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RESEARCH ARTICLE

Prevention of Cerebral Malaria Hypoxia through administration of Neem leaves extract (*Azadirachta indica*) in Mice C57BL

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ABSTRACT:

Background: Cerebral malaria is the most serious complication of malaria infection. *Plasmodium falciparum* is the most common cause of cerebral malaria. Pathomechanisms underlying the severity of cerebral malaria include parasite ability, parasitemia degree, host inflammatory response, sequestration, disruption of the blood brain barrier (BBB), and brain hypoxia. Hypoxia causes cells to produce transcription factors such as the HIF-2α protein. The development of antimalarial drugs is based on fatal complications caused by hypoxia in cerebral malaria. Thus, it is necessary to investigate the mechanism of antihypoxia in cerebral malaria using natural materials, one of which is leaves (*Azadirachta indica*). **Methods:** Inoculation of *Plasmodium berghei* strain ANKA in C57BL mice aged 13-16 weeks. Parasitemia calculations were performed every day from the blood of the mouse tails. Treatment was given using 96% ethanol extract from neem leaves with dose of 8mg, 12mg, and 16mg orally for 6days. As treatment comparisons, there were also negative controls, positive controls, and healthy controls. Brain tissue was isolated on the seventh day to study the expression of p>0.05). The hypothesis is tested using a one-way ANOVA test with post-hoc LSD test and Pearson's correlation test. **Results:** The administration of neem leaf extract significantly reduced parasitemia and hypoxia (p<0,000). Meanwhile, the correlation test revealed a very strong relationship (r=+0.732) between parasitemia and hypoxia. **Conclusion:** Neem leaf extract administration reduces parasitemia and prevents hypoxia in mice induced by cerebral malaria

KEYWORDS: Hypoxia, Parasitemia, Cerebral malaria, Neem leaves, *Azadirachta indica*.

INTRODUCTION:

Malaria is a parasitic infection caused by *Plasmodium sp*. and transmitted by the female *Anopheles sp*. vector. Worldwide malaria cases in 2021 reached 227million[1]. Indonesia unable to reduce the target of malaria incidence and mortality compared to countries in Southeast and South Asia (with decrease <37%)[2]. This is due to the vector's ability, the environment, host immunity, and the parasite's pathogenicity[3]. *Plasmodium falciparum* is a parasite genus that can cause severe malaria complications. Serious complications of malaria

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include nephrotic syndromes, anemia, and cerebral malaria[4].

Cerebral malaria manifests clinically like malaria in general but with neurological disorders[5]. Neurological disorders in malaria infection can cause fatal brain injury[6]. The pathomechanism that causes brain injury in cerebral malaria is brain hypoxia[7]. Hypoxia is caused by cytoadherence/attachment between *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP-1) ligand and the brain endothelial receptor Intercellular Adhesion Molecule-1 (ICAM-1)[7,8,9]. The cytoadherence induces sequestration/remaining of infected Red Blood Cells (iRBC) in the brain microvascular. The two processes above can cause an inflammatory response, induces apoptosis, reactive oxygen species (ROS), and platelet aggregation thereby reducing tissue perfusion in the brain[7,10].

Cells experiencing hypoxia have a cellular adaptive response to produce Hypoxia Inducible Factor (HIF-2α). Hypoxia can induce ICAM-1 protein levels in brain endothelium[11]. Studies that have been conducted on Human Cerebral Malaria (HCM) show the presence of HIF-2α expression in the nucleus and microvascular cytoplasm[12]. Brain microvascular damage is a major contributor to worsening cerebral malaria. To date, there has been no research on antimalarial drugs with antihypoxia mechanisms in cerebral malaria. In addition, many antimalarial drugs have experienced resistance[13]. So, it is necessary to develop drugs from natural resources such as neem (*Azadiracta indica*). Every part of this plant has been utilized for traditional medicine cures and mosquito repellent including leaves, oils, fruits, seeds, bark, and roots[14,15]. Neem are widely known as a plant that regulate free radical scavengeing, angiogenesis agent, transcription factors, anti-microbial agent, and anti-inflammatory agent[16,17]. This plant also reported to have antimalarial and neuroprotective activity[18,19]. However, the specific mechanism of this ability in plants is unknown. Therefore, this study was conducted to determine the effect of neem leaf extract in preventing cerebral malaria hypoxia.

