

(RESEARCH ARTICLE)



Effect of neem leaf therapy (*Azadirachta indica*) on TNF- α expression and hepatocyte apoptosis of balb/c mice infected with *Plasmodium berghei* anka

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Abstract

Introduction: Malaria infection induces the release of pro-inflammatory cytokines. High concentrations of TNF- α are a bad sign of malaria. Additionally, TNF- α can induce apoptosis. Currently, there is resistance to malaria drugs so it is necessary to look for other alternative therapies. This study aims to determine the effect of *Azadirachta indica* on the expression of TNF- α and apoptosis of mice infected with *Plasmodium berghei*.

Methods: thirty BALB/C mice aged 13-16 weeks were inoculated with *Plasmodium berghei*. The treatment used was 96% ethanol extract of *Azadirachta indica* leaves at a dose of 4 mg, 8 mg, and 12 mg orally for six days. As a comparison of treatments, there is a negative control and a positive control. Liver tissue was isolated on the seventh day, and TNF- α expression and apoptosis were examined using immunohistochemistry. The hypothesis was tested using one-way ANOVA with the Least Significant Difference (LSD) and Pearson's correlation test.

Results: The ANOVA test showed that there were differences in TNF- α expression and apoptosis in each treatment (P=0.000). The results of the LSD test on TNF- α expression and apoptosis showed significant differences in the five groups. The results of the Pearson correlation test showed that there was a relationship between the expression of TNF- α and the expression of apoptosis (P = +0,893) in the hepatocyte cells of BALB/C mice.

Conclusion: *Azadirachta indica* leaf extract can reduce TNF- α expression and hepatocyte cell apoptosis in mice infected compared to the negative control group without therapy

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Keywords: Malaria; TNF- α ; Apoptosis; *Azadirachta indica*

1. Introduction

Malaria is a deadly disease of approximately 241 million cases and around 627,000 deaths occur [1]. In Southeast Asia, Indonesia is an endemic region that ranks second after India [2]. The symptom of malaria infection is the replication of the *Plasmodium* parasite in erythrocytes and the rupture of infected erythrocytes [3]. *Plasmodium falciparum* infection is very dangerous because if it is not treated it can cause severe disease and even death [4]. This death is caused by occlusion that occurs due to the cytoadherence process in vital organs. The cytoadherence process is the attachment of ligands and receptors to the surface of infected erythrocytes [5]. Ligands and receptors cause mature stage erythrocytes to stimulate Th-1 cells to produce IFN- γ which triggers monocytes to produce TNF- α [6]. High concentrations of TNF- α cause inflammatory and coagulation reactions which can cause occlusion in malaria [7]. High concentrations of TNF- α are a bad sign of malaria cases [8].

After the *Anopheles* mosquito bites a human, the sporozoites enter the bloodstream and reach the liver cells. In the liver cells, sporozoites turn into merozoites which can attack liver cells directly. Infected liver cells will burst and enter the circulation to attack erythrocytes [9,10]. Infected erythrocytes will rupture and release toxins such as GPI (Glycosylphosphatidylinositol), hemozoin, uric acid, and parasite DNA [11]. This toxin can stimulate the TNF- α which triggers the formation of reactive oxygen species (ROS). This can cause high oxidative stress [12]. Oxidative stress triggers apoptosis (programmed cell death) by increasing Bax and decreasing Bcl [13].

Currently, traditional medicine can be a treatment option for treating malaria. This is because there is an increase in drug resistance and it is difficult for residents of remote areas to access antimalarial drugs. One of the traditional antimalarial medicines that can be found in abundance in Indonesia is *Azadirachta indica* leaves, the compounds contained therein include quercetin, alkaloids, flavonoids, triterpenoids, and the most biologically active compound is Azadirachtin [14]. Therefore, this research was conducted to determine the effect of neem leaf extract in preventing TNF- α expression and apoptosis in hepatocyte cells of mice infected with malaria.

