THE EFFECT OF Pandanus conoideus Lamk EXTRACT TO THE PARASITEMIA RATE OF Plasmodium berghei INFECTED IN MICE

by Blestina Maryorita

THE EFFECT OF Pandanus conoideus Lamk EXTRACT TO THE PARASITEMIA RATE OF Plasmodium berghei INFECTED IN MICE

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Abstract

Research on the effects of red fruit has been conducted with the various result. In this study, the effect of red fruit extract (EBM) on the level of parasitemia in Swiss mice infected with Plasmodium berghei was well assessed. The study used a laboratory quasi-experimental design with pre and post-test-only control groups. Sixty male Swiss mice of 8 weeks old and weighing 20-30 g, was simply randomized into four treatment groups. Group I (K1) was stimulated with EBM for 2 weeks before and 2 weeks after infection with P.berghei. K2 was stimulated with EBM for 2 weeks before infection, K3 was stimulated with EBM for 2 weeks after infection and K4 is negative control was given 0.6% tween 40. The dose of EBM was 7.8 mg/30 g BW mice / PO once a day. The number of parasitemia was examined microscopically. The differences levels of parasitemia of each treatment group were analyzed by t-test, one-way ANOVA, and honestly significant difference (HSD), and also multivariate analysis (manova). There are significant differences number of parasitemia in the treatment group were stimulated with EBM for 2 weeks after infection, if compared with a negative control group, for day-6 is 13,735% and day-9 is 1,054%, and also to the treatment group was stimulated with EBM for 2 weeks before infection, which day-6 is 13,141% and day-9 is 32,455%. But negative control group shows, day-6 is 58,180 % and day-9 is 78,506%. The extract of red fruit can reduce the number of parasitemia. The effects of EBM are more significant, especially when administered after malaria infection occurred.

Keywords: *Red Fruit Extract (EBM); number of parasitemia; immunomodulator*

1. Introduction

Red fruit (*Pandanus conoideus* Lamk) is a Papuans traditional food that is well-known for its efficacy and benefits. Empirically, red fruit oil has been used to treat various diseases such as cancer, stroke, hypertension, hepatitis, liver cirrhosis, diabetes mellitus, sinusitis, ovarian cysts, and epilepsy (Budi & Paimin, 2005). In the case of HIV / AIDS, after being administered the red fruit oil jointly consuming 80% of animal protein each day, it can increase the number of $CD4^+$ T cells (Budi, Hartono, & Setyanova, 2014). Content of red fruit dominated by unsaturated fatty acids such as, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, and some compounds of tocopherol (vitamin E), β-carotene (pro-vitamin A), omega, omega 3, omega 6, and omega 9 (Budi & Paimin, 2005; Waspodo & Nishigaki, 2007) is a complex compound of high anti-oxidant that also has an immunostimulant with an increased number of components of immunity, both cellular and humoral immunity, such as increase cell proliferation of lymphocytes, T helper cell activity and antibody production (Palupi, Andarwulan, & Herawati, 2007; Sakinah, Sukrasno, & Immaculata, 2007; Waspodo & Nishigaki, 2007). However, several studies contradict studies' results and empirical evidence above, including the effect of red fruit ekstraks in improving human immune response. Until now the effect of red fruit on cell culture experimental animals and the human immune response is still controversial.

To solve those problems basic research at the level of experimental animals using an infectious agent capable of inducing complex immune responses is essential. Malaria parasites are an appropriate agent for their ability to stimulate natural as well as immune response and adaptive immune response (Harijanto, 2000; Malaguarnera & Musumeci, 2002; Warren, 1993). Plasmodium berghei infection in swiss mica is one of the animal malaria methods that have properties that resemble *P. falciparum* in humans. This animal model of infection will be used to study the effect of red fruit ekstrak on cellular as well as a humoral immune response during *P.*

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berghei infection (Harijanto, 2000; Miller, Baruch, Marsh, & Doumbo, 2002). The increased cellular immune response of the body that mediated Th1 ($CD4^+$), will be accompanied by proinflammatory mediator release by macrophages and Th1 such as, cells cytokine interferon-gamma (IFN-γ), interleukin-1 (IL-1), IL-2, IL-12, and tumor necrosis factor-alpha (TNF-α) (Abbas & Lichtman, 2007; Kresno, 2010; Miller et al., 2002).

