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CONTENTS

The Red Fruit (*Pandanus Conoideus* Lam) Ethanol Extract Decreased the Nitric Oxide (NO) Levels of *Plasmodium Berghei* Infected Swiss Mice Malaria Model but not the *Interferon Gamma* (IFN-γ)

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ABSTRACT

Introduction: The immunity against malaria infection was very complex, involving humoral immunity and cellular immunity. One of the methods to improve body immune response was using antioxidant. Red fruit (*Pandanus conoideus*Lam) containing carotenoid and tocopherol that have antioxidants effects increase phagocytosis activity of macrophage and proliferation activity of lymphocyte as immune response againts parasite infection.
Objectives: This study was intended to know the effect of red fruit (*P. conoideus*Lam*)* ethanol extract on IFN-γ and nitric oxide (NO) levels in *Plasmodium berghei* infected Swiss mice malaria model.
Methods: This quasi experimental study used post test only control group design. Sixty eight-week aged male Swiss mice were devided into 6 groups. Group I was not given red fruit ethanol extracts neither infected by *P.berghei*, group II was given 260mg/kg/day of red fruit ethanol extracts, and it was not infected by *P.berghei*, groups III, IV and V were given red fruit ethanol extracts at 130, 260 and 520mg/kg/day respectively and infected by *P.berghei*. Group VI was not given the extract, however it was infected by *P.berghei* as control group. The red fruit ethanol extracts or the carrier was given for 28 days and the mice were infected by *P.berghei* in the beginning of week 3. On the day 3 and 9 after infection, 2 mL blood was taken from 3 mice of each group for serum isolation.
Results: There was not any significant difference ($p>0.05$) mean of IFN-γ levels in all groups neither on day 3 nor day 9 after infection. The nitric oxide levels mean of mice group which have received red fruit extracts at 130 and 260mg/kg/day on the day 9 and in mice group which have received red fruit extracts 260mg/kg/day on day 3 were lower than they were in control group without any extract (group VI). However, the nitric oxide levels mean of mice group which have received 520mg/kg/day did not show any significant difference ($p>0.05$) compared with control group.
Conclusion: The red fruit (*P. conoideus*Lam) ethanol extract did not show any significant effect on the IFN-γ levels, however at doses of 130 and 260mg/kg/day it decreased the nitric oxide level of *P. berghei* infected Swiss mice malaria model significantly.

Keywords: *Pandanus conoideus* Lam, *Interferon gamma* (INF-γ), *Nitric oxide* (NO), *Plasmodiumberghei,* and Malaria Model

INTISARI

Pendahuluan: Imunitas terhadap infeksi malaria sangat kompleks, melibatkan imunitas humoral dan imunitas seluler. Salah satu cara untuk meningkatkan respon imun tubuh adalah dengan pemberian antioksidan. Buah merah (*P. conoideus* Lam) diketahui mengandung karotenoid serta tokoferol yang berfungsi sebagai antioksidan, dan pada hewan coba terbukti dapat meningkatkan aktivitas fagositosis makrofag dan aktivitas proliferasi limfosit sebagai respons emun terhadap infeksi parasit.