2. Material and methods

2.1. Preparation of experimental animals

This purely experimental research was carried out in vivo using a posttest control group design. The experimental animals used were BALB/C mice aged 13-16 weeks with a body weight of between 20-30 grams get it from Universitas Islam Negeri Maulana Malik Ibrahim Malang. The number of mice was 6 per 5 treatment groups. The groups consisted of negative control (no treatment), positive control (Dihydroartemisinin + Piperaquine (DHP) 0.02496 mg), treatment 1 (*Azadirachta indica* leaf extract dose 4 mg), treatment 2 (*Azadirachta indica* leaf extract dose 8 mg), treatment 3 (*Azadirachta indica* leaf extract dose 12 mg). In this study, therapy was carried out for 7 days. The acclimatization and treatment process was carried out at the experimental animal laboratory, Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang.

2.2. Collection and extraction

Fresh insect-free *Azadirachta indica* leaves were collected from Tanjung East Java, Indonesia. Leaf identification is carried out by taxonomist Materia Medica Batu number: 000.9.3/2779/102.20/2023. Extraction of *Azadirachta indica* leaves was carried out at Materia Medica. A total of 100 grams of neem leaf powder was extracted using the maceration method using 96% ethanol solvent.

2.3. Experimental animals and parasite inoculation

The *Plasmodium berghei* strain ANKA used for this research was obtained from the Parasitology Laboratory, Faculty of Medicine, Universitas Brawijaya, Indonesia. Inoculation was carried out by intraperitoneal injection of 1×10^6 /ml *Plasmodium berghei* strain ANKA per mL of blood.

2.4. Examination and measurement of TNF- α and apoptosis in the hepatocyte cells

TNF- α was examined using the Bioss USA AG04212645 kit and the amount of apoptosis using the Santa Cruz, Inc Caspase 3 CPP324-1-18:sc-56052 kit with immunohistochemical staining at the Anatomical Pathology Laboratory, Brawijaya University, Indonesia. The hepatocyte cells slides were then examined by two independent examiners using a light microscope (1,000x). The expression of TNF- α and the amount of apoptosis were calculated based on the amount

of TNF- α expression and the amount of apoptosis in the cell nuclei and extracellular areas of liver tissue from BALB/C mice.

2.5. Data 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 TNF- α and apoptosis using *Pearson's* correlation test.

2.6. Ethical considerations

Ethical permission was granted by the research committee of the Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang (No.051/EC/ KEPK-FKIK/2022).

3. Results

3.1. Phytochemical screening

Phytochemical screening of *Azadirachta indica* leaf extract confirmed that it contains phenols, flavonoids, tannins, saponins, and alkaloids in 96% ethanol and air solvents but does not contain steroids, is seen in Table 1.

Table 1 Phytochemical screening of leaf extracts of *Azadirachta indica*

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

+, present; -, absent

3.2. Effect of *Azadirachta indica* Leaf extract on TNF- α expression in hepatocyte cells

Observation of TNF- α expression in hepatocyte cells for each treatment using 5 fields of view. Calculation of TNF- α expression for each field is by adding up the cells that express TNF- α in the TNF- α expression section and healthy cells then multiplying by 100%. Then each treatment is averaged. The average is shown in Figure 1.

