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LEMBAR
HASIL PENILAIAN SEJAWAT SEBIDANG ATAU *PEER REVIEW*
KARYA ILMIAH : JURNAL ILMIAH

Judul Artikel Ilmiah	:	EFFECTIVITY OF Annona muricata AND ARTEMISININ COMBINED THERAPY ON BRAIN CXCL10 EXPRESSION (Study in swiss mice during severe Plasmodium berghei ANKA infection)
Penulis Artikel Ilmiah	:	Abdulhakim Sulayman A. A.1, Kis Djamiatun, **Muflihatul Muniroh**
Status Pengusul	:	Penulis pertama/**penulis anggota**/penulis korespondensi
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Kecukupan & Kemutakhiran Data & Metodologi	:	Metode penelitian lengkap termasuk ethical approval. Procedure dan Instruments dijelaskan dengan interpretasinya.
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C-16

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a. Kelengkapan dan Kesesuaian unsur isi artikel (10%)		2.5		2.5
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KOMENTAR/ULASAN PEER REVIEW

Kelengkapan dan Kesesuaian Unsur	:	Sistematika unsur isi artikel lengkap dan sesuai, diuraikan pada setiap bagiannya.
Ruang Lingkup dan Kedalaman Pembahasan	:	Penelitian menggunakan tikus untuk mengetahui kombinasi AME dan ACT dapat menurunkan ekspresi otak CXCL 10 pada tikus. Hasil penelitian signifikan dibahas dengan detail dan didukung oleh jurnal terkini <10 tahun.
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Kelengkapan Unsur dan Kualitas Penerbit	:	Jurnal ilmiah nasional yang terakreditasi SINTA 2.

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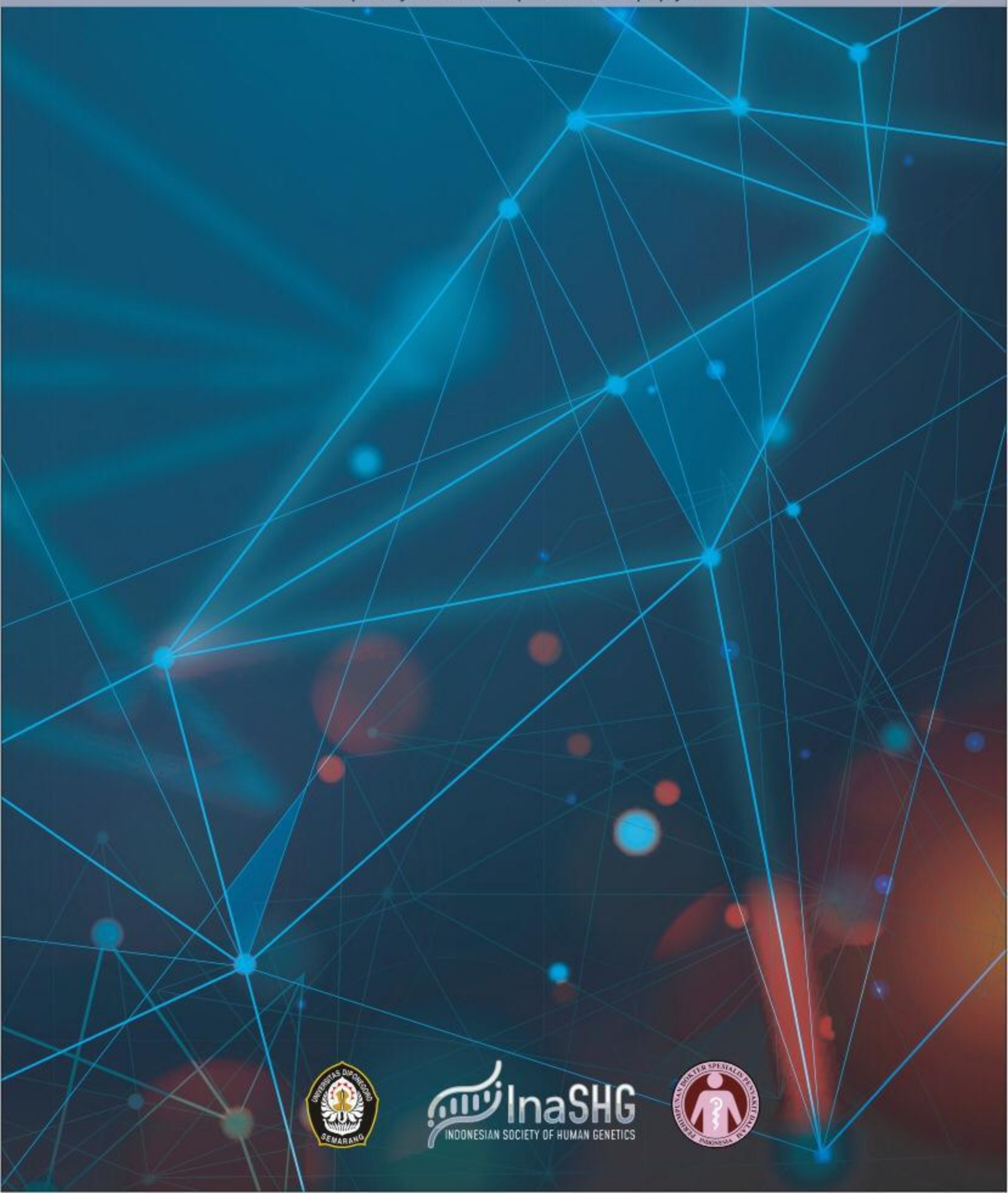

