RESEARCH ARTICLE

THE IMPACT OF NEEM LEAF EXTRACT (*AZADIRACHTA INDICA*) ON RENAL HYPOXIA BALB/C MICE INFECTED WITH *PLASMODIUM BERGHEI*

Zainabur Rahmah[1]*, Tafakur Bagus Religious[2], Putri Wulan Akbar[3], Erryk Kosbandhono[4], Muhammad Amiruddin[5], Ach Nashichuddin[6]

[1]Department of Parasitology, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim, Malang, Indonesia.
[2]Bachelor of Medicine, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim, Malang, Indonesia.
[3]Department of Biochemistry and Clinical Pathology, Faculty of Medicine, UIN Maulana Malik Ibrahim, Malang, Indonesia.
[4]Faculty of Syariah, UIN Maulana Malik Ibrahim, Malang, Indonesia.
[5]Department of Pharmacy, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim, Malang, Indonesia.
[6]Faculty of Science and Technology, UIN Maulana Malik Ibrahim, Malang, Indonesia.

*Corresponding Author : zainabur.rahmah@kedokteran.uin-malang.ac.id

ABSTRACT

Background: Millions of people die from malaria every year in tropical areas, making it a severe public health concern. *Plasmodium*, a genus of protozoa with several species, is the main cause of the disease. Humans contract malaria when bitten by female *Anopheles sp.* mosquitoes. Malaria is one of the world's deadliest diseases. The WHO's 2020 World Malaria Report indicates that there were 229 million cases of malaria globally in 2019, with 409,000 fatalities resulting from the disease. In nine Southeast Asian countries, malaria is endemic. After India, Indonesia is the second most endemic region. Transcription factors such as the HIF-1α protein are produced by hypoxic cells. Patients with malaria have a blockage of the cytoadherence pathway. Hypoxia occurs due to vascular disorders that prevent oxygen flow to the kidney tissue. Antimalarial drugs are developed in response to the lethal consequences of hypoxia.

Methods: BALB/c mice aged sixteen weeks were infected with the *Plasmodium berghei* ANKA strain. For six days, the neem leaf 96% ethanol extract was administered orally at doses of 8 mg, 12 mg, and 16 mg. As comparisons, negative, positive, and healthy controls were also included. The kidneys were isolated, and the mice were surgically removed on the seventh day. HIF-2α expression in kidney tissue, which was measured immunohistochemically using Abcam's anti-HIF-2α (H1α67) ChIP Grade antibody, was a characteristic of kidney hypoxia. One-way ANOVA, post-hoc LSD, and Pearson correlation tests were used to assess the hypotheses.

Results: Treatment with neem leaf extract significantly decreased oxygen levels ($p<0.000$). The correlation test showed a very strong relationship between neem leaf extract and HIF-2α expression in the kidney ($r=16.057$).

Conclusion: Twelve milligrams of neem leaf extract can reduce the risk of renal hypoxia.

Keywords: Neem leaves, malaria, degree of parasitemia, hypoxia, *Azadirachta indica.*

INTRODUCTION

Plasmodium sp, the parasite that causes malaria, is spread by the female *Anopheles sp* vector. In 2021, there were 227 million malaria cases registered worldwide.[1] The eastern part of Indonesia, specifically the provinces of Papua, West Papua, East Nusa Tenggara, and North Penajem Paser Regency, has the highest endemic rate.[2] With 86.022 cases overall, Papua is among the provinces with the greatest number of malaria cases in the nation, and this pattern is still present today. A parasite genus called *Plasmodium falciparum* is capable of causing malaria, which can be lethal. Nephrotic syndromes, anemia, and cerebral malaria are among the severe side effects of malaria.

Immune-mediated glomerular lesions, fluid loss with many pathogenetic processes, and obstruction of the renal microcirculation as a result of sequestration of infected erythrocytes are the causes of severe renal malaria.[3] The three main pathogenic mechanisms of *Plasmodium falciparum* are agglutination, rosetting, and cytoadherence.