MATERIALS AND METHODS:

Preparation of Experimental Animals:

This purely experimental study was conducted in vivo using a post test control group design. The experimental animals used were C57BL mice aged 13-16 weeks, weighing between 20-30grams. This mouse is routinely used as a model for cerebral malaria because it has a clear symptom and pathological presentation. The number of mice was 4 per 6 treatment groups. The group consisted of positive control with Dihydroartemisinin + Piperaquine (DHP) 0,02496mg, negative control, treatment 1 (neem leaf extract dose of 8mg), treatment 2 (neem leaf extract dose of 12mg), treatment 3(neem leaf extract dose of 12mg), and healthy control (not infected and treated). The process of acclimatization and treatment was carried out at the Experimental Animal Laboratory of FKIK UIN Malang.

Preparation of Neem Leaf Extract *(Azadirachta indica):*

Extraction was carried out by drying the neem leaves, and then macerating 100grams with water and 96% ethanol for 48hours. The sample is filtered, then the filtrate is evaporated to form a concentrated extract. The concentrated extract is stocked with 0.5% CMC dilution. Calculations were adjusted based on concentration of the preparations, the number of mice, and the length of treatment. The treatment was given orally. Neem leaf extract is made at the Materia Medica Batu.

Phytochemical Study of Neem Leaf Extract Preparation of Neem Leaf Extract *(Azadirachta indica):*

Weighing a sample of 2grams of neem leaf extract then put in a beaker glass and given 20ml of distilled water. Heat for ±15 minutes over a bunsen and pour into 2 test tubes. Then divided into 2 parts to be tested with flavonoids (quercetin) and triterpenoids (azadiractin). In the sample tested with flavonoids, 3 drops of concentrated HCl were added and a little magnesium powder, the positive results of the flavonoid (+) showed by color changing of the solution to orange/brick red/orange/dark red. Meanwhile, in the samples tested with triterpenoids, 3 drops of Bouchardat reagent were added and the positive results of the triterpenoids (+) were indicated by the presence of brown precipitate (Table 1).

Inoculation of *Plasmodium berghei* Strain ANKA:

Donor mice were inoculated with *Plasmodium berghei* strain ANKA from $1x10^6$/ml liquid nitrogen intraperitoneally. Then the parasitemia was calculated from the Giemsa-stained blood smears. If the parasitemia has reached 5-8%, it is ready to be donated to treated mice with a parasite count of $1x10^6$/ml blood. Inoculation was carried out at the Parasitology Laboratory of FK University of Brawijaya.

Parasitemia Examination:

Calculation of parasitemia degree in the treated mice was carried out for 6 days. Examination was obtained from the blood of mice tails which were thinly smeared on a glass object. Then stained using Giemsa. Parasite observations were counted for every 1000 healthy erythrocytes, then multiplied by 100%.

HIF-2α Examination in Brain Tissue:

The brain was isolated and stored in 10% formalin after being given treatment with the control group (on the 7th day). Gross tissue was cut to ±2-3 mm and blocked with

paraffin. Then deparaffinized with xylol and rehydrated with alcohol. The slides were blocked by endogenous peroxidase with methanol, PBS, and H2O2 30%. Slides were incubated with PBS, Triton X-100 0.1%, and BSA blocking buffer to remove specific antibody binding. Then, slides were treated with a primary antibody, namely anti-HIF-2α (BiossUSA: bs-1447R) in BSA blocking buffer. Slides were washed with PBS, incubated with DAB, stained with hematoxylin, and covered with mounting media. The slides were observed using a light microscope with a magnfication of 1000x. The expression of HIF-2α was calculated in the nucleus and cytoplasm. The HIF-2α expression is marked in blackish brown. Making slides was carried out at the Anatomical Pathology Laboratory of RSUD dr. Soetomo.