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Research Articles

Effectivity of Annona Muricata and Artemisinin Combined Therapy On Brain CXCL10 Expression

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Abstract

Background: Malaria, caused by Plasmodium spp infection, is a major global cause of morbidity and mortality. Most experimental cerebral malaria (ECM) studies show increase number of Th1 cells and CTLs in the brain, due to increase chemokine expression, including CXCL10, a potent chemokine involved in cerebral malaria (CM). Recent studies show that CXCL10 provokes apoptosis of human brain micro-endothelial cells and in vitro neuroglia cells.

Objective: To determine whether combination of Annona muricata-leaf-extracted-by-water (AME) and artemisinin-combination-therapy (ACT) reduce brain-CXCL10-expression of Swiss-mice inoculated with P. berghei ANKA (PbA).

Methods: This was an experimental-study with post-test-only-control-group-design. Twenty-four Swiss-mice (PbA-inoculated) were randomly divided into 4 groups. Control group (C) was PbA inoculated only. X1, X2 and X3 groups received AME, ACT and combination of AME and ACT treatment, respectively. CXCL10 was stained with in immunohistochemistry, which then observed by light microscope in order to determine Allred-score. Kruskal-Wallis test was used to statistically analyze the differences among groups, then followed by a Mann- Whitney U test.

Result: C and X1 groups had severe-PbA-infection when the study was end on day-7-PbA-infection, while X2 and X3 groups entered recovery-stage. The AME-ACT-treatment-group had significantly lower of brain-CXCL10-expression than AME-group (p=0.008) and nearly significantly lower than control-group (p=0.058). Group that received ACT alone had no different value of brain-CXCL10-expression than control-group (p=0.502) and combination AME–ACT group (p=0.335).

Conclusion: The combination of AME–ACT treatment decreases brain-CXCL10-expression of Swiss-mice during PbA-infection-recovery-stage, indicating the effectivity of AME–ACT combined therapy is better prevention of cerebral malaria than AME alone..

Keywords: *Annona muricata, Artemisinin, P. berghei ANKA, CXCL10*
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INTRODUCTION

Malaria, caused by *Plasmodium spp* infection that is transmitted by mosquitoes, is a major global cause of morbidity and mortality.[1] *Plasmodium spp* life cycle in the vertebrate host are including a clinically silent early stage in the liver. Followed by erythrocytic stage, which is responsible for the pathology of malaria.