Cytokine products of Th1 cells and macrophages tend to work together to stimulate the immune response to suppress the activity of parasites (Kresno, 2010; Malaguarnera & Musumeci, 2002) [8, 11], however, the excessive response may result in certain pathological conditions, which can be fatal such as severe malaria, cerebral malaria (Harijanto, 2000; Kurtzhals et al., 1998). As a protective mechanism, Th2 cells produce several anti-inflammatory mediators such as IL-4, IL-5, IL-9, IL-10, and IL-13 (Harijanto, 2000; Kresno, 2010). The number of anti-inflammatory mediators can inhibit the production of TNF-α inpatient with falciparum malaria which may or reduce the pathological effect of Th1 cell pathology, probably by inhibiting the secretion of IL-12, IFN-γ, and TNF-α (Kresno, 2010; Malaguarnera & Musumeci, 2002), and has also been reported to damage the brain tissue caused by cerebral malaria (Baratawidjaja & Rengganis, 2009; Kurtzhals et al., 1998; Miller et al., 2002).

In the present study swiss mice infected with the rodent malaria parasite, Plasmodium berghei were used to determine the effect of red fruit extracts during as well as parasitemia during and parasitemia during the lower of infection.

2. Method

This research uses quasi-experimental designs in vivo laboratory in experimental animals of mice with the pattern of pre and post-test only control group design.

Experimental animals

Swiss Mice was chosen because it has the same similarities in their immunity components with human (Lodish et al., 2000), is quite sensitive to *Plasmodium* infection, and is more resistant to *Plasmodium berghei* infection (*P.berghei*) than other strains, although not treated (Suwarni, Tuti, Ilahi, & Marwoto, 1994). A total of 60 male Swiss mice of 8 weeks old and weighing 20-30 grams were selected after a period of acclimatization for 2 weeks, randomized into 4 groups of 15 mice/ group.

Red fruit extract preparation

The preparation of long red fruit (ogi or mbarugum; Wamena) with 1.3 m length per piece, 11,5 cm diameter, and 6.4 kg weight without the fruit stalk. Extracted by maceration using hot distilled water with a ratio of 100-150 ml / 250 grams of fruit meat [17]. The EBM results were then adjusted to the following doses: safe Dose EBM following literature, 2 g / kg day/70 BB (Budi et al., 2014). With the dose conversion factor to mice, 20g is 0.0026, resulted from 5.2 mg/20 g BW or 7.8 mg/30 g BW mice. 7.8 mg x 500 times to be taken (rounded) = 3900 mg EBM materials will be needed. Tween 40 solution with a concentration of 0.6% is used as a solvent in preparing EBM.

Inoculums of *Plasmodium berghei*

Plasmodium Berghei ANKA was chosen because it is haemoprotozoa that causes malaria in mice, even in molecular analysis of Plasmodium falciparum that man infects (Jekti, Sulaksono, & Sundari, 2002). The parasite density of *P. berghei* used was 1x107sel / 0.2 ml. This concentration is obtained by performing the inoculum (culture *P.berghei* in vivo) in two male Swiss mice donors. Blood donor mice containing parasites 20%, isolated by cardiac puncture technique, diluted with 990 ml RPMI-1640 entering-in the Eppendorf tube, added 10 ml of blood donor mice, forming dilution 102 (stock A). Then, it added 10 ml into 990 ml stock A (B stock, dilution 104). And so on until it reaches a concentration $1x10^7$sel / 0.2 ml (Ash & Orihel, 1991). Stock *P.berghei* obtained from the Laboratory of Parasitology University of Gadjah Mada.

Examination of parasitemia rate

Examination of parasitemia rate using thin blood smear techniques with cutting mice's tip tails. Microscopic observation of the parasite was conducted using a light microscope with a magnification of 100x and selected in each field of view of the parasite and erythrocyte not overlapping. Determination of parasite numbers performed quantitatively, by counting the number of parasites per 1000 cells eritrosit (Ash & Orihel, 1991)

Data analysis

Content comparison of parasitemia rates from each treatment group was analyzed using a one-way ANOVA statistical test of two-pairs by the significance of $p < 0.05$ and followed by a test

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of *honestly significant difference* (HSD) to determine the treatment group and the days with the higher contribution in rejecting the null hypothesis. T-test was also conducted to determine the differences among treatment groups (t-independent), and differences of everyday examination in each treatment group (t-dependent). To avoid bias due to a separate test on the ANOVA test above, other statistical t-tests will be done well with statistical manova or multivariance analysis (Jones, 2010; Riyanto, 2009).

