spleen which was removed from animals, was then crushed until smooth on petri dish containing phosphate buffer saline (PBS). The homogenate, as a result, was put into a 15-propylene tube and added 10 ml of PBS, centrifuged at 2500 rpm for 5 minutes in 4 °C in temperature, respectively. The supernatant was removed and the pellet was resuspended with 1 ml of sterile PBS.

20 µL of the suspension was mixed with 80 µL of trypan blue, then observed using hemocytometry to calculating the number of lymphocyte cells. The suspension stained with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8 (BD Pharmigen^{TM}) in ratio 1:50. Cells were an analyzed based on the parameters set in the flow cytometry machine (FACS Calibur). The cells which had an expression of $CD4^+$ and $CD8^+$ were counted in the $CD4^+$ and $CD8^+$ cell gates by CellQuest software with the formula:

$$\text{Percentage gated } CD4^+ CD8^+: \frac{\text{event quadrant}}{\text{gated event}} \times 100\%$$

$\sum$CD4/CD8 = percentage gated x $\sum$ lymphocytes cells

2.4. Data analysis

The data analysis was conducted by a non-parametric analysis using Kruskal Wallis that caused the distribution data not normal and homogeneous. Post hoc test used Games Howell Test $P < 0.05$.

3. Results and Discussion

Based on the observation using hemocytometry, the calculation of lymphocyte cells in the spleen is shown in Table 1. These results were used to calculate the $CD4^+$ and $CD8^+$ population cells based on the formula written in the flow-cytometry analysis.

Table 1. The average of Lymphocyte cells in Spleen

Treatment	The average of Lymphocytes cells
N	6,062,500
C-	10,087,500
C+	6,400,000
T1	6,987,500
T2	5,637,500
T3	9,450,000

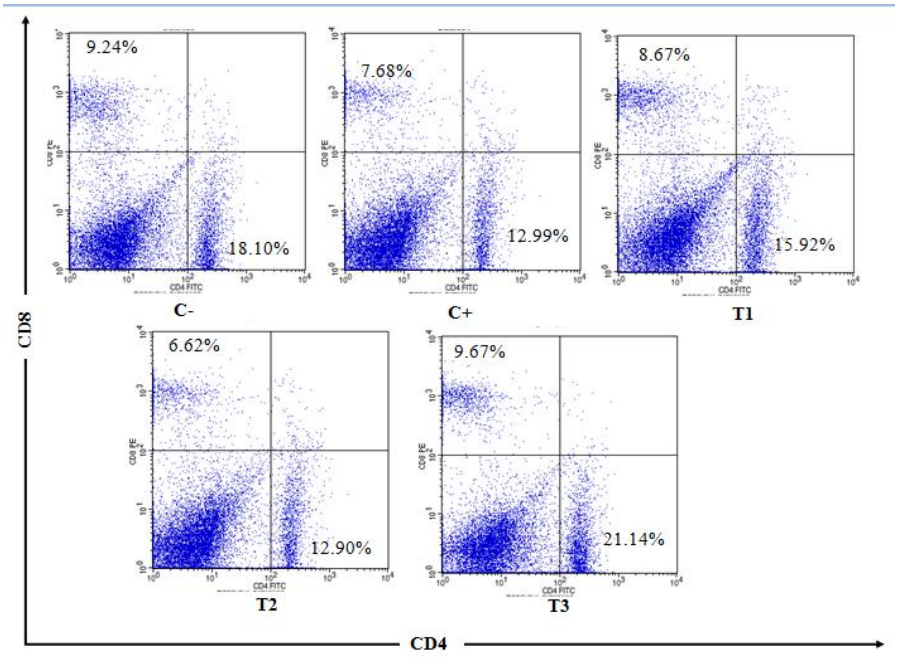

Figure 1. The population of $CD4^+$ and $CD8^+$ cells from flow-cytomery analysis. C-, RA model (negative control). C+, RA+aspirin (positive control). T1, RA+ red betel leaf extract at 100 mg/kg BW. T2, RA+red betel leaf extract at 200 mg/kg BW. T3, RA+red betel leaf extract at 400 mg/kg BW

Table 2. The average of the population $CD4^+$ and $CD8^+$ cells

Treatment	Average (cell/mL)	
	$CD4^+$ Cell	**$CD8^+$ Cell**
Normal	23,001,675[b]	2,799,831
C-	34,673,250[d]	4,450,725
C+	28,443,357[c]	4,226,530
T1	22,329,200[b]	3,105,069
T2	14,068,150[a]	1,760,994
T3	38,343,350[e]	4,452,213

The data were presented as mean. [a,b,..]considered as significantly different

The results showed that based on Kruskal Wallis test (Table 2), the amount of the $CD4^+$ cells was significantly different between groups. The amount of $CD4^+$ in the negative control group increased significantly compared to N groups. The positive control group decreased significantly compared to the amount of $CD4^+$ cells compared to the negative control group. The treatment groups using red betel leaf extract, T1 and T2, significantly decreased the population of $CD4^+$ and $CD8^+$ cells compared to negative and positive groups. T2 group had the lowest of $CD4^+$ cells between the groups. The $CD4^+$ cells in T3 treatment group had the highest population number between the groups.