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Malaria in patients may be caused by *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi* while some species of *Plasmodium* that infects animal, but not humans, are available for laboratory studies, including *P. berghei* ANKA (PbA) which allows the dissection of immune mechanisms of protection and pathology.[2]

Severe malaria including cerebral malaria might be fatal. Susceptible mice infected with PbA develop experimental cerebral malaria (ECM). Most ECM study

using C57BL6 mice showed that increase number of Th1 cells and CTLs in the brain. These cells migrate to brain due to chemokines.[3,4] Increase levels of chemokine ~~levels~~ are widely associated with cerebral malaria (CM). The most potent chemokines involved in CM are those that bind to the C-X-C motif 3 receptor (CXCR3), including CXCL9, and CXCL10. CXCL10 binding to high affinity CXCR3, is the first known chemokine that can direct the activated CD4+ and CD8+T-cells, natural killer (NK) cells and natural killer T (NKT) cells.[4] The importance of CXCL10 has been suggested by several studies on malaria. A study on CM in Ghanaian children shows that of 36 biomarkers, only CXCL10 is an independent serum marker associated with CM death. In addition, studies of cerebral spinal fluid (CSF) of CM patients show that CXCL10 is one of eight biomarkers significantly related with CM.[5] The ECM study in C57BL6 mice shows that CXCL10 is highly induced in the brain especially in infected mouse neurons of PbA. Interestingly CXCL10-deficient mice are protected in part from ECM death.[4] Recent-study shows that CXCL10 provokes apoptosis of human brain micro-endothelial cells and neuroglia cells *in vitro*.[6] In contrast to those found in CM and ECM studies, *in vitro* study on astrocytes shows a reduced CXCL10 expression during hypoglycemia and hypoxia which are two conditions found in severe malaria.[7] Astrocytes are important in maintaining brain homeostasis and preventing brain inflammation caused by T cells.[8] CXCL10 is expressed by many brain-cells including astrocytes. The production of CXCL10 by astrocytes is consistent with the detection of *in situ* CXCL10 mRNA in astrocytes during malaria infection.[2] The mechanisms underlying the different CXCL10 production in this model may be due to the relative magnitudes of microbial signals versus cytokine, such as IFN-γ. Most recent study shows that CXCL10 expression in the brain of severe-PbA-infected Swiss mice is modulated by ethanolic leaf extract of *Annona muricata*.[9] Parasitaemia, however is not affected by this extract.[9] Additionally, it has not been studied before whether artemisinin combination therapy (ACT), anti-malaria standard therapy commonly used, affect CXCL10 expressed by astrocyte during severe malaria. Therefore it is interesting to study the influence of combination of this herb and ACT toward CXCL10 expressed by astrocytes of Swiss mice during severe malaria.

After the emergence of widespread drug resistance to chloroquine and sulfadoxine-pyrimethamine, ACT is introduced as a highly effective treatment for uncomplicated malaria (caused by *P falciparum* and *P. vivax*), preventing the progression of severe illness and death.[10] The first-line treatment recommended by WHO for uncomplicated falciparum malaria in almost all endemic countries is ACT.[11] Funding for ACT procurement has increased rapidly and is now widely available from several manufacturers and in many formulations. Between year of 2003 and 2007, almost all countries in Africa changed their treatment policy to ACT as a first-line treatment of uncomplicated malaria, and since then, ACT procurement and distribution increase remarkably.[12]

Annonaceae comprising about 123 genera and 2,100 species, is draw attention because of their traditional use of malaria.[13] The anti-malarial treatment of *Annona muricata* leaf extract (AME) is demonstrated *in vivo* by using mice inoculated with PbA.[14] The CM study showed that tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine, contributed to the pathogenesis of CM.[15] Ethanolic-AME treatment is associated with decreased TNF-α and nitric oxide (NO) produced by splenocytes from Swiss inoculated with PbA as a model for ECM.[16] Water extract of *A. muricata* leaves is available in the market, this therefore it is interesting to focus a study in the combination of this herb and ACT an anti-malaria therapy used in Indonesia. The purpose of this research is to determine whether a combination of *Annona muricata* and ACT reduces CXCL10 expressed by astrocytes in the brain of Swiss albino mice inoculated with PbA.