Tissue hypoxia and microvascular blockage are the results of these processes.[4]

A transcriptional component that is active in hypoxic environments is hypoxia inducible factor-alpha (HIF-α). It is possible for proximal tubular epithelial cells to react in this way to hypoxia. It is well known that HIF-1α transfection causes differentiation development linked to alterations such as growth arrest and elevated CD11b expression. Furthermore, glomerular and tubular cells may change morphologically as a result of HIF-1α 1α mRNA overexpression in renal tissue. Additionally, this hypoxic state may increase blood vessel permeability, leading to microvascular dysfunction. The pathophysiology of severe malaria with acute renal failure brought on by ischemic circumstances includes microvascular dysfunction.[5]

Plasmodium, a parasite belonging to the phylum Apicomplexa, is the cause of malaria. Only five *Plasmodium* species, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *and Plasmodium knowlesi*.[3] The malaria parasite *Plasmodium berghei* affects mammals besides humans. In order to study malaria in mice, *Plasmodium berghei* is used as an experimental model. In key areas like anatomy, physiology, and life cycle, it has been demonstrated that *Plasmodium berghei* infection in mice results in an infection that is comparable to malaria in people and primates.[6]

Blockage of the cytoaderenal pathway is the cause of this death. The binding of ligands and receptors to the surface of infected erythrocytes is known as the cytoadrenal process. Hypoxia, a disorder that prevents blood vessels from delivering oxygen to the tissues, is caused by rosetting cytoadherence and sequestration in the microvasculature.[7,8]

Hypoxia causes the release of inflammatory mediators and an increase in the expression of mediators that regulate homeostasis. Hypoxia can be induced by hypoxia-inducible factor 1α, also known as HIF-1α.[6] Acute renal failure, which is defined by a quick reduction in kidney function, is one of the outcomes of improperly treating hypoxia. In cerebral malaria, antimalarial medications with antihypoxic actions have not been investigated. Furthermore, resistance to certain antimalarial medications has been noted.[7,8] The HIF-1 and HIF-2 proteins are overexpressed by cells in response to hypoxia. On the other hand, post-mortem malaria findings lack the HIF-1 protein. This is because, as a coregulator during hypoxia, HIF-2 dominates transcription and has a lengthy half-life (chronic hypoxia). HIF-1α remains stable in hypoxic environments, as it is not degraded. Additionally, HIF-2α expression serves as another indicator of hypoxia. According to studies, HIF-1α is primarily active during the first two to twenty-four hours of hypoxic/anoxic triggers (<0.1% O_2), whereas HIF-2α is still active after two to three days of physiological hypoxia (<5% O_2). This suggests that, in some situations, HIF-1α contributes to the early stages of hypoxia, while HIF-2α controls the response to chronic hypoxia.[9]

The neem plant contains active compounds that may have beneficial medical effects. All parts of the neem plant, including the leaves, stems, and seeds, contain bioactive compounds. It has been demonstrated that neem leaf extract works well against malaria parasites.[10,11] It is crucial to use natural resources like neem (*Azadirachta indica*) to create medications.[12] In addition to being employed as a mosquito repellent, this plant's leaves, oil, fruit, seeds, bark, and roots have all been utilized in traditional medicine.[12-13] The neem plant has also been reported to have antimalarial and neuroprotective activities.[14,15] Azadirachtin, quercetin, gedunin, and other phytochemicals are important in neem plants.[16] However, the specific mechanisms of these properties in the plant remain unknown. Therefore, this study was conducted to determine the effect of neem leaf extract in preventing hypoxia in malaria-infected kidneys.

METHODS

Research Animal

The experimental animals used were BALB/c mice aged 13-16 weeks with a body weight between 20 and 30 grams. These mice are routinely used as a model of renal malaria because they have clear symptoms and pathological presentations. The number of mice was 4 per 6 treatment groups. The groups consisted of a positive control with Dihydroartemisinin+Piperaquine (DHP) 0.02496 mg, a negative control, treatment 1 (neem leaf extract dose 8 mg), treatment 2 (neem leaf extract dose 12 mg), treatment 3 (neem leaf extract dose 16 mg), and a healthy control (not infected and given treatment). The acclimatization and treatment processes were carried out in the Experimental Animal Laboratory of the Faculty of Medicine and Health Sciences, UIN Malang.

Neem Leaf (*Azadirachta indica*) Extract Preparation

The neem leaves were dried before being macerated for 48 hours with water and 96% ethanol to do the extraction. A concentrated extract is created by filtering the sample and then evaporating the filtrate. A 0.5% CMC dilution is used to stock the concentrated extract. The number of animals, treatment duration, and preparation concentration all affected the calculations. The medication was administered orally. Materia Medica Batu produces an extract from neem leaves.