Pathway

The course of study can be seen in the flow scheme following research:

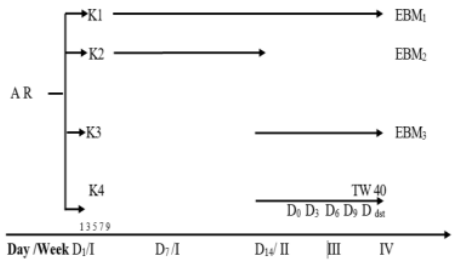

Figure 1. *Schematic Flow Research*

Information

A- R : The period of adaptation, 80 tails of mice acclimatized for 2 weeks.

R - K1 – K4 : 60 mice were selected and randomized into 4 (four) treatment groups i.e. groups 1 through 4, each consisting of 15 mice tails

Hr_1 - Hr_{14} : 1-14 Day / the day of EBM as Immunostimulant for 2 weeks

Hr_{14} /II/ D_0 : Week 2 or days 0, when Plasmodium berghei infection started in each treatment group are IP with Plasmodium density 1x107 cells / 0.2 ml. However the 3 tails of mice of each treatment group, not infected, because the insulation will be done (collection) serum.

$D_{0, 3, 6, \&}$ D_9 : Day 3 serum in isolation tails of mice from each treatment group.

D_{dst} : The next day, 3 tails of mice of each group are allowed to live to be evaluated the survival caused by the treatment administered.

1,3,5,7 & 9 : It is the day of examination of parasitemia rate on 3 mice from each treatment group.

EBM1 : Delivery of red fruit extract for 4 weeks of treatment.

EBM2 : Delivery of red fruit extracts only 2 weeks before infection.

EBM3 : Delivery of red fruit extract at 2 weeks after infection.

TW 40 : Negative control (Tween 40 0,6 %)

3. Result and Discussion

Comparison of parasitemia rate

The comparison means of parasitemia rate, from each treatment group, can be seen in the following tables 1 and figure 2.

Table 1. Mean of parasitemia From Each Treatment Group delivered after *P.berghei* infection and Red Fruit Extract (EBM) During Treatment

Group Treatment	Day	Parasitemia (%)	
		Mean	SD
K 1	0	0,497	0,201
Prev EBM	3	33,193	13,992
Infection	6	-	-
	9	-	-
K 2	0	0,463	0,210
EBM before	3	13,208	3,158
Infection	6	13,141*	6,883
	9	32,455*	-
K 3	0	0,299	0,057
EBM After	3	18,364	18,571
Infection	6	13,735*	1,714
	9	1,054*	1,826
K 4	0	0,533	0,251
Negative control	3	27,304	15,506
(Tween 40 0,6 %)	6	58,180	25,777
	9	76,506	6,406

* = There are significant differences in K4 at p <0.05

- = Average (Mean) can not be coun 34 (dead mice)

The number of parasitemia day 1 = day 0, day 5 = day 3 and day 7 =day 6.

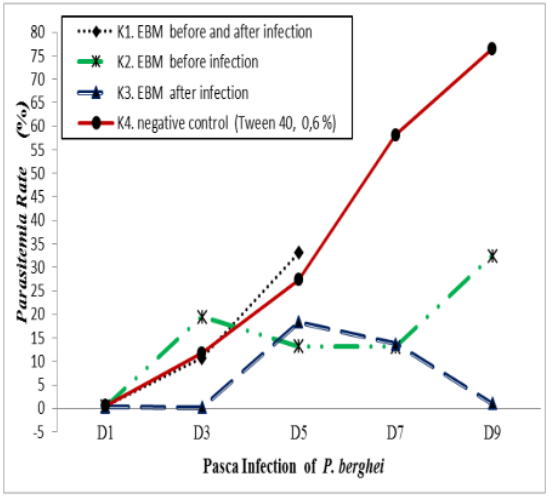

Figure 2*. Parasitemia rate pasca infection*

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In the analysis of the mean number of parasitemia, a one-way ANOVA test resulted in day 0 (D0), D3, D6, and D9, respectively, to indicate the significance of $p < 0.05$. The Tunkey HSD test when compared to K4 (negative control), it found more significant differences in the mean number of parasitemia on D3, D7, and D9 as the contribution from K3.