Research Ethics:

Ethical clearance in this study was submitted and approved by KEPK (Health Research Ethics Committee) Faculty of Medicine and Health Sciences State Islamic Universitas Islam Negeri Maulana Malik Ibrahim Malang with the following ethical clearance number: No.090/EC/KEPK-FKIK/2022.

Statistical Analysis:

Analysis used SPSS version 26. Data were tested for normality and homogeneity ($p>0.05$). Hypothesis testing using *one-way* ANOVA with post-hoc LSD ($p<0.05$). Correlation test between parasitemia and hypoxia using *Pearson's* correlation test.

RESULT:

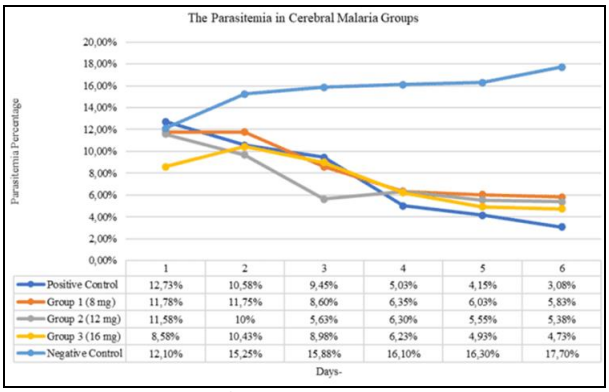
Table 1: Phytochemical Screening of Leaf Extracts of Azadirachta indica A. Juss

Phytochemicals	96% ethanol	Aqueous
Phenols	+	+
Flavonoids	+	+
Tannins	+	+
Saponins	+	+
Alkaloids	+	+
Terpenoids	+	+
Steroids	-	-

+, present; -, absent

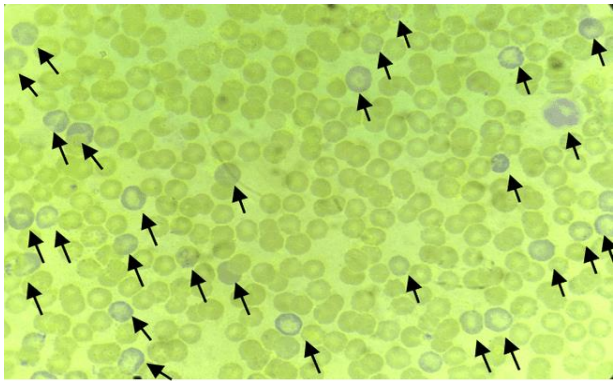
The Effect of Neem Leaf Extract on Cerebral Malaria Parasitemia:

The effect of the treatment was observed through a decrease in the degree of parasitemia from the difference between the last day and the first day. The greatest decrease in parasitemia was in treatment 2(6.20%) when compared to the positive control (9.65%). Treatments 1 and 3 decreased sequentially (5.95%) and (3.85%) (Figure 1). Meanwhile, the negative control experienced an increase in parasitemia (5.60%). Observation of cerebral malaria parasitaemia, it is characterized by the predominance of schizont formation (mature parasites). In addition, there are also trophozoite/ring forms and banana-shaped (Figure 2).