Materials and Methods

This study was experimental study, by using randomized post test only control group design. PbA-mouse donor was purchased from Parasitology Department Universitas Gajah Mada. The PbA-infection was done by intra peritoneal injection of 10^7 parasitized-red blood cells (pRBCs) in 0.2 ml solution to all experimental mice used in this study. The treatment used was *A. muricata* leaf-extracted using water (AME), ACT and combination both of AME and ACT. AME was obtained for free from SudoMuncul-company. AME was obtained from *A. muricata* leaves which were sorted, washed and dried. Percolation was then done by using water at 60°C for two hours; subsequently evaporation was done in 60°C temperature. The filler was then added to the wet granulation of *A. muricata* leaf-extract. The next was oven dried the granules and then milled them. Finally the AME was ready to use. ACT used in this study was Dihydroartemisinin Piperaquine (DHP). The DHP dose for malaria patients was adjusted for PbA-infected mice. The calculation of ACT dose used was 0.546 multiply by (mouse body-weight (BW) divided by 20).

This study used 24 female-Swiss albino mice, which were purchased from the private animal breeding at Bandung (certified by Food and Agriculture Department of Bandung City Government). The mice were grouped in four and were PbA inoculated. Control (C) group was PbA-inoculated only and did not receive any treatment, while X_1, X_2 and X_3 groups were treated with AME, ACT and combination of AME and ACT. AME-prevention dose of 4.68mg/ 30g mice-weight was given for 7 days before PbA-infection which then continued until day 3 PbA-infection. AME-therapeutic dose of 9.36mg/ 30g mice-weight was then administered until day 6 PbA-infection. ACT dose used was 0.819mg/ 30g mice-weight which administer since day 4 until day 6 PbA-infection. All mice were terminated on day 7 PbA-infection. The animals were housed in Parasitology Department, Faculty of Medicine Diponegoro University. This study had been approved by the medical research ethics committee, Faculty of Medicine Diponegoro University-RSUP Dr. Kariadi Semarang with No. 86/EC/H/FK-RSDK/XII/2017. This study was a continuation of our previous study which evaluated the parasitaemia and number of lymphocytes at the brain perivascular in severe malaria infection. The brains were collected from all groups for CXCL10-expression study. The brain-