Tujuan: Penelitian ini bertujuan untuk mengetahui efek pemberian ekstrak etanol buah merah pada kadar interferon gamma (IFN-γ) dan nitritoksida (NO) mencit Swiss yang di infeksi *Plasmodiumberghei*.
Metode: Enampuluh ekor mencit Swiss jantan umur 8 minggu dibagi menjadi 6 kelompok. Kelompok I tidak diberi ekstrak etanol buah merah, tidak di infeksi *P.berghei*, kelompok II diberi ekstrak etanol buah merah 260 mg/kg BB/hari, tidak di infeksi, kelompok III, IV, V diberi ekstrak etanol buah merah berturut turut 130, 260, dan 520 mg/kg BB/hari dan di infeksi *P.berghei*. Kelompok VI diberi bahan pembawa dan di infeksi *P.berghei*. Ekstrak etanol buah merah atau bahan pembawa diberikan selama 28 hari, infeksi *P.berghei* dilakukan pada awal minggu ke-3 (hari ke-15). Pada hari ke-3 dan hari ke-9 setelah infeksi, 3 ekor mencit tiap kelompok diambil darahnya sebanyak 2mL melalui vena orbitalis untuk diambil serumnya.
Hasil: Reratakadar interferon gamma (IFN-γ) pada semua kelompok mencit yang diberi ekstrak etanol buah merah, tidak menunjukkan perbedaan (p>0,05) baik pada hari ke-3 maupun hari ke-9 setelah di infeksi. Rerata kadar NO kelompok mencit yang diberi ekstrak etanol buah merah 130mg/kg BB/hari pada hari ke-9, dan kelompok mencit yang diberi ekstrak etanol buah merah 260mg/kg BB/hari pada hari ke-3 maupun hari ke-9 setelah di infeksi *P. berghei,* menunjukkan lebih rendah (p<0,05) dibandingkan mencit yang diinfeksi dan tidak diberi buah merah. Namun kelompok mencit yang diberi ekstrak etanol buah merah dosis 520 mg/Kg BB/hari tidak menunjukkan perbedaan bermakna (p>0,05).
Simpulan: Ekstrak etanol buah merah (*P. conoideus* Lam) tidak memberikan perbedaan yang bermakna pada kadar IFN-γ, namun pemberian ekstrak etanol buah merah 130 dan 260 mg/kg BB/hari, dapat menurunkan kadar nitritoksida (NO) pada mencit Swiss model malaria yang di infeksi *P. berghei.*

Kata kunci: *Pandanus conoideus* Lam, *Interferon gamma* (INF-γ), Nitritoksida (NO), *Plasmodiumberghei,* dan Model Malaria

INTRODUCTION

Immune system was necessary for body to defend against various environmental exposed. Natural immunity mechanism represented physiologic mechanism of non-specific immunity in the form of normal body components that were always found in healthy individuals and ready to prevent microbe entrance into body and to remove it quickly.[1]

In malaria infection the immune system was exposed by various parasite stages and stimulated both humoral and cellular immune mechanism. Host defense that directly or indirectly worked in parasite replication could destroy or kill infected cells.[2] The immunity to the malaria infection was very complex because it involved humoral immunity that was mediated by specific antibody and cellular immunity that worked in cellular components of body immune system.[3,4]

Plasmodium falciparum was the most dangerous cause of malaria of other causes as *P. vivax, P. malariae,* and *P. ovale,* because it caused high morbidity and mortality.[5,6] The infection by *P. falciparum* often caused complication affected some organs such as brain, lung, kidney and peritoneum.[1,7] *Plasmodium berghei* represented *haemoprotozoa* of the kind of malaria parasite which was able to infect and to cause malaria in mouse. The infection by the *P. berghei* could cause cerebral malaria symptom similar to human cereberal malaria resulting from the infection by the *P. falciparum.*[8]

When the *P. falciparum* infected the body, parasite antigen triggered (cellular) lymphocyte T activation as main controller of body immune system that subsequently activated T helper cell (CD4+) and cytotoxic T cell (CD8+).[1] The T helper cell (CD4+) activated Th-1 and secreted proinflamatory cytokine (TNF-α,TNF-β, IL-1, IL-1, IL-12, and IFN-γ) that activated cellular immune, and Th-2 excreted anti-inflamatory cytokine (IL-4,IL-5, IL-6 and IL-10) that activated humoral immune system.[9] The cytotoxic T

cell (CD8+) would recognize and eliminate the parasite at pre-erythrocytic stage or hepatotoxitic phase with the help of class I MHC/HLA molecule. The class I MHC/HLA molecule would bind parasite antigen on cell surface.[7,10] Proinflamatory cytokine and NO overproduction were resulted in adhesive molecule expression in endothel so that cytoadherens and parasite sequestration increased and it was expected that it was also resulted in disorder of cell function and certain organ function. The exclusion of the mediator actually aimed at killing the parasite, but because free radicals were of non-specific nature it could cause damage in the adjacent tissues, including endothel cells.[11]