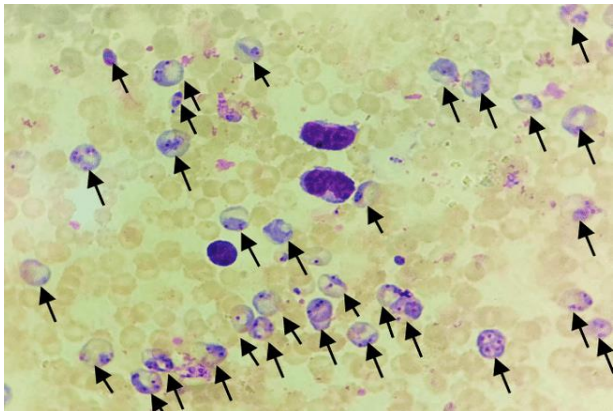


	1	2	3	4	5	6
Positive Control	12,73%	10,58%	9,45%	5,03%	4,15%	3,08%
Group 1 (8 mg)	11,78%	11,75%	8,60%	6,35%	6,03%	5,83%
Group 2 (12 mg)	11,58%	10%	5,63%	6,30%	5,55%	5,38%
Group 3 (16 mg)	8,58%	10,43%	8,98%	6,23%	4,93%	4,73%
Negative Control	12,10%	15,25%	15,88%	16,10%	16,30%	17,70%

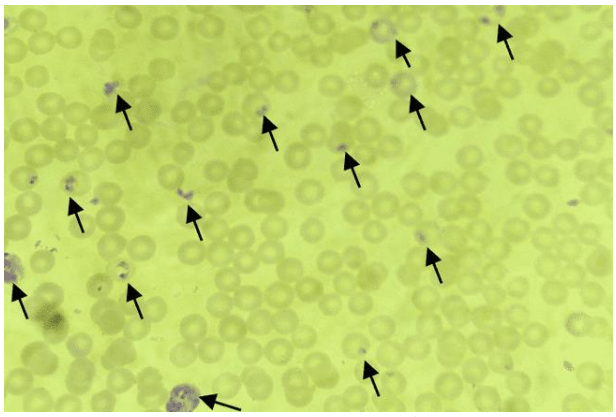
Figure 1. The decrease of parasitemia



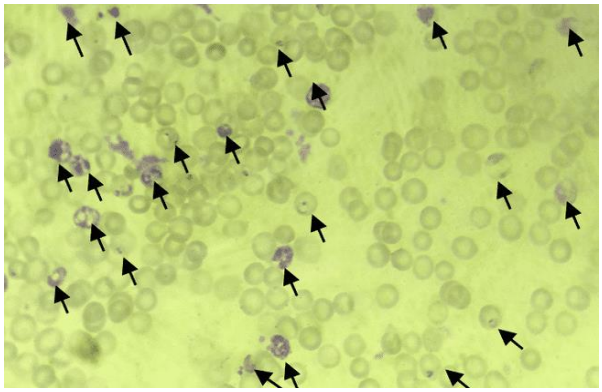
Positive Control (DHP)



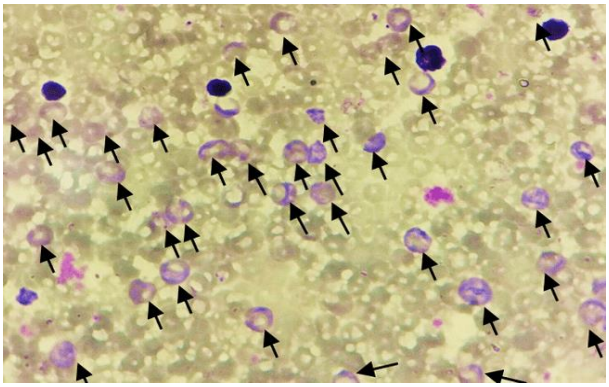
Treatment Group 1-Neem leaves extract dose of 8 mg



Treatment Group 2-Neem leaves extract dose of 12 mg



Treatment Group 3-Neem leaves extract dose of 16 mg



Negative Control

Figure 2: Observation of *Plasmodium berghei*-induced mice parasitemia. Black arrows indicate erythrocytes infected by parasite.

The normality and homogeneity tests on the variable degree of parasitemia have a significant value (p>0.05). These results meet the assumptions of the one-way ANOVA comparative test with a significance value (p<0.000). This value means that there is a significant difference between the administration of neem leaf extract and the control group towards parasitemia. Then in the LSD post-hoc test it was shown that there was no significant difference in treatment 3 (p=0.987), treatment 2 (p=0.823), and treatment 1 (p=0.050) (Table 2). Hence, these 3 groups had almost the same ability as the positive control, and treatment group 2 had a greater effect.