The population of $CD8^+$ cells had no significant difference between groups, but they had a tendency of the $CD8^+$population cells. The negative control group had a higher amount of and $CD8^+$ compared to N groups. The positive control group showed the decrease in $CD8^+$ cells compared to negative control group. The treatment groups using red betel leaf extract, T1 and T2 showed the decrease in $CD8^+$cells compared to negative and positive groups. T2 group had the lowest of $CD8^+$ cells between the groups. The $CD8^+$ cells in T3 treatment groups had the highest amount between the groups. Based on the results, the T2 treatment group is the effective dosage to reduce the amount of $CD4^+$ and $CD8^+$ cells.

RA is an autoimmune disorder caused by inflammation in the synovial joint membrane. The mechanism of inflammation begins with the presence of antigens processed by Antigen Presenting Cells (APC), enabling activation of Cluster of Differentiation 4^+ ($CD4^+$). The activation of $CD4^+$ secretes other various cytokines such as TNF-α, IL-3, IL-4, IL-6. On the other hand, the activation of $CD4^+$ will trigger the activation of $CD8^+$ cytotoxic cells [2,18]. Based on data analysis, the $CD4^+$ and $CD8^+$ cells in negative control groups were higher than normal groups. The RA research used animal models of RA induced using adjuvants. An adjuvant is classified as an immunomodulatory. The aims of using Adjuvant is to increase the recruitment of various components of immune cells both innate and adaptive immune responses and enhance antigen binding capacity [19]. Adjuvants are divided into 2 namely Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA). CFA contains an oil-in-oil emulsion and added heat-killed Mycobacterium. The IFA contains water-in-oil emulsion that contains antigen in liquid form, paraffin oil, and surfactants such as mannide monooleate which can simultaneously extend the release of antigens injected [19]. IFA can help CFA to produce T helper 1 (Th1) and T helper 2 (Th2) cell responses [20,21]. $CD4^+$ T helper cells are an essential marker in autoimmune disease such as RA. The differentiation of $CD4^+$ T helper cells will promote secreting pro-inflammatory cytokines such as IL-6 and TNF-α [2]. Fontes *et al.* found that CFA expanded monocytes in spleen which could cause inflammation [19]. Geboes *et al.* also reported that administration using CFA could bring about the inflammation in both fore and hind limbs [21]. The immune cells found in the inflamed joints such as macrophages, neutrophils, and lymphocytes including $CD4^+$ T helper cells and $CD8^+$ T cytotoxic cells could produce free radicals such as ROS [7].

T1 and T2 groups showed the decrease in the $CD4^+$ and $CD8^+$ cells compared to negative control group. The treatment that used red betel leaf extract also showed better results compared to the positive control group. The T2 group had the lowest CD4+ and CD8+ cells between groups. Red betel leaf extract consists of an active compound which comes from secondary plant metabolite such as, tannin, alkaloid, phenolic, and especially flavonoids [17]. Flavonoid functions as free radicals scavenger so that the flavonoids were characterized as primer antioxidant. Flavonoids could prevent the generation of ROS by the involvement of binding metal ions (chelating) [22]. The lowering mechanism of ROS could decrease the immune system activity such as as$CD4^+$ and $CD8^+$ cells. Hence, the decrease in $CD4^+$ and $CD8^+$ cells would reduce the inflammation caused by the low level of cytokine pro-inflammation.

Phenolic contents in red betel leaves also could induce the endogenous antioxidant activity of glutathione peroxide up to 50% [23]. Flavonoids could function as primary antioxidants by donating hydrogen ions so that ions experiencing free radicals became stable. The stable state of ions causes a decrease in oxidative stress in the tissue. The flavonoids could also function as secondary antioxidants by increasing the synthesis of endogenous antioxidant enzymes superoxide dismutase (SOD) [24]. Abrahim *et al.* reported phenolic content such as flavonoids were found in Piper betel leaf extract that could increase both catalase and SOD as an endogenous antioxidant in the case treated MCF-7 human breast cancer [25]. The ethanol extract from P. crocatum can be a free radical scavenging determined on inhibiting of 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) as a radical [15]. The decrease in free radicals improved oxidative stress as the one of the factors causing RA.

4. Conclusion

The treatment using red betel leaf extract decreased the profile population of $CD4^+$ and $CD8^+$cells in the induced-RA mice. T1 and T2 groups decreased the profile population of $CD4^+$ cells significantly compared to negative and positive groups. Based on the results, the red betel leaf extract had potential as an alternative medicine for RA disease. The effective dosage that could be used for RA treatment was 200 mg/kg BW.

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