Plasmodium berghei Strain ANKA Inoculation

Plasmodium berghei strain ANKA was injected intraperitoneally into donor mice at a dose of $1x10^6$/ml liquid nitrogen. The parasitemia was then determined using the Giemsa-stained blood smears. It is prepared for donation to

DOI: 10.15408/ tjems.v12i1.44758

treated mice with a parasite count of $1x10^6$/ml blood if the parasitemia has decreased to 5-8%. The infection was carried out at the Parasitology Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia.

The Amount of HIF-2α Expression in Hypoxic Kidney Cells

HIF-2α analysis was carried out at Brawijaya University Faculty of Medicine Anatomical Pathology Laboratory utilizing the immunohistochemistry method using anti-HIF2 antibody Polyclonal (Biossusa). In order to prepare the slides for immunohistochemical staining, deparaffinization was done using xylol twice for ten minutes, 100% ethanol once for five minutes, 90% ethanol once for five minutes, 80% ethanol once for five minutes, 70% ethanol once for five minutes, and sterile distilled water three times for five minutes. After drying and dropping the slides with 3% H_2O_2 in methanol and incubating for 15 to 20 minutes, they were again washed with sterile PBS three times for five minutes. Antigen retrieval (AR) was performed using Heat Receptor Epitope Retrieval (HIER) by heating the samples in a citrate buffer with a pH of 6.0 for 20 minutes at 95 degrees Celsius in a water bath. The slides were handled gently throughout the process. Dripping 0.25% Triton-X100 in BSA blocking buffer for an hour at room temperature and then washing with sterile PBS three times for five minutes was how non-specific protein blocking was accomplished. The slide was then incubated at 40^C throughout the entire night after being dripped with primary antibody (primary antibody: 5% FBS = 1:100) in blocking buffer BSA. The slide was cleaned with sterile PBS for three by five minutes the next day. After 60 minutes of room temperature incubation with the secondary antibody anti-rabbit anti-mouse IgG, three 5-minute washes were performed using sterile PBS. Subsequently, the slide was dripped with SAHRP (SAHRP in sterile PBS 1:500), allowed to sit at room temperature for 40 minutes, and then rinsed with sterile PBS three times and sterile aquadest three times. DAB chromogen (1:50) was then added, allowed to sit at room temperature for 30 minutes, and then rinsed with sterile PBS three times. Mayer's hematoxylin was used to counterstain the last slide, which was then allowed to sit at room temperature for five to ten minutes before being rinsed three times with sterile tap water. HIF-2α expression in the kidneys of each treatment was monitored using six fields of view. To calculate hypoxic cells in each field of view, the total number of hypoxic cells was divided by the total number of hypoxic cells and healthy cells. The result was then multiplied by 100%, and then averaged out. HIF-2α expression is characterized by a blackish-brown color. The slides were observed using a light microscope at 1000x magnification.

Statistical Analysis

Version 26 of SPSS was used to examine statistical information. The data underwent tests for homogeneity and normality ($p<0.05$), utilizing Mann-Whitney, Shapiro-Wilk, and Kruskal-Wallis H hypothesis testing.

ETHICAL APPROVAL

This study was approved by the Health Research Ethics Committee (KEPK) of the Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang (Ethical Clearance No. 64/EC/KEPK-FKIK/12/2023).

RESULTS

Benefits of Neem Leaf Extract on Hypoxic HIF-2α Expression

HIF-2α expression in the kidneys of each treatment group was monitored using six fields of view. To calculate the number of hypoxic cells in each field, the total number of hypoxic cells was divided by the total number of hypoxic and healthy cells. The result was then multiplied by 100%. Then determined the average for each treatment. The average is shown in Figure 1.

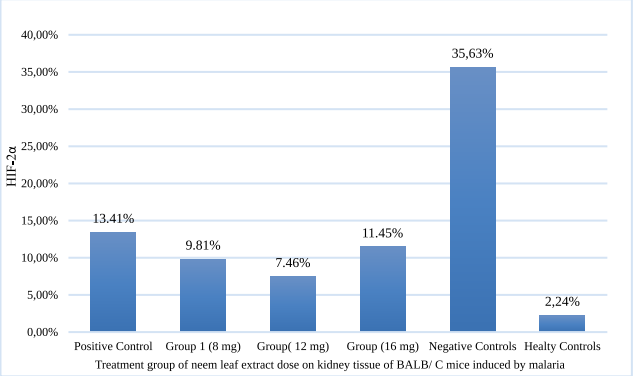

Figure 1. Results of the percentage of HIF-2α expression in malaria-induced kidney tissue.
The percentage ratio of HIF-2α expression in neem leaf extract doses in kidney tissue was significantly lower compared to positive and negative controls and higher than healthy controls (*$p<0.05$; Mann-Whitney's).