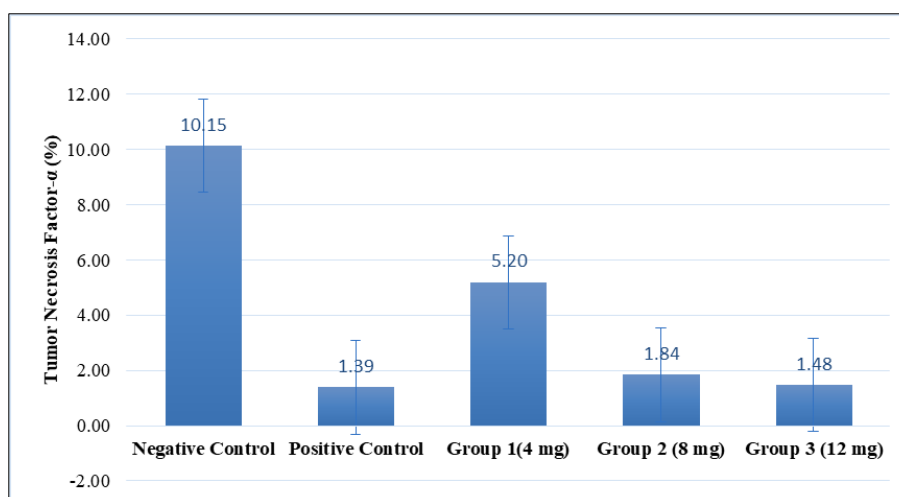
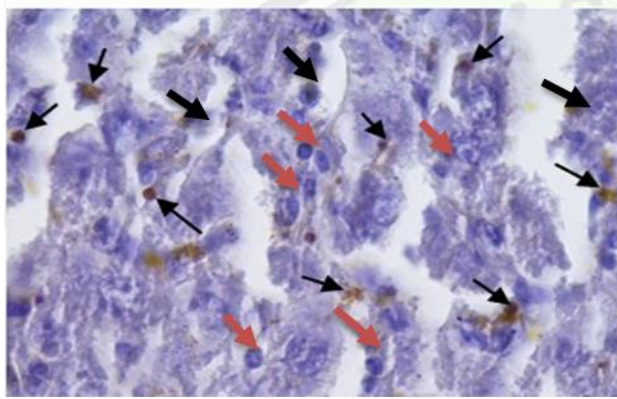
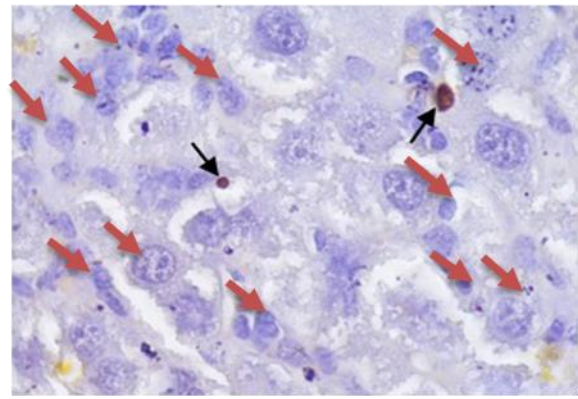


Figure 1 Graph of the average Tumor Necrosis Factor- α (TNF- α)

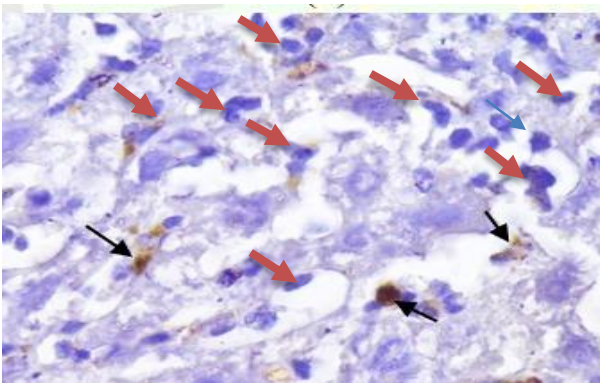
TNF- α expression is shown by the color of the nucleus and cytoplasm changing to blackishbrown. The highest expression was in the negative control (10.15%). The treatment that was able to reduce TNF- α expression the most was treatment 3 (1.48%). Meanwhile, the minimum expression occurred in the positive control (1.39%). The histopathological picture of liver tissue TNF- α expression is shown in Figure 2.