The results of independent t-test, comparison of treatment group 3 (K3) with treatment group 4 (K4), has a significant value of $p = 0.004$, and K2 - K4 have $p = 0.019$. However, K1 and K4 comparisons have a significance of $p = 0.127$. On the dependent t-test, results from the comparison of D1 with D3, D5, D7, and D9 are more significantly shown by K1, K2, and K4.

Effect of Different Time of Red Fruit Extract Treatment Against Parasitemia Rate

EBM treatments were given for 28 days, i.e. 14 days prior- and post-infection (K1), resulting in a higher level of pro-inflammatory immune response and cannot be regulated by existing anti-inflammatory mediators. Although the initial infection can slightly decrease the level of pro-inflammatory mediators as the indication of normal regulatory mechanisms of the immune system against higher levels due to EBM stimulation. Similarly, under physiological conditions, when the body is exposed to an infectious agent of malaria parasites, the immune response is activated with the cellular immune response. Immune cells, especially macrophages will release pro-inflammatory mediators and anti-inflammatory simultaneously. This condition is consistent with Bratawidjaja's statement, that the effect of cytokine antagonists is insignificant because the compensation of other cytokines, as well as the same cytokines, can be produced for specific and nonspecific immune response (Baratawidjaja & Rengganis, 2009).

EBM treatment groups were given for 14 days before infection (K2) and did not show significant immunostimulatory effects. At the beginning of the infection, the increased level of pro-inflammatory mediators is already quite high due to stimulated EBM, as shown in Figure above. This condition has been inferred by previous researchers, the red fruit can improve macrophage phagocytic activity, lymphocyte proliferation, and activate the cellular immune response (Kumala, Kusmardi, & Inreiatmoko, 2007; Ratnawati, 2005; Wahyuniari, Soesatyo, Ghufron, Yustina, & Wiryawan, 2009).

Despite the increased activity of cellular immune response with the release of pro-inflammatory mediators, but it did not completely suppress the proliferation of parasites, on the contrary, lead to pathological conditions due to the death of mice that began D6 after infection with P. berghei. Although the death of mice is also caused by ANKA factors of infected P. berghei, because it is a Plasmodium to cause lethal phase in experimental animals D6 to D8 mice after infection (Pelmann & Troye-Blomberg, 2002). Discontinuance of EBM will optimize the protection mechanisms on excessive cellular immune response and pathological impact. A few days after P.berghei infection, or before reaching the highest peak of parasitemia, pro-inflammatory mediators could be reduced by anti-inflammatory mediators, including cytokines product of macrophages, Th 2 and Th3 will increase. Although the process is very slow that affects pathophysiological mechanisms and the death of mice on the previous day, the result is in line with Bratawidjaja's statement, that cytokines will be active at a very low level 10^{-10}-10^{-15} mmol / l to stimulate the targeted cell (Baratawidjaja & Rengganis, 2009). A different and unique condition is shown by the treatment group that was given EBM for 14 days after infection with P. *berghei* or K3.

The results of statistical analysis showed an increase in the real humoral immune response, as noted in previous studies, the administration of red fruit oil was shown to reduce cellular immune responses (Sakinah et al., 2007). From D0 to D3, the parasitemia infection increased significantly but rather associated with the parasite virulence and the peak breeding of *P. berghei* ANKA (eritrositer phase) which causes a lethal phase in D6 to D8 after infection (Pelmann & Troye-Blomberg, 2002). The results showed that the new deaths occurred in D8 mice after infection with P. berghei and only 1 mice death. After the D3 infection, the parasitemia rate decreased significantly. K3 parasitemia decreased significantly on D9, reaching 1.054%, from the previous 18.365% (D3) and 13, 735% (D6). The above conditions indicate that administration of EBM after malaria infection can be accompanied by the significant decrease of parasitemia rate.