The kidney's color changes to a blackish brown when HIF-2α is expressed. The negative control had the highest expression (35.63%). Neem 12 mg (7.46%) is the medication that can most effectively lower hypoxia. Conversely, healthy controls exhibited the least amount of expression (2.24%). The histological appearance of kidney hypoxia is shown in Figure 2.

DOI: 10.15408/ tjems.v12i1.44758

Print ISSN: 2716-3628, Online ISSN: 2720-9563

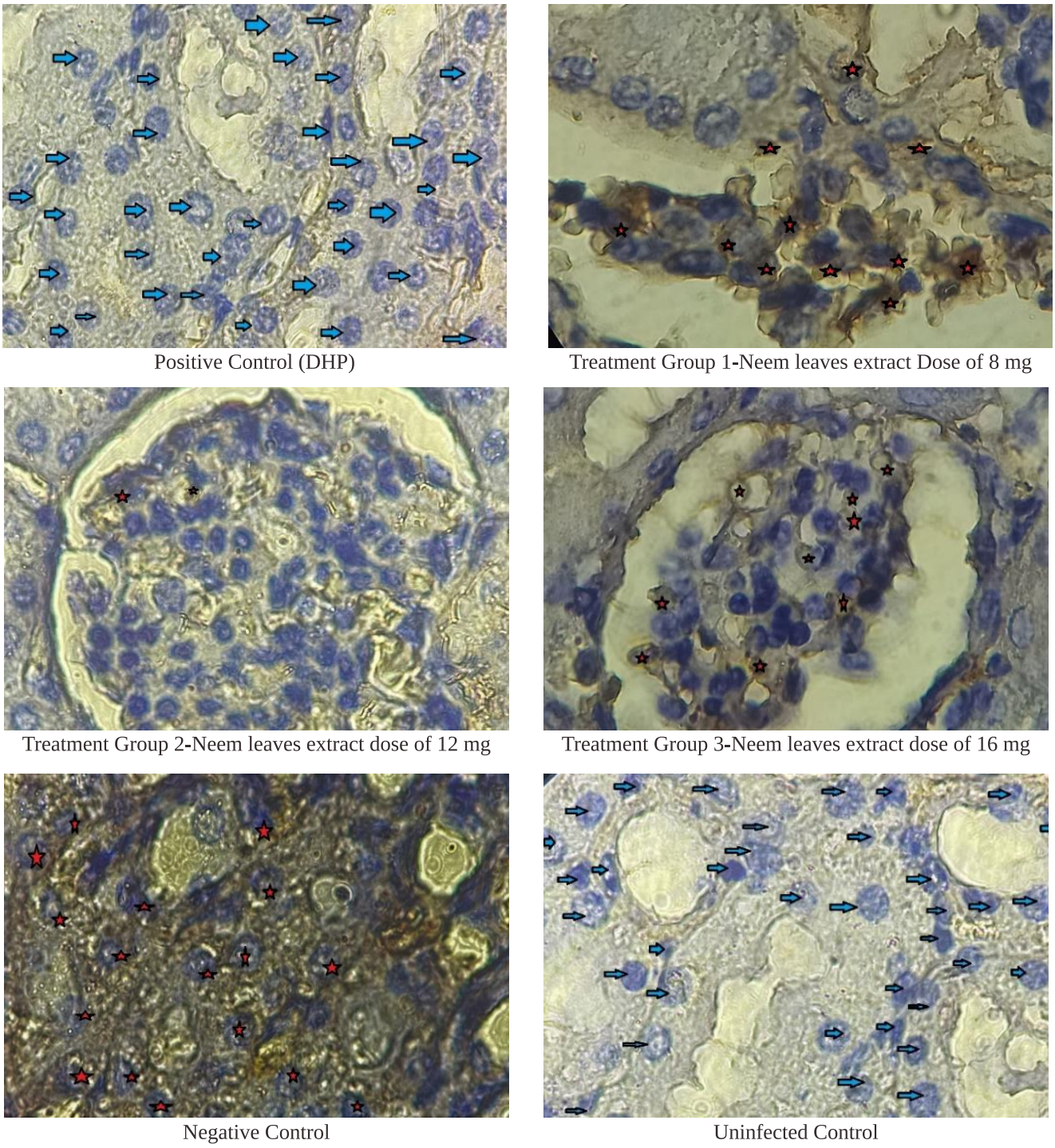

Figure 2. Observation of *Plasmodium berghei*-induced renal hypoxia in mice. Black arrows indicate healthy renal proximal tubule cells, and red arrows indicate hypoxic renal proximal tubule cells.