Table 2. The LSD post-hoc test results (parasitemia variable)

Control Group	Mean ± Standard Deviation	+	T1	T2	T3	-
+	7,3325 ± 0,45485	-	0.050	0.823	0.987	0.000*
T1	8,3150 ± 1,01930		-	0.077	0.052	0.000*
T2	7,4375 ± 0,41620			-	0.836	0.000*
T3	7,3400 ± 0,53148				-	0.000*
-	15,5525 ± 0,65708					-

Description: (*) indicates a significance value

The Effect of Neem Leaf Extract on HIF-2α Expression of Cerebral Malaria

Observation of HIF-2α expression in the brain of each treatment using 5 visual fields. Calculation of hypoxia for each field is summing hypoxic cells divided by hypoxic and healthy cells and then multiplied by 100%. Then averaged each treatment. The average is shown in Figure 3.

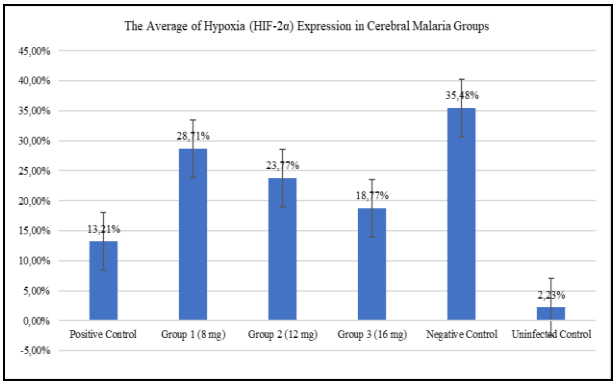
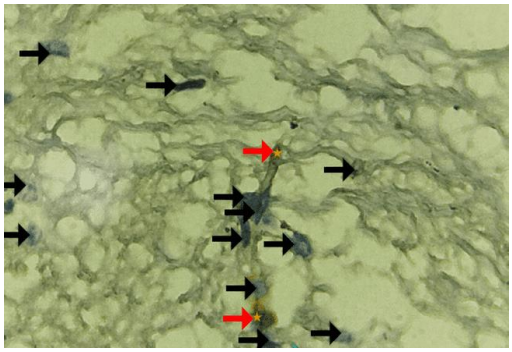
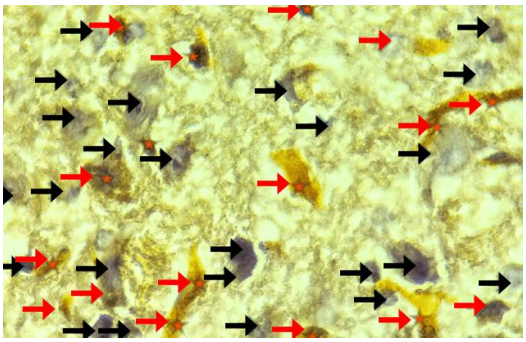


Figure 3: The average of hypoxia (HIF-2α) expression in cerebral malaria groups

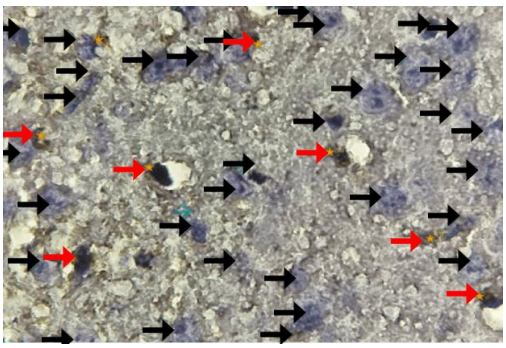
The HIF-2α expression is indicated by the color of the nucleus and cytoplasm turning blackish brown. The highest expression was in the negative control (35.48%). The treatment that can reduce the greatest hypoxia is treatment 3 (18.87%). While the most minimal expression occurred in healthy controls (2.28%) and positive controls (13.21%). The histopathological appearance of hypoxia in the brain is shown in Figure 4.



