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The Effect of Annona Muricata Leaves Towards Blood Levels of CXCL9 and Lymphoblast (Study in Cerebral Malaria Phase of Swiss Mice)

Pengaruh Daun Annona Muricata pada Kadar CXCL9 dan Lymfoblas Darah (Studi Kasus pada Mencit Dengan Cerebral Malaria)

✉**Mohamed M.Y. Gadalla[1], Edi Dharmana[2], Kiss Jamiatun[2], Budi Laksono[3]**

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[1]Health Department of Libya, Libya
[2]Medical Faculty, Diponegoro University, Semarang, Indonesia
[3]Central Java Health Department, Semarang, Indonesia

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Abstract

Cerebral malaria (CM) forms part of the spectrum of severe malaria, with a case fatality rate ranging from 15% in adults in southeast Asia to 8.5% in children in Africa. A.Muricata was used to cure Malaria in traditional medicine. The research will examine the effect of it in the chemokine (C-X-C motif) receptor 3 (CXCR3) binding chemokines, including chemokine (C-X-C motif) ligand 4 (CXCL4), CXCL9. The intervented mice group were infected then the it's spleen were cultured , incubation 72 hours and then analyzed the result. The CXCL9 level of PbA-infected mice treated with A. muricata are lower than group of infected mice without treatment. Lymphoblast level of PbA-infected mice treated with A. Muricata are higher than group of infected mice without treatment. A. Muricata treatment cure in the CM in the mice and may be a potential treatment in human CM.

Abstrak

Cerebral malaria (CM) adalah keadaan infeksi malaria yang berat dengan tingkat kefatalan dari 15% di Asia tenggara dan 8% di Afrika. A. Muricata secara tradisional dipakai mengobati CM. Riset ini meneliti pengaruh A. Muricata pada ikatan chemokine (C-X-C motif) reseptor 3 (CXCR3)termasuk chemokine (C-X-C motif) ligand 4 (CXCL4) dan CXCL9. Kelompok mice intervensi diinfeksi dan limfanya di culture dalam inkubator 72 jam untuk dianalisis. Kadar PbA CXCL9 pada mencit intervensi yang diberi A. Muricata lebih rendah dari pada kontrol. Kadar PbA limfoblast intervensi lebihtinggi dari pada kontrol. A. Muricata memperbaiki CM pada mencit dan berpotensi sebagai pengobat pada CM manusia.

✉ Correspondence Author:
E-mail: mohamed.gadallah80@ yahoo.com

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INTRODUCTION

Cerebral malaria (CM) forms part of the spectrum of severe malaria, with a case fatality rate ranging from 15% in adults in southeast Asia (Dondrop, 2005) to 8.5% in children in Africa (Fanello, 2010). Clinical signs of acidosis carry a higher risk of death but nevertheless CM accounts for a significant proportion of malaria mortality, as well as the potential for neurological deficits in survivors. The standard clinical definition of CM centers on a state of unarousable coma partnered with the presence of malaria infected red blood cells in the peripheral circulation and a lack of other potential causes of coma such as other infections or hypoglycemia (WHO,2000 ; Idro 2005 ; WHO 2010). More recently, ophthalmic observations of retinopathy have been added to this definition in both adults and children to increase the specificity of the clinical diagnosis (Maude 2009; Beare 2011). Most observations of the pathophysiology of disease come from postmortem observations of *Plasmodium falciparum* (Pf) infections, which are thought to account for the vast majority of CM cases, and show a common feature of vascular sequestration of infected erythrocytes (IE) in the brain (Mac Pherson 1985). There are also some differences, particularly between CM in adults and children, broadly separable into a 'pure' sequestration pattern and IE sequestration with variable (and moderate) vascular pathology. The latter varies from the accumulation of proinflammatory cells such as leukocytes and platelets to localized vascular damage (e.g., vessels partially denuded of endothelium) (Dorovini 2011).

A lymphoblast is a different form of a naive lymphocyte that occurs when the lymphocyte is activated by an antigen (from antigen-presenting cells) and increased in volume by nucleus and cytoplasm growth as well as new mRNA and protein synthesis. The lymphoblast then starts dividing and making clones of its original naive cells. Finally the dividing cells differentiate into effector cells, known as Plasma Cells (for B cells), Cytotoxic T cells, and Helper T cells.