The hypoxia variable satisfies the premise of the normalcy test ($p>0.05$), despite data variety ($p<0.05$). A significant difference or result ($p<0.000$) is found using the Non-Parametric Kruskal-Wallis H test. Results of the Kruskal-Wallis Non-Parametric Test between the Administration of Neem Leaf Extract and Hypoxia can be seen in Table 1.

Table 1. Kruskal-Wallis Non-Parametric Test between Administration of Neem Leaf Extract and Hypoxia

Variables	Kruskal-Wallis	Asymp. Sig.
Hypoxia	16.057	0.007

DOI: 10.15408/ tjems.v12i1.44758

The Mann-Whitney test findings show that neem 12 mg has the least significant difference from the positive control (p=0.021) (Table 1). As a result, neem 12 mg is thought to be just as efficient as Dihydroartemisinin+ Piperaquine (DHP) at lowering renal hypoxic episodes.

Table 2. Results of HIF-2α Expression Percentage in Kidney Tissue Induced by Malaria.

Treatment Group	Mean±Standard Deviation	T+	Neem 8 mg	Neem 12 mg	Neem 16 mg	T-	Healthy control
Positive control	13.412±6.832	-	0.086	0.149	1	0.021*	0.057
Neem 8 mg	9.812±4.758	0.086	-	0.386	0.686	0.021*	0.081
Neem 12 mg	7.462±2.962	0.149	0.386	-	0.083	0.021*	0.081
Neem 16 mg	11.445±1.329	1	0.686	0.083	-	0.021	0.020*
Negative control	35.630±10.062	0.021*	0.021*	0.021*	0.021	-	0.020*
Healthy control	2.235±3.772	0.057	0.081	0.081	0.020*	0.020*	-

The ratio of the percentage of HIF-2α expression in the administration of neem leaf extract doses, in kidney tissue, compared to control (*p<0.05/significant; Mann-Whitney's).

DISCUSSION

The active ingredient in neem leaves, azadirachtin, can prevent excessive inflammation and ROS, preventing tissue damage, lowering hypoxia, and lowering HIF-2α expression. Hypoxia in the kidneys can result from an imbalance of vasoactive chemicals, chronic ischemia, anemia, and abnormalities of the peritubular capillaries. Cells with this condition generate more reactive oxygen species (ROS). A sustained increase in ROS can damage macromolecules such as lipids, DNA, and RNA, leading to changes in metabolism and medical disorders.[16] Hypoxia can also activate fibrolase and change how the extracellular matrix of kidney cells is metabolized. A fibrogenic consequence of hypoxia in peritubular capillaries is obliteration. In renal tubular cells, prolonged exposure to severe hypoxia can result in mitochondrial malfunction, which either induces death or causes a persistent energy deficit.[17-18]

A previous study has found that ethanol, methanol, and aqueous extracts from all parts of the neem plant can prevent malaria caused by several strains of *Plasmodium falciparum* and *Plasmodium berghei*.[19] Azadirachtin, a terpenoid present in the neem plant, is believed to possess antimalarial properties. It inhibits the development of motile gametes from malaria parasites in vivo.[20] Additionally, alkaloids, flavonoids, tannins, and steroids have all demonstrated anti-plasmodium activity.[21] The study observed a 7.46% decrease in parasitemia after treatment with a 12 mg dose of neem leaf extract, indicating the antimalarial efficacy of *Azadirachta indica*.

High systemic parasitemia can lead to parasite attachment/cytoadherence and sequestration of the remaining infected erythrocytes in the endothelium. Because *Plasmodium falciparum* obstructs the vasculature of internal organs, making malaria infection more difficult, it can induce hypoxia. The processes in this blocking process are cytoadherens, sequestration, and rosetting. The third mechanism of malaria infection is hypoxia, which is caused by reduced blood flow.[22]

The body's physiological reaction to a malaria infection is the production of HIF-1α (Hypoxia-Inducible Factor-1α). A protein called HIF-1α is produced in tissues when there is hypoxia, or a shortage of oxygen.[23] Damage to blood arteries and elevated blood pressure can result in hypoxia in malaria infections by decreasing blood supply to the kidneys. The body's defensive mechanisms are activated, and hypoxia is overcome when HIF-1α is formed in kidney tissue.[24] HIF-1α is crucial for controlling how the body reacts to hypoxia and preventing damage to kidney tissue. But too much HIF-1α production might exacerbate the patient's illness and lead to major side effects like kidney failure and nephropathy.[25-26]