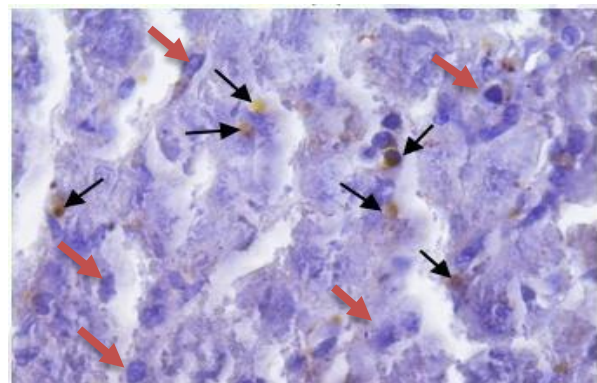
1. Negative Control



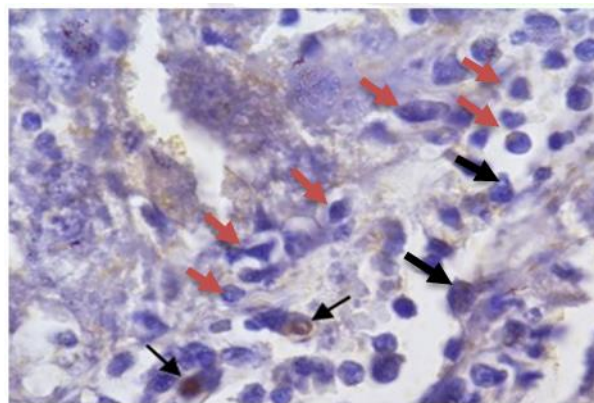
2. Positive Control



3. Group 1 (4 mg)



4. Group 2 (8 mg)



5. Group 3 (12 mg)

Figure 2 Observation of TNF- α expression in hepatocyte cells

shows that it was induced by *Plasmodium berghei*. Black arrows indicate healthy hepatocytes and orange arrows indicate hepatocytes expressing TNF- α .

The TNF- α variable meets the assumptions of the normality test ($p > 0.05$), but there is data variability ($p < 0.05$). The one-way ANOVA parametric test gave significant results ($p < 0.000$) or there were significant differences. The results of the LSD follow-up test showed that the smallest significant difference to the positive control was treatment 2 ($p = 0.555$)

(Table 2). Thus, treatment 3 is considered to have the same effectiveness as DHP in reducing the incidence of TNF- α expression in hepatocyte cells.

Table 2 The LSD post-hoc test results (TNF- α variable)

| Treatment Group | Average \pm Standard Deviation | BNT Test Significance Value | | | | |
|-----------------|----------------------------------|-----------------------------|--------|--------|--------|--------|
| | | N | N+ | T1 | T2 | T3 |
| N | 9.49 \pm 2.02 | - | 0.000* | 0.000* | 0.000* | 0.000* |
| N+ | 2.56 \pm 0.97 | 0.000* | - | 0.000 | 0.555 | 0.901 |
| T1 | 3.29 \pm 1.10 | 0.000 | 0.000 | - | 0.000 | 0.000 |
| T2 | 1.70 \pm 0.37 | 0.000 | 0.555 | 0.000 | - | 0.641 |
| T3 | 1.90 \pm 0.59 | 0.000 | 0.901 | 0.000 | 0.641 | - |

Description: (*) indicates a significance value

3.3. Effect of *Azadirachta indica* leaf extract on the amount of apoptosis in hepatocyte cells

Observation of apoptotic cells in hepatocyte for each treatment using 5 fields of view. Calculation of apoptosis for each field is by adding up the apoptotic cells in the apoptotic cells and healthy cells section and then multiplying by 100%. Then each treatment is averaged. The average is shown in Figure 3.