Human studies and mouse models of CM have independently implicated that cytokines, adhesion molecules, and chemokines have important roles in the disease pathogenesis. The tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) cytokines are two proinflammatory molecules that clearly contribute to CM pathogenesis (Grau 1995). The ICAM-1 (intercellular adhesion molecule 1) adhesion molecule has long been linked to CM due to its role in IE engagement and in T-cell adhesion and transendothelial cell migration through the brain endothelial cells. Many chemokines have been noted to exhibit increased expression in cerebral malaria, but the chemokines most strongly implicated to have a role are the chemokine (C-X-C motif) receptor 3 (CXCR3) binding chemokines, including chemokine (C-X-C motif) ligand 4 (CXCL4), CXCL9 (Armah 2007 ; Luster 2005).

The important of CXCL9 and CXCL is this antimicrobial gene encodes a protein thought to be involved in T cell trafficking. The encoded protein binds to C-X-C motif chemokine 3 and is a chemoattractant for lymphocytes but not for neutrophils. Increase of those chemokines protective or pathologies (eg.related with severe clinical manifestation or related with the mortality or reduce survival days. So it is important and interesting to study those chemokines. The effect of *Annona muricata* on CXCL9 and the lymphoblasts number in spleenocyte infected mice.

Leaves extract of *Annona muricata* has antiplasmodial effect based on *P. falciparum in vitro* study. Beside that *A. muricata* has anti-inflammatory effect. This open possibility to further study *A. muricata* in CM. Endothelial dysfunction due to inflammation occur in CM and ECM is a proposed as target of therapy. Leaves extract of *A. muricata* treatment associated with both a significant reduce parasitemia and TNF- levels during cerebral malaria phase of *swiss* mice inoculated *P. berghei* ANKA (PbA). The doses used in those studies were 200 and 100 mg/kg/day. Two out of five mice receiving 200 mg/kg/day were died during experiment. Therefore the doses of 150 and 100 mg/kg BW/ day will be used in this following study.

METHODS

The research was conducted from june 12, 2015 – August 8, 2015

Research places was in University of Diponegoro – Semarang Indonesia. Population of this study was Female Swiss albino mice. Sample was Female Swiss albino mice

Instruments: 1) Instrument for inoculated, 2) Instrument for caring mice : plastic container 45 cm x 35,5 cm x 14,5 cm, iron container 36,5 cm x 28 cm x 15,5 cm, bottle, 3) Scale to observe mice weight.

Consumable materials: 1) Swiss albino mice, 2) Extract *Annona muricata,* 3) Inoculated *Plasmodium berghei* ANKA, 4) Mice serum, 5)

Spleen cell

Mice divided into 6 groups

1. Negative control is a group without treatment *Annona muricata* and without inoculated *Plasmodium berghei* ANKA
2. Positive control is a group without treatment *Annona muricata* and with inoculated *Plasmodium berghei* ANKA
3. Group receive *Annona muricata* 100 g but not PbA inoculation.
4. Group receive *Annona muricata* 150 g but not PbA inoculation.
5. Group receive *Annona muricata* 100 g and PbA inoculation.
6. Group receive *Annona muricata* 150 g and PbA inoculation.

Before treatment and/ or PbA inoculation, mice will be adapted for 7 days, observed for healthy condition and was weighted, and then randomly alocated into 6 groups. Each mice will be treated in the separate plastic cage. Every day, researcher scale the mice's feed to know the intake of mice/ day. During *Annona muricata* treatment, mice will be inoculated with PbA. Measurement variable e.q. level of CXCL9 and number of lymphoblast produced by spleen.

Data collection. Take the blood from mice's eye after mice anaesthezed. Mixture the blood with lymphoblast in the tube to analyze erythrocyte. Make the spleen culture with stimulate CXCL9, incubation 72 hours and then collect the cell to efipendorf tube, spin the tube with microcentrifuge. Keep the sample to the refrigerator on -80°C to analyze the result.