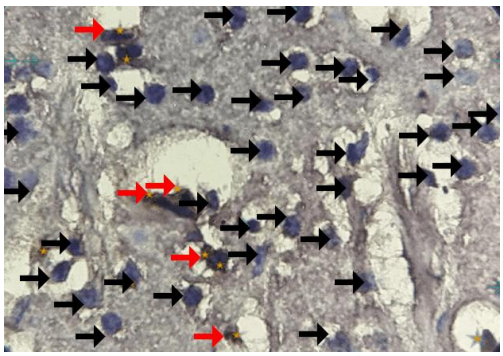
Positive Control (DHP)



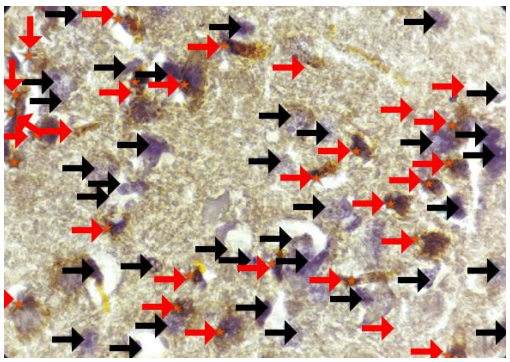
Treatment Group 1-Neem leaves extract dose of 8 mg



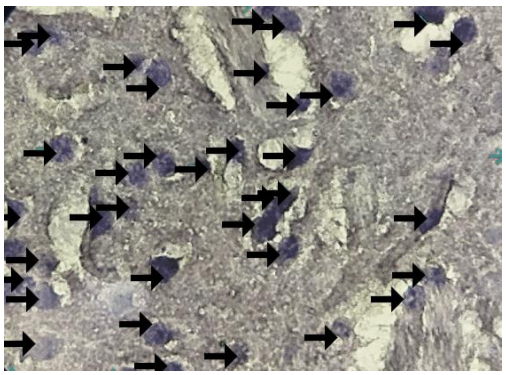
Treatment Group 2-Neem leaves extract dose of 12 mg



Treatment Group 3-Neem leaves extract dose of 16 mg



Negative Control



Uninfected Control

Figure 4: Observation of *Plasmodium berghei*-induced mice brain hypoxia. Black arrows indicate healthy neurons and red arrows indicate hypoxic neurons.

The hypoxia variable meets the assumptions for the normality test (p>0.05), but there is a diversity of data (p<0.05). The one-way ANOVA parametric test has significant results (p<0.000) or there is a significant difference. The results of the LSD follow-up test showed that the smallest significant difference to the positive control was treatment 3(p=0.110) (Table 3). Thus, treatment 3 is considered to have the same effectiveness as DHP in reducing hypoxic events in the brain.

Table 3: The LSD post-hoc test results (hypoxia variable)

Control Group	Mean ± Standard Deviation	+	T1	T2	T3	-
+	13,2100 ± 1,72770	-	0.000*	0.006*	0.110	0.000*
T1	28,7100 ± 0,23509		-	0.152	0.008*	0.056
T2	23,7725 ± 1,99154			-	0.146	0.003*
T3	18,7650 ± 8,99335				-	0.000*
-	35,9870 ± 8,92036					-
Description: (*) indicates a significance value						

Correlation of Parasitemia and Hypoxia in Cerebral Malaria:

There is a very strong correlation in a positive direction between the degree of parasitemia and hypoxia (r=+0.732) and (p=0.000) (Table 4). Thus, a high parasitemia correlates strongly with a high incidence of hypoxia.

Table 4: *Pearson's* correlation test results between parasitemia and hypoxia

Correlation Coefficient (rs)	*p-Value*	N
+0.732	0.000	20

DISCUSSION:

The presence of cerebral malaria is determined by severe or moderate type of thrombocytopenia and achieving a parasitemia level at 5-8%[20,21]. In this study, the treatment that was able to reduce the highest degree of parasitemia was the positive control (9.65%). Parasite elimination by administering DHP to positive controls was carried out at the asexual stage[22,23]. Whereas in the administration of neem leaf extract, treatment 2 had the greatest ability to reduce parasitemia (6.20%). This is supported by the ability of *Azadirachta indica* to inhibit schizont maturation in the asexual stage[24]. Maturation is an early sign of severe malaria[25]. The increase in parasitemia in malaria is associated with the release of approximately 20–24 merozoites from mature schizonts. Thus, at the vascular level the schizonts can sequestered in the brain microvascular[26,27].