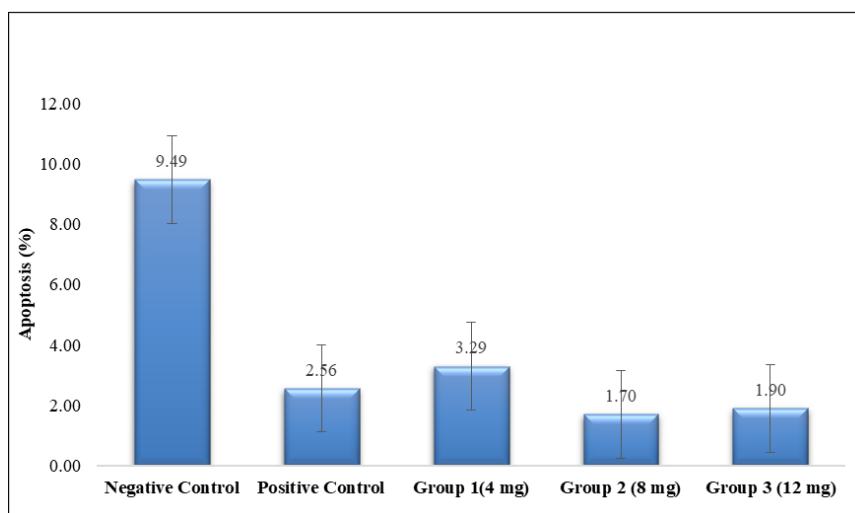


Figure 3 Average Apoptosis in Mice hepatocyte cells with Various Treatments

Apoptotic cells are indicated by the color of the nucleus and cytoplasm turning yellowish. The highest expression was in the negative control (9.49%). The treatment that was able to reduce cell apoptosis in liver tissue was treatment 3 (1.70%). The histopathological picture of hr apoptosis is shown in Figure 4. Expression of apoptosis in hepatocyte cells by immunohistochemical staining.

The apoptosis variable met the assumptions of the normality test ($p > 0.05$), but there was variability in the data ($p < 0.05$). The one-way ANOVA parametric test gave significant results ($p < 0.000$) or there were significant differences. Then the post-hoc LSD test showed that there were no significant differences in treatment 1 ($p = 0.285$), treatment 2 ($p = 0.210$), and treatment 3 ($p = 0.338$) (Table 3). Therefore, this third group has almost the same ability as the positive control (DHP-treated group) in preventing apoptosis in hepatocyte cells.