Variables of Study: Independent Variables: *Annona muricata* , Dependent Variables : Level of CXCL9 and Number of lymphoblast produced by spleen

Data Analysis for level CXCL9 and number of lymphoblast Produced by Spleen

Statistical analysis by using Oneway ANOVA, if homogeneity and distribution of data analysis is normal Post hoc (Tukey HSD) analysis is used if Oneway ANOVA have statistical differences.

If the data is not normally distributed, the non parametris statistic (Kruskal Wallis) will be useed and followed by Mann Whitney test and post hoc.

RESULT AND DISCUSSION

Data Description Results CXCL 9. After make experiment with CXCL 9 in mice the result as follows:

The highest mean of CXCL 9 concentration among the treatment groups were observed in Control (-) and P2 group healthy mice or healthy mice with 150 mg/kg BW/day *A.muricata* treatment (Graph 4.1). The mean of CXCL 9 concentration will be lower in positive control group rather than negative control group. P3 group that is PbA- inoculated mice with 100mg/ kg BW/day *A.muricata* treatment showed higher number of mean CXCL 9 rather than P4 which PbA- inoculated mice with 150mg/kg BW/day *A.muricata* treatment (Table 4.1.).

This showed that CXCL 9 will reduce the level of CXCL9 produced by spleen and lymphoblast treated in mice inoculated with *Plasmodium berghei* ANKA given *Annona muricata* compared to those without *Annona muricata*

Statistic Analysis of CXCL 9. Normality test showed that the distribution of all the data on the concentration of CXCL9 inoculation group with *P. berghei ANKA* normally distributed (p = 0.947), with treatment 100 mg/kg BW/day *A. muricata* (p = 0.457) and treatment 150 mg/kg BW/day *A. muricata* (p = 0.182). As well as in those not inoculated using *P. berghei ANKA* (p = 0.588), with treatment 100 mg/kg BW/day *A. muricata* (p = 0.697) and treatment 150 mg/kg BW/day *A. muricata* (p = 0.451).

Because the data are normally distributed, then the test continued with One Way Anova.

Table 1. The CXCL 9 Concentration Research Group With or Without Inovulation of *P. berghei* ANKA and Giving Leaf Extract *Annona muricata*

Treatment Group	CXCL 9 Concentration (pg/ml)	
	Mean ± SD	**Median (min – max)**
Control (-)	311.80±106.33	185.26-500.48
P1	128.52±122.35	27.07-262.19
P2	303.736±289.1724	28.666-304.85
Control (+)	99.0895±92.2525	11.316-217.4
P3	235.56±154.556	62.737-454.93
P4	129.386±80.42027	35.884-257.78

Based One Way Anova test showed that the extract of *Annona muricata* on samples by inoculation of *P. berghei ANKA* significant effect (p=0.032) on the CXCL 9 concentration. Based on further testing using the Tukey HSD indicate that there are significant differences between the positive control with P3 and P4, there is a significant difference between P3 and P4.

The *Post hoc* test indicate that control negative the number of CXCL 9 significant different with control positive. No significant different between control negative and P1, P2. Also no significant different between P1 and P2. From treatment with inoculated PbA showed that not significant different between positive control and P3, P4. Also not significant different between P3 and P4 (Table 2).

From the Table 2. it can be say that there was no significant correlation between P1 and P2 this means there is no association between the increase dose of CXCL9 and there is no association between A. M 150mg/kgBW/day and lymphoblast in healthy swiss mice. CXCL 9 in all dose studied had no association with the lymphoblast in healthy swiss mice.

Meanwhile, P3 and P4 also not significant, this means that the PbA inoculation was not associated lymphoblast in those treated with CXCL9 in any dose studied.

Data Description Results Lymphoblast of Spleen. The highest mean of Lymphoblast percentage among the treatment groups were observed in P3 group mice or healthy mice with 100 mg/kg BW/day *A.muricata* treatment (Table 2). The mean of percentage Lymphoblast will be lower in negative control group rather than positive control group. P3 group treated PbA- inoculated mice with 100mg/kg BW/day *A.muricata* treatment showed higher number of mean Lymphoblast rather than P4 group treated with PbA- inoculated mice with 150mg/kg BW/day *A.muricata* treatment.