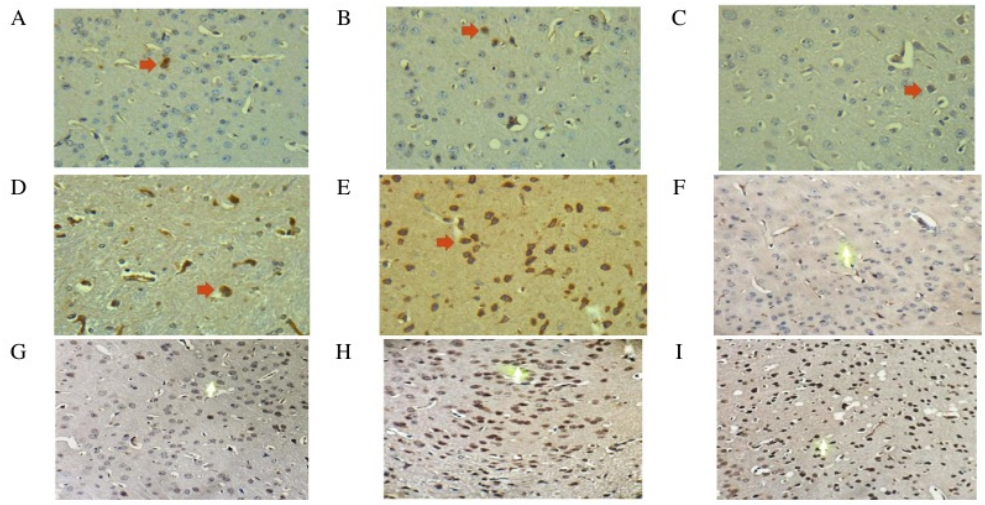

Figure 1. IHC of brain-CXCL10 used to determine Allred score
Allred score = proportion score + intensity score. A= Proportion ≤ 1/100 colored cell (score 1); B= Proportion ≤ 1/10 colored cell (score 2); C= Proportion ≤ 1/3 colored cell (score 3); D=Proportion ≤ 2/3 colored cell (score 4); E= Proportion all cells are coloured(score 5); F=Intensity (-) (score 0); G=Weak Intensity (score 1); H= Moderate Intensity (score 2); I= Strong Intensity (score 3).

CXCL10 expression was stained by using IHC method, and was evaluated based on Allred score which were determined by two experts from Pathology Anatomy Department Faculty of Medicine Diponegoro University, Semarang, Indonesia. Because of the CXCL10 data were not homogen and not distributed normaly, statistical analysis used was *Kruskal-Wallis* test and then followed by *Mann-Whitney U* test.

RESULTS

Parasitaemia level was observed at day 3 and 7 post inoculation (p.i). All groups were positive of PbA-infection at day 3 p.i. The mean parasitaemia at day 7 p.i of C and X_1 groups were 19.88% and 18.40%, while those of X_2 and X_3 groups were 0.55% and 0.41%. Those of C and X_1 groups indicated severe-PbA-infection at day 7 p.i. X_2 and X_3 groups entered recovery-phase of PbA-infection as indicated by very low parasitaemia. No difference in parasitaemia level at day 7 p.i was found between either C and X_1 groups or X_2 and X_3 groups (*Mann-Whitney U* test; $p > 0.05$).

The IHC showed the different proportions and intensity of CXCL10 expression in the cytoplasmic cells in the brain (Figure 1). The higher the proportion and intensity is showed with slightly hematoxylin (blue).

The brain-CXCL10 expressions in Swiss Mice

Allred score was determined based on the calculation both proportion and intensity scores (Figure 1). Kappa test was done to identify the similarity of the two assessors to determine Allred score of CXCL10-expression on the same area of the IHC-slide. This test indicated a moderate agreement between the two assessors (Kappa value = 0.6). The CXCL10-expression of X_1 group was higher than other groups (C, X_2 and X_3 groups) (Figure 2).

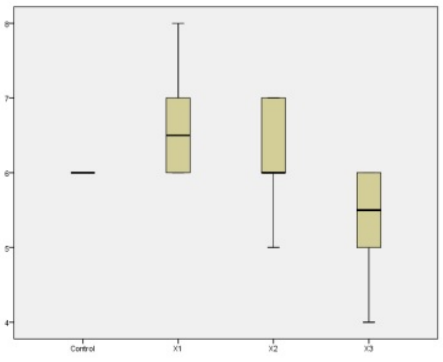

Figure 2. Graph Box plot was Allred score of brain-CXCL10-expresssion.
All mice used were PbA-infected, and were divided in four groups which were C group: without any treatment; X_1 group: treated with AME; X_2 group: treated with ACT; Group X_3: treated with AME and ACT.

Statistical analysis indicated that CXCL10 Allred score was normally distributed in all studied group. Analysis of variance, however, showed that the data were not homogen (p=0.015), .therefore, *Kruskal-Wallis* test was performed. This test showed that CXCL10 expression was significantly different among the studied groups (p = 0.011). Then *Mann-Whitney U* test was performed to see the difference of CXCL10 expressions between two groups (see Table 1). The X_1 group expressed significantly higher CXCL10 than C (p = 0.022), X_2 (p = 0.017) and X_3 (p = 0.008) groups. The CXCL10 expression of X_3 group was lower than C group, but the difference was not significant (p = 0.058).