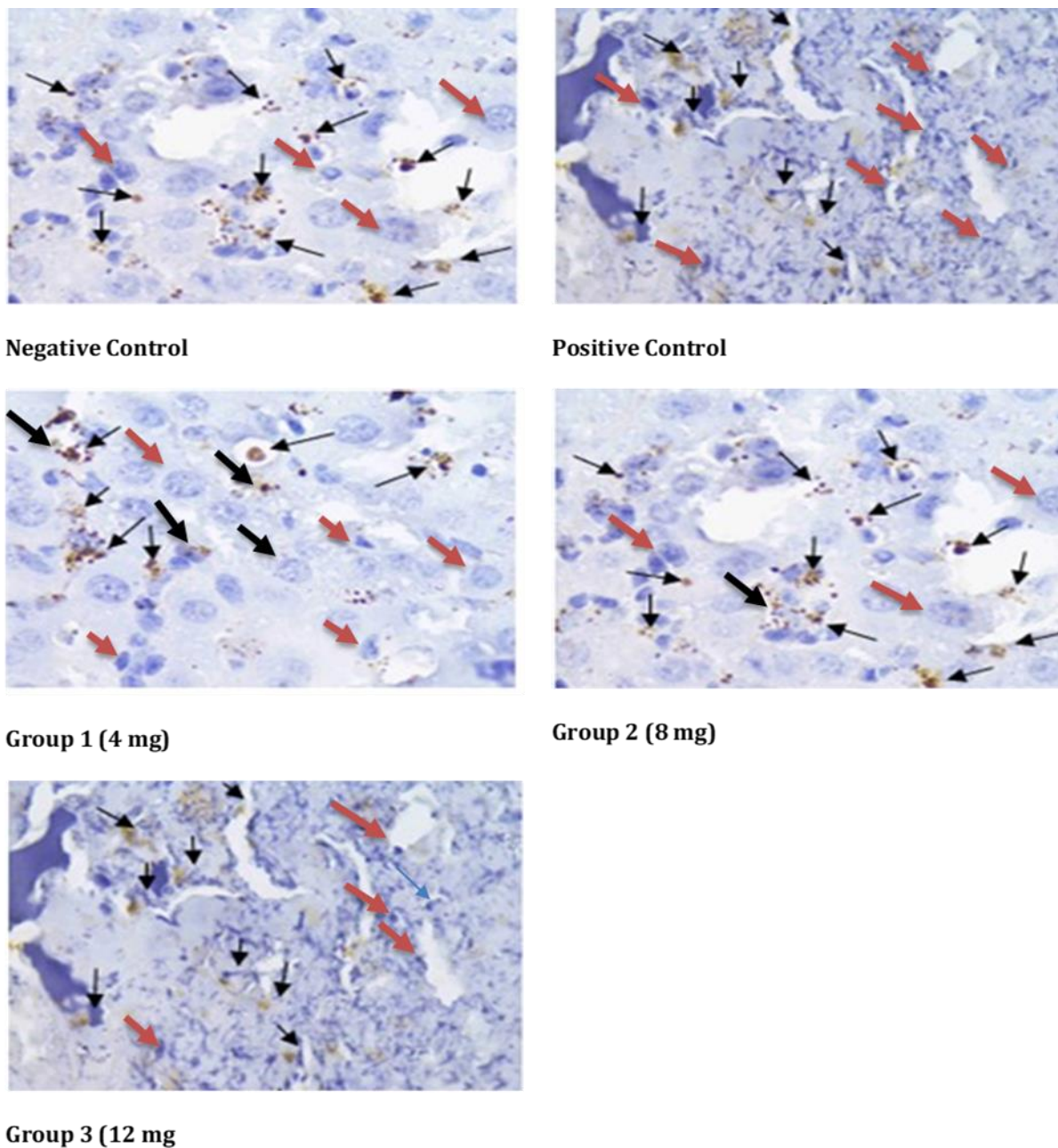


Figure 4 Observation of cell opoptosis in mice hepatocyte cells induced by *Plasmodium berghei*. Black arrows indicate healthy hepatocytes and orange arrows indicate hepatocytes undergoing apoptosis.

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

| Treatment Group | Average±SD | LSD Test Significance Value | | | | |
|-----------------|------------|-----------------------------|--------|--------|--------|--------|
| | | N | N+ | T1 | T2 | T3 |
| N | 9.49±2.02 | - | 0.000* | 0.000* | 0.000* | 0.000* |
| N+ | 2.56±0.97 | 0.000 | - | 0.285 | 0.210 | 0.338 |
| T1 | 3.29±1.10 | 0.000 | 0.285 | - | 0.025 | 0.049 |
| T2 | 1.70±0.37 | 0.000 | 0.210 | 0.025 | - | 0.759 |
| T3 | 1.90±0.59 | 0.000 | 0.338 | 0.049 | 0.759 | - |

Description: (*) indicates a significance value

3.4. Correlation of TNF- α expression and hepatocyte cell apoptosis in BALB/C mice infected with *Plasmodium berghei*

There is a very strong correlation in the positive direction between TNF- α and apoptosis ($r=+0.893$) and ($p=0.000$) (Table 4). Thus, high TNF- α is strongly correlated with a high incidence of apoptosis

Table 4 Pearson's correlation test results between TNF- α and apoptosis

| Correlation Coefficient (rs) | P-Value | Information |
|------------------------------|---------|-------------|
| +0.893 | 0.000 | Significant |

4. Discussion

High levels of TNF- α are a bad sign of malaria, because they can cause inflammatory reactions and coagulation. This reaction can cause occlusion in cerebral malaria [7]. High TNF- α concentrations of up to two to ten times were found in cerebral malaria patients and in fatal cases [16]. This is proven by attachment to the brain occurring when the concentration of TNF- α is high [17]. However, appropriate levels of TNF- α will provide protection and healing from malaria parasites [8]. In this study, the expression of TNF- α in the negative control group (10.15%) was an immune system response to high parasitemia [8]. Meanwhile, Treatment group 1 (5.20%), group 2 (1.84%), and group 3 (1.48%) showed an anti-malarial effect on neem leaf extract. This is in line with research [16], that the content of neem leaves has anti-*Plasmodium* activity against *Plasmodium falciparum* in vitro.