Statistic Analysis of Lymphoblast of Spleen. Normality test showed that the distribution of all the data of lymphoblast inoculation group with *P. berghei ANKA* normally distributed (p = 0.296), with treatment 100 mg/kg BW/day *A. muricata* (p = 0.964) and treatment 150 mg/kg BW/day *A. muricata* (p = 0.765). As well as in those not inoculated using *P. berghei ANKA* (p = 0.386) or the healthy mice.

Because the data are normally distributed, then the test continued with One Way Anova. Based One Way Anova test showed that the extract of *Annona muricata* on samples by inoculation of *P. berghei* ANKA significant effect (p=0.043) on Lymphoblast of Spleen. Based on further testing using the Tukey HSD test showed that there were significant differences between the positive control with P3 and P4, there is significant difference between P3 and P4.

The *Post hoc* test indicate that control negative of lymphoblast not significant different with control positive. From treatment with inoculated PbA showed that not significant different between positive control and P3, P4. Also not significant different between P3 and P4 (Table 4).

From the Table 4. it can be say that there was no significant correlation between P3 and

Table 2. Mann-Whitney Test of CXCL 9 Concentration

Treatment	Neg	Pos	P1	P2	P3	P4
Neg		p = 0.041*	p = 0.865^{ns}	p = 0.908^{ns}	p = 0.165^{ns}	p = 0.610^{ns}
Pos			p = 0.168^{ns}	p = 0.021*	p = 0.202^{ns}	p = 0.798^{ns}
P1				p = 0.925^{ns}	p = 0.335^{ns}	p = 0.750^{ns}
P2					p = 0.938^{ns}	p = 0.543^{ns}
P3						p = 0.772^{ns}
P4	p = 0.610^{ns}	p = 0.789^{ns}	p = 0.608^{ns}	p = 0.338^{ns}	p = 0.708^{ns}	

Table 3. The Percentage of Lymphoblast Research Group With or Without Inovulation of *P. berghei* ANKA and Giving Leaf Extract *Annona muricata*

Treatment Group	**Lymphoblast of Spleen**	
	Mean ± SD	**Median (min – max)**
Control (-)	0.5522±0.028355	0.523-0.595
Control (+)	0.5530±0.013435	0.513-0.621
P3	0.5535±0.005657	0.511-0.633
P4	0.5523±0.00345	0.592-0.625

Table 4. Mann-Whitney test of Lymphoblast

Treatment	Neg	Pos	P3	P4
Neg		$p = 0.076^{ns}$	$p = 0.062^{ns}$	$p = 0.562^{ns}$
Pos			$p = 0.711^{ns}$	$p = 0.808^{ns}$
P3				$p = 0.582^{ns}$
P4	$p = 0.566^{ns}$	$p = 0.265^{ns}$	$p = 0.445^{ns}$	

P4 this means there is no association between the increase dose of CXCL9 and there is no association between A. M 150mg/kgBW/day and lymphoblast in healthy swiss mice. CXCL 9 in all dose studied had no association with the lymphoblast in healthy swiss mice.

Meanwhile, P3 and P4 also not significant, this means that the PbA inoculation was not associated lymphoblast in those treated with CXCL9 in any dose studied.

Concentration of CXCL9

As we know that in CM literature and study, both experimental animals and in vitro models has been central to our understanding of the events that cause brain pathology. CM is the main lethal complication of *P. berghei* ANKA infection in mice that involve multi-process and multi-system disorder very complex presents a variety of clinical features. It is characterized by the absorption of PRBC as the main important feature, especially in the deep brain microvasculature and with increased levels of pro-inflammatory cytokines. In this study the decline in the concentration of CXCL 9 after inoculation using *P. berghei* ANKA. Rapid onset of malaria occur as early as on day 1 following inoculation with the parasite. A high degree of parasitemia with death indicates a severe level of infection in this model. Lost large amounts of normal RBC on this model showed characteristic signs of severe anemia which is one of the major clinical manifestation of severe malaria in humans.