Free radicals overproduction in the malaria infection by *P. falciparum* caused oxidative stress. It was clearly observed in the increase in serum lipid through the increase in ROS (Reactive Oxygen Species) production and in peroxide supply in the form of hemoglobin and free Fe resulting from hemolysis.[12,13] The increase in the free radicals was resulted among others from neutrophils. The neutrophils tried to eliminate the parasite through phagocytosis and released the free radicals along with proteolytic enzimes. The phagocytosis ability of the neutrophils and its ability to exclude free radicals were induced by the presence of parasite opsonization antibody and cytokines as IFN-γ, TNF-α and IL-1β, and granulocyte macrophage-colony stimulating factor (GM-CSF).The oxidative stress in the pathomechanism of malaria with complication required that the management of the malaria must not only be organized by giving malaria medicines but also by giving adjuvants such as antioxidants.[7,14]

One of potential antioxidant sources was *P. conoideus*Lam or well-known as red fruit. In the daily life of Papua people the red fruit was used as food related to custom ceremony and as traditional medicine.[15] The red fruit (*P. conoideus*Lam) was well-known to have high content of active compounds such as antioxidant and useful nutrients.[16,17] The active compounds with *immunomodulator* effect were β-carotene and α-tocopherol.[18,19] It was expected that the antioxidant was able to reduce free radicals activities in the malaria infection and to alleviate parasitemia, to decrease apoptosis and to accelerate recovery.[20] In general, the red fruit as antioxidant could play an important role in repelling the effect of the free radicals by inhibiting lipid peroxidation so that erythrocyte wall became stronger and was not prone to ruptures and subsequently it caused the decrease in the spread of *Plasmodium* or in the parasitemia level.[21]

It was expected that the antioxidant of the red fruit could improve stamina and increase lymphocyte T cell helper activity that played an important role in activating cellular and humoral immunities. The cellular immunity of the cell T helper responded the Th1 excreting cytokine INF-γ and the TNF-α triggered the macrophage to excrete NO that played an active role in acting against parasite infection both at hepatocyte and erythrocyte stage.[22,23] This study was intended to investigate the effect of red fruit (*P. conoideus* Lam*)* ethanol extract on IFN-γ and nitric oxide (NO) levels in *P. berghei* infected Swiss mice malaria model.

MATERALS AND METHODS

This quasi-experimental study used post test only control group design. The sixty eight-week aged male Swiss mice with 20-30g of body weight were devided into 6 groups. The pellets food AD2 and drinking water were controlled daily. The mice were obtained from LPPT (*Laboratorium Pengujian dan Penelitian Terpadu*/ Integrated Research and Testing Laboratory), Universitas Gadjah Mada, Yogyakarta. The protocol has been approved by the Medical and Health Research Ethics Committee Faculty of Medicine Universitas Gadjah Mada Yogyakarta. Group I was not given red fruit ethanol extracts neither infected by *P.berghei*, group II was given 260mg/kg/day of red fruit ethanol extracts, and it was not infected by *P.berghei*, groups III, IV and V were given red fruit ethanol extracts at 130, 260 and 520mg/kg/day respectively and infected by *P.berghei*. Group VI was not given the extract, however it was infected by *P.berghei* as control group. The red fruit ethanol extracts or the carrier was given

orally once daily for 28 days. The mice were infected by *P.berghei* in the beginning of week 3 with the parasite density of $1x10^7$/0.2 mL intraperitoneally. On the day 3 and 9 after infection, 2 mL blood was taken from 3 mice that have determined for parasitaemia of each group for serum isolation.

The red fruit was obtained from Wamena region of Papua. Identification and determination was conducted at Department of Biology Faculty of Pharmacy Universitas Gadjah Mada. The seeds of the red fruit were removed from their stem and macerated in 70% of ethanol.[24]

The day 3 and 9 after the infection by the *P. berghei* blood samples of 2mL/ mouse were drawn from 3 mice of each of the groups under anesthesia of ketamine 0.1 mL/mouse. The serum isolated were kept at 4 ^oC and INF-γ level was examined using INF-γ ELISA kit for mice and the NO level was examined using ELISA kit with spectrophotometer at 450 nm wave legth. The data was analized using non-parametric analysis.