Table1. Statistical analysis of CXCL 10 expressed in the brain

Groups	CXCL10 expression median (min-max)	*p*		
		X1	X2	X3
Control	6.00 (6.00 – 6.00)	0.022*	0.502	0.058
X1	6.75 (6.00 – 8.00)	(-)	0.017*	0.008*
X2	5.75 (4.00 – 7.00)		(-)	0.335
X3	5.33 (4.00 – 8.00)			(-)

* Mann-Whitney U test with significant difference ($p < 0.05$)

Additionally, X_2 and X_3 groups showed no difference of CXCL10-expression ($p = 0.335$).

DISCUSSION

The present study showed that the X_1 group which received water extract of *Annona muricata* leaves had significant higher CXCL10 expression in the brain than C group (PbA-infected only) (Table 2). This occurred during severe PbA infection in both C and X_1 groups. All together suggest that water extract of *Annona muricata* leaves associates with the increase the brain-CXCL10-expression of Swiss mice during severe PbA-infection. This finding is in line with previous study using ethanolic extract of *Annona muricata* leaves.[9] The ethanolic extract of *Annona muricata* leaves in dose of 100 mg/Kg BW/day for total period of 14 days increases the brain-CXCL10-expression in severe PbA infected Swiss mice. The present study showed that the brain-CXCL10 expression of X_2 and X_3 group was not different than C-group. Both of X_2 and X_3 groups had very low parasitaemia level indicating that they were entering recovery stage. This suggests that the low parasitaemia shows no observable influence on the brain-CXCL10-expression during recovery stage of PbA-infection. This present finding was not as expected before. The previous study showed that the brain-CXCL10-expression in controls with severe PbA infection was significantly lower than healthy control groups.[9] This indicates that reduce the brain-CXCL10 expression associates with severe PbA infection when the highest parasitaemia levels occur.

The groups that received ACT and AME-ACT-combination showed comparable brain-CXCL10 expression on day 7 PbA-infection (Table 1). This was in line with the study comparing artemether (ART) alone and the combination of ART and immunomodulator treatment, showing that CXCL10 of those treated groups was not different in day 11 PbA-infection.[6] Parasitaemia percentage was significantly lower in those of treated either ACT or AME-ACT-combination than control group. This indicates that the relation between brain-CXCL10-expression and parasitaemia percentage in treated groups is different than that of controls. Brain-CXCL10-expression in those treated groups might relate to the re-convalescent phase of severe PbA-infection, while those in positive controls might relate to the immunopathological condition. This was supported in some part by study observing parasitaemia and CXCL10 expression. The previous study using PbA-infected C57BL/6J, showed lower parasitaemia percentage in those treated groups (either ART or combination ART-immunomodulator) than that of day 11 PbA-infected-control group.[6] Reduce CXCL10 expression and parasitaemia occurred at the same time in those treated groups.[6] This indicates that decrease CXCL10 expression comes about at the recovery phase of PbA-infection. Brain-CXCL10 expressed in the control-group in the present study, however was not in line with previous study showed that brain-CXCL10 mRNAs of day 11 PbA-infected control group were significantly higher than those receiving treatments.[6] Because of the difference of mouse-strain used and method used to evaluate a CXCL10 expression, the previous finding was different than ours. The Kappa value of 0.6 on the Allred score of CXCL10 expression indicates that the CXCL10 mRNA examination or CXCL10 IHC examination using more advanced methods are needed to provide more accurate information.