Azadirachta indica leaves contain various compounds such as triterpenoids (azadirachtin), saponins, alkaloids, and phenols. The azadirachtin compound can inhibit the exflagellation of *Plasmodium falciparum* and *Plasmodium berghei* microgametes in vitro [18]. Azadirachtin is a triterpenoid group compound [19]. Azadirachtin can disrupt the incorporation of microtubules into the axoneme and mitotic spindle during microgametosis [20]. Saponin can increase immunity against malaria infection which is characterized by an increase in lymphocytes, besides that saponin has hemolytic properties which can cause hemoglobin lysis [21]. This hemolytic property causes the cleaning of damaged erythrocytes before the release of hemoglobin [22]. Alkaloids can inhibit the growth of *Plasmodium* by forming bonds with DNA or inhibiting its protein synthesis [23]. In addition, the alkaloid content can suppress the production of TNF- α and Nitric oxide (NO) [24]. Phenol can inhibit *Plasmodium* by increasing erythrocyte oxidation and inhibiting protein synthesis [25].

Apoptosis examination was carried out using the immunohistochemical method. It is known that the highest average apoptosis in the negative control group was (9.49%), while in the treatment group, the lowest was in treatment group 2 at (1.70%). The results of the ANOVA test obtained a calculated F value of 46.954 with a significance of 0.000. This states that neem leaf extract (*Azadirachta indica*) reduces hepatocyte apoptosis in BALB/C mice infected with *Plasmodium berghei*. Apoptosis is programmed cell death which is a general process to eliminate potentially harmful cells and cells that have finished their functional period [26]. Apoptosis in *Plasmodium* serves to prevent the early death of vectors and control their density. During malaria infection, several effector T cells were found that were activated but many were eliminated through apoptosis. This aims to maintain a balance between the remaining cells and the apoptosis of immune cells during infection. On the other hand, in malaria infection, CD4+ T cells are often depleted due to the parasite's strategy to avoid immune cells [27].

Apoptosis occurs due to the activation of caspase enzymes, one of which is caspase-3. The caspase-3 enzyme is the most frequently activated death protease enzyme for the apoptosis of various tissues [28]. Two different pathways will unite to activate caspase, namely the intrinsic pathway and the extrinsic pathway [25]. The extrinsic pathway is caused by the triggering of cell surface death receptors by the tumor necrosis factor (TNF) receptor superfamily, including CD95 and TNF-related apoptosis-inducing ligand (TRAIL-R1/R2), while the intrinsic pathway is induced by stress in mitochondria which triggers the release of cytochrome- c [29].

The results of the ANOVA test show that there is a relationship between the effect of neem leaves and hepatocyte apoptosis, this is in line with research [30]. In this study, neem leaf extract was able to reduce caspase-3 expression at the highest dose compared to the untreated group ($P<0.05$). The optimal dose in this study was shown at the dose in treatment group 2 (T2), namely 8 mg/gr. This dose was able to reduce the amount of apoptosis most significantly compared to the other groups.

Triterpenoid compounds in neem leaf extract such as azadirachtin and nimbin have anti-inflammatory and antioxidant effects. This content can significantly inhibit caspase-3 and caspase-9 at a dose of 500 mg/kg [31, 32, 33]. Another content of neem leaves, namely saponin, has been proven to fight oxidative stress by inhibiting apoptosis [33].

5. Conclusion

There is antimalarial activity in neem leaf extract (*Azadirachta indica*) which is characterized by a decrease in TNF- α expression and the occurrence of apoptosis in mouse hepatocyte cells infected with *Plasmodium berghei*

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest regarding this paper.

Statement of ethical approval

Before the start of participant involvement in the research, the participant's consent to participate was also agreed. This research has received research ethics approval from Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim Malang.

Author contributions

All authors contributed significantly to the design and development of this work.

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