In a previous study reported that both aqueous/ methanol/ ethanol extracts from all parts of the neem plant can inhibit malaria from several strains of *P. falciparum* and *P. berghei*[28]. The plant is believed to have antimalarial activity from a specific compound,

namely azadirachtin (terpenoid). Azadirachtin can inhibit the development of motile gametes of malaria parasites (*in vivo*)[24]. Other compounds such as alkaloids, flavonoids, tannins, and steroids are reported to have antiplasmodium activity[29]. The antimalarial activity of *Azadirachta indica* in this study was proven by a decrease in parasitemia at treatment 2 (neem leaf extract dose of 12 mg) of 6.20%. According to Akin-Osanaiya *et al.* (2013), there was a significant reduction in parasitemia (51-80%)[30]. The difference in reduction rate is due to differences in administration doses, experimental models/ animals, and treatment administration. Oral administration can reduce antimalarial ability because it passes through various enzymatic systems before being absorbed systemically[29,30].

High parasitemia in the systemic can cause parasite attachment/cytoadherence and the remaining infected erythrocytes in the endothelium/sequestration[31]. Sequestration is mediated by the release of chemokines from the cytoadherence process, resulting in inflammation by CD8+ T cells, TGF-β, and IFN-γ[5,31,32]. The occurrence of sequestration is often found in the cerebral cortex rather than in the brainstem and midbrain because it has a high level of vascularization[33]. These anatomical conditions can support leukocytes to infiltrate and platelets to aggregate in brain endothelial cell[31]. The complex pathomechanisms described above can cause disruption of the blood-brain barrier (BBB), cerebral edema, and obstruction in the microvascular brain, resulting in decreased blood flow and hypoxia[5,31,34]. This condition provides statistical support in the form of high parasitemia, which strongly correlates with hypoxic events.

When cells are exposed to hypoxia, they adapt by expressing HIF-1 and HIF-2 proteins[35,36]. However, HIF-1 protein was not found in cerebral malaria post-mortem findings[37]. This is due to the fact that HIF-2 has a long half-life (chronic hypoxia) and predominates in the transcription process as a coregulator during hypoxia[37,38]. Hypoxia Inducible Factor-2 is a brown-black protein found in the nucleus and cytoplasm of rat brain endothelial cells[12,37,39,40]. In this study, the negative control had the highest HIF-2 expression (35.84%), followed by treatment 1 (28.71%). Meanwhile, treatment group 3 experienced the least hypoxia compared to other treatments (18.37%). Low levels of hypoxia may be due to the neuroprotective ability of *Azadirachta indica*. This ability is known to come from compounds (flavonoids) contained in these plants. Quercetin can suppress the expression of intracellular adhesion molecule (ICAM-1), white blood cell adhesion, and the severity of chronic hypoxia through the production of superoxide dismutase (SOD)[41]. This antioxidant mechanism can be efficient because of its ability to quench the hydroxyl radicals created in the reaction mixture[42]. Quercetin also have abilities to inibits hypoxia by maintaining vascular endothelial function and promoting angiogenesis[43]. This is consistent with the post-hoc test results, which showed that treatment group 3 (neem leaf extract dose of 16 mg) was not significantly different from the positive control (p=0.110). Furthermore, *Azadirachta indica* aqueous extract can reduce nervous symptoms due to central nervous system (CNS) depressant activity in the form of analgesic and sedative-hypnotic effects[44].

CONCLUSION:

There is antimalarial activity in neem leaf extract, indicated by a decrease in parasitemia and brain hypoxia in cerebral malaria.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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