Among many cell types in the brain, astrocytes express CXCL10 at the latest stage of infection.[17] Other infectious disease induce an increase CXCL10 expressed by astrocytes, which are the main CXCL10 source in CNS.[18] Our study might complete with evaluation IHC of glial fibrillary acidic protein (GFAP) as active-astrocyte-biomarker. Many factors may influence astrocyte-CXCL10 expression in the brain of those with malaria. Apoptotic-astrocytes were found in PbA-inoculated C57BL/6 mice, and reduce astrocyte-CXCL10-expression was found in the condition of hypoxia and hypoglycemia, which were both presents in severe malaria.[19] Whether these factors influence astrocyte-CXCL10-expression in the present study are needed to be further evaluated.

It remains a hope that AME-ACT-combination protects severe malaria. Lower brain-CXCL10-expression found in during recovery stage of PbA-infected Swiss-mice treated with AME-ACT-combination than the PbA-infected-controls (Table 2). This was almost statistically different ($p = 0.058$), therefore additional protective and pathologic marker should be assessed. CXCL10 has immunopathologic effect in malaria. CXCL10 levels were higher in the cerebrospinal fluid of CM patients, and those in plasma correlate with mortality of those patients.[20] Genetic CXCL10-polymorphism relates to higher CXCL10 plasma is associated with CM susceptibility.[21] This may be explained by study shows that CXCL10 is capable to induce apoptosis of neuroglia.[6] CXCL10 may not be the only pathologic factor. Both pRBCs and their soluble factors induce apoptosis of vascular endothelial cells and neuroglia.[22] Therefore, it is warrant to be evaluated that a reduce brain-CXCL10-expression found in group treated

with ACT-AME-combination reduces neuronal apoptosis during recovery phase of PbA-infected-Swiss-mice.

AME treatment associates with a significant increase brain-CXCL10-expression during severe-PbA-infection (Table 2). Based on astrocyte studies, the protective effect of AME treatment in severe malaria stays possible. Astrocytes are important cells to limit central nervous system (CNS) inflammation induced by T-cells.[8,23] By releasing CXCL10, astrocytes attract Th1-cells expressing CXCR3, the receptor of CXCL10. Beside that astrocytes are able to suppress the activation, proliferation, and function of Th1-cells. The AME beneficial for those with severe malaria needs to be evaluated in further studies. Whether any association between increase astrocyte-CXCL10-expression and reduce function of Th1-cells is found in AME-treatment of severe PbA infection. Additionally, whether a reduced astrocyte-CXCL10-expression associates with a reduced function of Th1-cells in the recovery stage of PbA-infection in Swiss mice treated with combination of AME-ACT. A difference of CXCL10 expressed by astrocytes was found in a virus-infection susceptible and resistant mouse.[24] The present study evaluated the effect of AME and ACT in PbA severely infected Swiss mice which were ECM susceptible mice. Evidence in this present study, therefore, is completed by comparing the ECM-susceptible and resistant mice.

The evaluation of TLRs and CTLA expressed by astrocytes would enrich the present study. Astrocytes express TLR2 and TLR4.[25,26] The ligand binding-TLR2 and TLR4 induces astrocyte-CXCL10 and IL-10 production.[25] Those TLRs are capable of binding glycosylphosphatidylinositols (GPIs) of *Plasmodium sp*. TLRs binding to their ligands induce anti-malaria immune response.[27] Th-effectors cells after cross the blood-brain-barrier, are initially encountered astrocytes which capable of inhibiting the activation, proliferation and effectors function of those effectors' cells. The inhibitory action of astrocytes is mediated by the induction of increase cytotoxic-T-lymphocyte-associated antigen-4 (CTLA-4, CD152) expression on the surface of those Th-effectors while Transforming growth factor-beta (TGF-β) secreted by astrocytes or T cells has only minor role in this inhibition.[8] The previous study showed that CTLA-4 expressed on Th1 cells regulate migration of these cells.[28] TLRs and CTLA-4 determined in this present study will give more understanding in the mechanism utilized by AME or AME-ACT-combination in preventing immunopathology in severely PbA infected Swiss mice.

CONCLUSION

The combination of *Annona muricata* leaf extract (AME) and artemisinin combined therapy (ACT) lowers brain-CXCL10-expression more than AME alone.

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