HIF-2α regulates cellular adaptation to hypoxia, involved in several biological processes such as angiogenesis, cell survival/proliferation, energy metabolism, erythropoiesis, extracellular matrix function, invasion/metastasis, iron metabolism, pH regulation, multidrug resistance, and stem cell properties. HIF-2α expression is regulated through hypoxia-dependent protein stabilization with the help of PHD (prolyl hydroxylase-enzyme domain) proteins that degrade HIF-2 via pVHL (von Hippel-Lindau protein). PHD activity is inhibited under hypoxic conditions, leading to HIF-2α accumulation in cells, followed by its nuclear translocation. Hypoxia-responsive genes are activated when nuclear HIF-2α and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear) form a dimeric complex

DOI: 10.15408/ tjems.v12i1.44758

that constitutively binds to HIF-1α hypoxia response element/HRE. It is now known that HIF-2α and HIF-1α have both comparable and different functions, particularly in malignancies, while sharing 48% of their amino acid sequences. Research has indicated that whereas HIF-2α is active even after two to three days of physiological hypoxia (<5% O_2), HIF-1α (hypoxia inducible factor-1α) is primarily active during two to twenty-four hours of hypoxic/anoxic stimuli (<0.1% O_2). Indicating that in some situations, HIF-2 controls the response to chronic hypoxia, whereas HIF-1 takes a role in the early phases of hypoxia.[9]

The study's negative control had the highest HIF-2α expression (35.63%), while group 3 displayed a figure of 11.45% after receiving excessive doses of neem leaf extract, which can impair hepatocyte and respiratory function by reducing cell size and number, which can raise the risk of hypoxia. Compared to the other groups, group 2 had the lowest expression of HIF-2α (7.46%).

This number is explained by the component *Azadirachta indica's* antimalarial properties. Among these is the substance azadirachtin, which is known to have antimalarial properties, namely lowering the level of parasitemia in *Plasmodium berghei*-infected mice (reduction varies from 51-80%). Low hypoxia levels could be caused by the *Azadirachta indica* neuroprotective properties. Flavonoids, which are chemicals found in various plants, are known to provide this ability.[27]

CONCLUSION

Neem leaf extract can reduce hypoxia by reducing the percentage of HIF-2α in kidney tissue in BALB/C mice with malaria induction. A dose of 12 mg neem is effective in reducing the percentage of HIF-2α to close to healthy controls.

IMPLICATION

The findings of this study highlight the potential of neem leaf extract (*Azadirachta indica*) as a complementary therapy in malaria management, particularly in addressing renal complications such as hypoxia. The observed reduction in HIF-2α expression at an optimal dose of 12 mg suggests that neem extract not only has antimalarial properties but also provides renal protection by mitigating tissue hypoxia. This is especially relevant considering that renal dysfunction is a frequent and severe complication of Plasmodium falciparum infection.

STRENGTHS AND LIMITATIONS

This study utilized HIF-2α expression, which remains elevated in hypoxic tissue for two to three days, as a reliable marker. However, the trial was limited to a single treatment using neem leaves; future research should explore the potential benefits of combination therapies.

CONFLICT OF INTEREST

Regarding this inquiry, the writers have no conflicts of interest.

ACKNOWLEDGMENTS

We are grateful for the research support provided to Bantuan Operasional perguruan Tinggi Negeri (BOPTN) in 2022 by the Republic of Indonesia's Ministry of Religion via UIN Maulana Malik Ibrahim Malang. Thank you for your assistance with this research, Imam Subandi, S.Si.

DECLARATION OF USING AI

The authors affirm that artificial intelligence (AI) tools were used to assist in the writing process solely for language enhancement purposes, such as grammar checking, paraphrasing, and improving clarity. No AI tools were employed to generate original content, conduct data analysis, or interpret research findings. The authors take full responsibility for the content, interpretations, and conclusions presented in this manuscript.

FUNDING SOURCES

This research was supported by BOPTN 2022 funding from the Ministry of Religious Affairs of the Republic of Indonesia through Maulana Malik Ibrahim State Islamic University Malang.

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DOI: 10.15408/ tjems.v12i1.44758

Print ISSN: 2716-3628, Online ISSN: 2720-9563

DOI: 10.15408/ tjems.v12i1.44758