RESULTS

The red fruit ethanol extracts or carrier was given for 28 days. The mice were infected by *P.berghei* in the beginning of week 3. The mean of IFN-γ level of all groups on the day 3 and 9 after the infection were summarized in Table 1.

Table 1. The mean of IFN-γ level of *Plasmodium berghei* infectred Swiss mice malaria model at day 3 and 9 after infection

	Group n=3	IFN-γ Levels (ng/mL) after *P. berghei* infection	
		Day 3	Day 9
I.	Extract (-), infection (-)	3.7600±3.0151	3.4367±3.0166
II.	260mg/kg/day of extract, infection (-)	2.0667±5.0767	2.3033±1.9646
III.	130mg/kg/day of extract, infection (+)	5.5200±1.8592	2.4167±3.8734
IV.	260mg/kg/day of extract, infection (+)	2.4433±1.0771	1.8567±2.1079
V.	520mg/kg/day of extract, infection (+)	4.5967±2.4832	1.5833±2.1519
VI.	Extract (-), infection (+)	4.9133±3.8630	2.9200±2.0192

It was clearly observed in the table 1 that there was not any significant difference ($p>0.05$) mean of IFN- between day 3 and day 9 after infection in all group. Also, there was not any significant difference mean of IFN- ($p>0.05$) among six groups neither day 3 nor day 9 after infection. These findings showed that giving the red fruit ethanol extracts at doses 130, 260 and 520mg/kg/ day for 14 days before infection to day 3 and continued day 9 after infection did not give any significant difference ($p>0.05$) mean of IFN- level with control group without any extract and infected by *P. berghei*. However, the IFN- level of group VI 3 days after infected by *P. berghei* was tend to higher than group I which was not infected. This result showed that infection of *P. berghei* tend to increase the IFN- level.

The mean of nitric oxide level of all groups at day 3 and day 9 after infection was summarized in Table 2.

Table 2. The mean of nitric oxide level of *Plasmodiumberghei* infectred Swiss mice malaria model at day 3 and 9 after infection

	Group (n=3)	Nitric Oxide Levels (ng/mL) after *P. berghei* Infection	
		Day 3	Day 9
I.	Extract (-), infection (-)	0.0286±0.0049	0.0315±0.0005*
II.	260mg/kg/day of extract, infection (-)	0.0311±0.0008	0.0287±0.0002*
III.	130mg/kg/day of extract, infection (+)	0.0332±0.0016	0.0304±0.0008*
IV.	260mg/kg/day of extract, infection (+)	0.0225±0.0004*	0.0271±0.0006*
V.	520mg/kg/day of extract, infection (+)	0.0254±0.0034	0.0348±0.0009
VI.	Extract (-), infection (+)	0.0268±0.0039	0.0353±0.0017

*$p < 0.05$ (compared to group VI)

In mice groups that were not infected by *P. berghei*, there was not any significant difference mean of nitric oxide level (p>0.05) between group I without any extracts and group II with 260mg/kg/day red fruit ethanol extract for 14 days before infection date to day 3 after infection date. However, when the red fruit ethanol extract was continued to day 9 after infection date, it showed decreasing of nitric oxide level. It showed that giving the red fruit ethanol extract for 23 days decreased the NO level (p<0.05) in mice groups that were not infected by the *P.berghei*.