Adequacy of both cellular immune response and humoral immunity comes with EBM after infection with P. berghei, mainly initiated by the changing role of the β-carotene

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compound in EBM that is from pro-oxidant to anti-oxidant compounds (Burlon & Ingold, 1984). In malaria parasite-infected conditions, a decline in the volume and capacity of oxygen (O_2) due to phagocytosis of erythrocytes parasites by the spleen lymphocytes and macrophages can cause anemia (Malaguarnera & Musumeci, 2002; Miller et al., 2002; Silbernagl & Lang, 2007), besides the plasmodium also invade erythrocytes of all ages (Harijanto, 2000; Jawetz, Melnick, & Adelberg, 2008). The situation is certainly steadily reduced availability of oxygen level in the circulation and lower partial pressure of oxygen (PO_2). Decreased PO_2 will spur changes in chemical structure and- the function of β-carotene from pro-oxidant to antioxidant compounds, as stated previously that β-carotene is an oxygen binder and as a potential anti-oxidant, but it is effective as a free radical binding when only 2-20% oxygen available and low PO2 (Burlon & Ingold, 1984; Murray, Granner, & Rodwell, 2003).

On the other hand, the content of tocopherols was previously functioning as phenolic hydrogen donors to neutralize β-carotene in pro-oxidant conditions, if the oxygen availability and PO2 are adequate, in pre-infection or early infection conditions. However, after infection with the parasite, many pro-oxidant compounds or oxyradical can be generated either by immune component and the consequences of such parasitic activity, ROS and ROI class, therefore, the availability of anti-oxidant compounds of tocopherols are not fulfilled. Instead of these conditions, it further enhances the ability of anti-free radical of tocopherol and β-carotene, because the chemical structure of β-carotene changes to pro-oxidant and anti-oxidant compounds due to the parasitic infection that led to the decreased PO2 pressure. In addition, several unsaturated fatty acids in EBM have functioned as an anti-free radical because the structure is susceptible to oxidation in the double bond, making the complex compounds a powerful anti-free radical (Burlon & Ingold, 1984; Murray et al., 2003). The biochemical reaction mechanism is similar to the statement of deMan, that the rate of oxidation of fatty acids is influenced by the amount of oxygen, the degree of unsaturated lipid, and the presence of antioxidants.

Provision of anti-oxidants contained in EBM, resulting in several free radical substances ROI and RNI of effector immune cells, especially macrophages, will neutralize the nonradical compounds. Neutralized free radicals substances of ROI and RNI group products on the immune effector cells, apparently weaken the protective functions of macrophages and Th1, in turn, it will decrease the production of TNF-α, IL-1, IL-2, IL-6, IL-8, IL-12, and IL-18. Instead, these conditions facilitate the activation and differentiation of CD4 + T cells by a subset of Th2 cells is initiated by cytokines autoregulation or anti-inflammatory that released by macrophages, such as IL-4, IL-5, IL-10, and IL-13, in addition to the interaction of β-carotene (retinol and retinoic acid) and immune effector cells through the binding with Retinol Binding Protein Celular (CRBP), including macrophages, B cells, plasma cells, Th2, Th3 (Treg), CTL and NK, which facilitates the proliferation and differentiation. Given vitamin A plays an important role in the regulation of the immune system both specific and non-specific as well as it plays a role in the process of Th2 cell differentiation, the growth, and differentiation of B cells into plasma cells, and antibody production by antigen-specific configuration and maintain normal antibodies in the circulation under influence Th2 cells (Baratawidjaja & Rengganis, 2009; Janeway, Travers, Walport, & Shlomchik, 2001; Murray et al., 2003).

IL-10 is also the product of Th2 and Th3 (T reg), which will increase the regulator of immune response and the sensitivity and specificity of effector cells. Several pro-inflammatory cytokines Th1 and macrophage products that are released at the beginning of the infection, will continue to circulate the blood for a few days to several months, and Th1 cytokine macrophage products include TNF-α, may increase the activity of effector cells in the immune response order. Specifically, with the mediation of antibodies, including increased activity of Plasmodicidal effector cells (Abbas & Lichtman, 2007; Kresno, 2010; Miller et al., 2002).

4. Conclusion and Sugestion

Red fruit extract (EBM) can reduce the parasitemia rate. The more pronounced decreased parasitemia rate was found particularly in the group administered EBM after malaria infection. Referring to the above conclusion, it should conduct a further study about 1) determination standards and the availability of oxygen partial pressure of oxygen in the blood circulation may affect changes in β-carotene compounds in EBM of pro-oxidant to be anti-oxidant compounds. 2) the influence of

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each bioactive substance or substance that has anti-oxidant EBM to the inhibition of growth of malaria parasites. 3) the influence of each bioactive substance or substance with anti-oxidant EBM in activating the body's immune response, both cellular and humoral. 4) impact of EBM and combination of anti-malarial drugs in patients with malaria.

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