Concentration of CXCL9 in P3 group significantly higher than P4. The reason is because P3 is the PbA- inoculated mice with 100mg/kg BW/day *A.muricata* treatment and P4 is PbA- inoculated mice with 150mg/kg BW/day *A.muricata* treatment, so with the higher dose of CXCL9 it will make the lower level of PbA-infected mice AM.

In this research, it is known that the extract of *Annona muricata* no significant effect on the group with *P. berghei* ANKA inoculation. In line with research conducted by Craig, et. al. (2011) adding the extract of *A. muricata* in mice that have been infected using the CXCL9 showed no significant results. IFN-β treatment leads to reduction of CXCL9 and ICAM-1 in the brain, reduction of T-cell CXCR3 expression, and downregulation of serum tumor necrosis factor alpha (TNF-α). In addition, IFN-β-treated *P. berghei*-infected mice also had fewer brain T-cell infiltrates, further demonstrating its protective effects.

The concentration of CXCL9 was no different between those with healthy and PbA inoculated mice because IFN-β treatment leads to reduction of CXCL9 and ICAM-1 in the brain, reduction of T-cell CXCR3 expression, and downregulation of serum tumor necrosis factor alpha (TNF-α). In addition, IFN-β-treated *P. berghei*-infected mice also had fewer brain T-cell infiltrates, further demonstrating its protective effects.

The factors influence the concentration of CXCL9 during PbA infection is Mice infected with P.berghei ANKA (PbA) provide a suitable experimental model to study the pathogenesis of human cerebral malaria as they recapitulate the vast majority of the immunological features. Pb-A infected susceptible mice develop behavioral changes indivative of cerebral involvement at approximately day 5 post-infection and ptogress to coma and death at approximately days 6-8. The influence of experimental mixed-Plasmodium-species infection on the expression of PbA induced cerebral jmalaria was completely inhibited by the simultaneous presence of the non-lethal parasite line and the concentration of CXCL9 during the infection.

As we know that in CM literature and study, both experimental animals and in vitro models has been central to our understanding of the events that cause brain pathology. CM is the main lethal complication of *P. berghei* ANKA infection in mice that involve multi-process and multi-system disorder very complex presents a variety of clinical features. It is characterized by the absorption of PRBC as the main important feature, especially in the deep brain microvasculature and with increased levels of pro-inflammatory cytokines. In this study the decline in the concentration of CXXL 9 after inoculation

using *P. berghei* ANKA. Rapid onset of malaria occur as early as on day 1 following inoculation with the parasite. A high degree of parasitemia with death indicates a severe level of infection in this model. Lost large amounts of normal RBC on this model showed characteristic signs of severe anemia which is one of the major clinical manifestation of severe malaria in humans. In this study, the result show that the spleen in CXCL 9 is lower rather than in CM higher.

Based on the results of the study, found that an increase in the Lymphoblast of spleen in mice that had been inoculated with *P. berghei* ANKA compared with mice not inoculated with *P. berghei* ANKA. This is consistent with research on the role of Spleenocyte in the pathogenesis of cerebral malaria and the results showed that the increase in the lymphoblast may be a risk factor for death in cerebral malaria patients[13]. Moreover, in patients with *P. falciparum* malaria and *P. vivax* infections showed a significant increase in the lymphoblast of spleen, TNF-α, IFN-γ, IL-12, IgM and MCP-1 in the peripheral blood. This response, in concert with other inflammatory cytokines and production of specific antibodies against the parasite, can cause pathological responses (Hansen 2007).

Increased levels of lymphoblast may play an important role in local immune response, possibly through the activation of macrophages to parasite clearance. Immunohistological studies have reported that, although the lymphoblast is found in skeletal muscle cell nuclei, it is not found in the walls of cerebral blood vessels of patients with cerebral malaria (John, 2006). The role of lymphoblast in malaria can reduce the infection of genetic variability from malaria infection. So hypothesis in this research accepted. This means Spleenocyte level of PbA-infected mice treated with *A. muricata* are higher than group of infected mice without treatment.

CONCLUSION

CXCL9 level of PbA-infected mice treated with *A. muricata* are lower than group of infected mice without treatment. Lymphoblast level of PbA-infected mice treated with *A. muricata* are higher than group of infected mice without treatment.

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