There was significant difference mean of nitric oxide level (p<0.05) between mice groups infected by the *P. berghei* and treated with red fruit ethanol extract at dose 130 mg/kg/day at day 9 after infection, at dose 260 mg/kg/day at day 3 and 9 after infection and control infected mice group without any treatment (group VI). However, there was not any significant difference mean of NO level (p>0.05) between mice group V which are given red fruit ethanol extract at dose 520mg/kg/day and control group (group VI). These finding showed that the treatment of the red fruit ethanol extract at doses of 130 and 260mg/kg/day decreased the NO level content after *P. berghei* infection. At higher dose of 520mg/kg/day, red fruit ethanol extract did not decrease the nitric oxide level at day 3 and 9 after *P. berghei* infection and it showed that there was other possible mechanism that influenced it. Thus, it was necessary to conduct further study. The concentrationof β-caroten in plasma worked as anti-inflammation and antioxidant and it was necessary for the dose of the antioxidant not to exceed than 260mg/kg/day to act as active antioxidant at PO_2 2 – 21 mmHg.[25]

DISCUSSION

The effect red fruit ethanol extract on IFN-γ level

The increase of IFN-γ level cytokine after *P. berghei* infection was resulted from the formation of the cytokine from endothelial cells, monocyte and macrophage after stimulation by malaria toxin in the form of *lipopolysaccharide* (LPS) and *glycosylphosphatydyl inositol* (GPI) that were recognized by CD4+ and CD8+. Th-1 (CD4+) served as regulator that helped produce and activate phagocytes, while Th-2 (CD8+) played the roles of direct effector of parasitic phagocytosis and inhibitor of parasitic development by producing IFN-γ.[26,27] The cytokine, especially the IFN-γ played its role to respond inflammation and to activate macrophage to produce TNF-. Collectively, the two cytokines induced the increase in the expression of adhesive molecules such as ICAM-1, E-selectin and MHC-1.[8] Proinflammation cytokine such as IFN-γ in its high/excessive content could cause damage to endothelial cells and it was beneficial for parasitic growth because of the increase in cytoadherens through the increase in the expression of adhesive molecules in the endothelial cells.[28]

There was not any significant difference in the IFN-γ (p>0.05) in the groups of the mice infected by the *P. berghei* given the red fruit ethanol extract at the doses of 130, 260 and 520mg/kg/day (groups III, IV and V) for 14 days before infection up to the day 3 and 9 after the infection. It was because the β-carotene and the α-tocopherol of the red fruit as antioxidants at the beginning of the infection by the *P. berghei* could not suppress the activation of immune cell T helper-1 in producing proinflammation cytokines such as TNF-α, TNF-β, IFN-γ, IL-1, IL-6, IL-8 and IL-12.[1,29] Though at later infection stage the Th2 cells became more active in producing the anti-inflammatory cytokines such as IL-4 and IL-10, the decrease in the IFN- content was necessary to maintain normal immune balance between Th-1and Th-2 in order to inhibit the proliferation and the spread of the parasite at erythrocyte stage.[28]

The compound of the red fruit (*P. conoideus*Lam) in the experiment animals infected by the *P. berghei* played the role of activating immune system that triggered macrophage and lymphocyte T helper to suppress excessive proinflammation cytokine secretionsuch as IFN- γ.[25,30] It showed that the infection by the *P. berghei* as immunogen and the adjuvant treatment of the red fruit (*P. conoideus* Lam) extract as antioxidant were simultaneously able to induce cellular immune response in the

form of macrophage phagocytosis activity to prevent and to inhibit more severe complication of parasitic infection.[31]

Intracellular parasitic infection included the infection by the *P. berghei* and it caused the proliferation of lymphocyte T that would in turn trigger cell-mediated immune response. The activated lymphocyte T and NK cells would secrete IFN-γ and it would increase the activity of macrophage in eliminating microbes. The increase in the activity of the lymphocyte T improved the ability of the macrophage in eliminating microbes.[32] On the contrary, the macrophage secreted IL-12 that improved the differentiation of the lymphocyte t helper. The lymphocyte T helper would also secrete IL-2 that stimulated the proliferation and the differentiation of the cytolytic T lymphocyte. Additionaly, the lymphocyte T helper would express CD40L that would bind CD40 in the macrophage and activate the macrophage to make it more efficient in stimulating the differentiation of the cytolitic lymphocyte T that could lyses parasite infected erythrocyte cells.[9]

The effect of red fruit ethanol extract on nitric oxide level

The increase in theNOcontent after the infection by the *P. berghei* resulted from the production of endothel from amino acid L-arginine as the response of the physiological stimuli of a reaction that catalyzed by *nitric oxide synthase* (NOS) enzyme.[37] There was significant difference in the nitric oxide content ($p<0.05$) between the mice groups infected by the *P. berghei* with the treatment of the red fruit ethanol extract at the doses of 130 mg/kg/day on the day 9 and groups given the red fruit at 260 mg/kg/day on the day 3 and 9 after the infection by the *P. berghe*and the infected mice group with no treatment of the red fruit ethanol extract (group VI). It showed that the treatment of the red fruit ethanol extract at the doses of 130 and 260mg/kg/daycould decrease the NO content after the infection by the *P. berghei*. Thus, it could be concluded that the decrease in the expression of the NOon the day 3 and 9 after the infection by the *P. berghei* took place because of the treatment of the red fruit ethanol extract after the infection.

The decrease in the NO content was indicative of the important role of the compounds of β-carotene, α-tocopherol, vitamin C as antioxidant capable of binding free radicals.[34,35] Additionally, the active compound of the of β-carotene and the α-tocopherol could activate cell T helper and macrophage through the subset Th-1 by suppressing the secretion of proinflammation cytokine TNF-α and INF-γ so that *inducible nitrit oxide synthase* (iNOS) enzyme was triggered to release NO through the subset Th-2 by producing anti-inflammation cytokine IL-10 to reduce the suppression of the production of NO.[36]

The protectic medium and proper NO content could play an important role in reducing adhesive molecular expressions (CD 36, ICAM-1, ELAM-1 and VCAM-1), in inhibiting parasitic growth and in degenerating parasites through oxidative stress and it could inhibit the production of the TNF and its pathological effect on the endothelial cells by reducing the adhesive molecular expression.[37] The red fruit as antioxidant activated cellular immune through phagocytes (macrophage, monotion and lymphocyte secreting TNF- and IFN-. The phagocyte would produce free radicals of NO, H2O2, O2 singlet and OH- that would inhibit the parasitic growth and degeneration through oxidative stress.[38]

The mice of the group V were given the red fruit ethanol extract at the dose of 520mg/kg/day and there was not any significant difference in the NO ($p>0.05$) both on the day 3 and the day 9 as compared to the mice of the groups that were not given the red fruit ethanol extract (group VI). It showed that the treatment of the red fruit ethanol extract at the higher dose of 520mg/kg/day did not decrease the NO content both on the day 3 and on the day 9 after the infection by the *P. berghei.* It was because the β-carotencompound of the red fruit as antioxidant could not play the role in regulating immune function in the development and the differentiation of the subset Th1 to induce the production of the proinflammation cytokine such as TNF-α and INF-γ. The proinflammation cytokine would activate endothel cells, macrophage

and other immune cells and also hepatocyte to continuously produce NO and it produced a big quantity of the NOwhen parasitic infection took place.[39]

The high content of the NO could bind free radicals of H_2O_2 produced by neutrophils and macrophages to form peroxinitric that was toxic for cels so that it caused excessive vasolidation and hypotension and tissue perfusion disorder, neuron transmission disorder that might play an important role in the severe malaria pathogenesis. The high content of the NO could also increase adhesive molecular expressions so that it increased *cytoadherens* and parasitic *sequestration*.[28,40]

It was highly possible that the insignificant IFN- and the significant NO content resulted from the use of the red fruit (*P. conoideus* Lam) as antioxidant rich of the β-caroteneand α-tocxopherol and when it was given at proper dose, it stimulated both cellular immune and humoral immune, macrophage and lymphocyte T helper. It was consistent with the prior studies suggesting that the β-caroteneandα-tocxopherol could increase the number and the function of the cells that played an important role in responding immune system.[41]

CONCLUSION

It could be concluded that the red fruit (*P. conoideus*Lam) ethanol extract did not show any significant effect on the IFN-γ levels, however at doses of 130 and 260mg/kg/day it decreased the nitric oxide level of *P. berghei* infected Swiss mice malaria model significantly.

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