JOURNAL OF BIOLOGY EDUCATION
J O B E
**E-ISSN 2656-3436/ P-ISSN 2615-3947**
**IAIN KUDUS**
**http://journal.iainkudus.ac.id/index.php/jbe**

# Effectiveness of *Azadirachta indica* Leaf Extract Against Transforming Growth Factor-β Levels in Swiss Mice Inoculated by *Plasmodium berghei* ANKA

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## ABSTRACT

Malaria is one of the health problems for the world's population in tropical and subtropical regions. Malaria infection is caused by protozoa *Plasmodium spp*. This study aims to determine the effectiveness of *A. indica* leaf extract against TGF-β levels in Swiss mice inoculated with *Plasmodium berghei* ANKA (PbA). This study used true experimental with a post-test design only control group design which used Swiss mice subjects weighing 30-35 grams as much as 24 heads and divided into four groups (K, T1, T2, T3); all groups were inoculated PbA. Group T1 (*A. indica* leaf extract therapy at a dose of 0.25 mg/grBB), Group T2 (*A. indica* leaf extract therapy at a dose of 0.5 mg/grBB), Group T3 (*A. indica* leaf extract therapy at a dose of 1 mg/grBB). Data analysis using the *Anova + posthoc test* with a significance value of $p < 0.05$ and CI of 95%. The results showed differences in TGF-β levels, both in the control and treatment groups after treatment, with a p-value of < 0.05. Greater increases in TGF-β levels occurred in the T3 group (average +38.46 pg/ml) compared to group K (average +14.37 pg/ml), group T1 (average +14.37 pg/ml), and group T2 (average +22.13 pg/ml).

**Keywords**: *Azadirachta indica extract*; transforming growth factor-β; *Plasmodium berghei* ANKA

## INTRODUCTION

*Malaria* is an infectious disorder due to a collection of protozoa of the *Plasmodium species*. The bite of an Anopheles mosquito transmits the disease. (Gusra et al., 2014). A global report by the world malaria report 2019 states there have been about 405,000 deaths out of 228 million cases caused by malaria. In 2018, malaria cases were 93% in Africa, 2.1%

in the Mediterranean, and 3.4% in Southeast Asia (Santoso et al., 2018). While in Indonesia, there are around 33% classified as low endemic (172 regencies/cities), 7% classified as medium endemic (37 regencies/cities), and 8% as high endemic (247 regencies/cities) found in West Papua, Papua, and NTT (Hasyim et al., 2018; Pava et al., 2017; Rosa et al., 2022; Santoso et al., 2018).

When malaria occurs, erythrocytes infected with schizont maturing will undergo lysis and secrete thousands of merozoites, hemozoin, and *Glycosyl phosphatidylinositol* (GPI). *Glycosyl phosphatidylinositol* (GPI) is a toxin compound produced by merozoites and acts as a PAMP (*Pathogen Associated Molecular Pattern*). The interaction between GPI and TLR (*Toll-like Receptor*) will activate T-Helper (Th) cells which trigger increased secretion from TNF-α (*Tumor Necrosis Factor-α*) (Sulistyawati et al., 2018). TNF-α is a pro-inflammatory cytokine produced by activated immune cells such as B cells, CD4+ cells, macrophages, and mast cells (Irawati, 2014). Th-2 cells will also be activated to secrete IL-10 (*Interleukin-10*) to offset the production of TNF-α to fight malaria infection (Sulistyawati et al., 2018). The increase in TNF-α production in the final phase of cerebral malaria is the cause of the severity, severity of tissue damage that occurs (Irawati, 2014).

Cytokines secreted by Th-1 cells will overcome the occurrence of malaria infection by affecting the phagocytic function of macrophages. On the contrary, cytokines produced by TNF-α will activate the phagocytosis function of macrophages in eradicating infected erythrocytes and parasites damaged in the blood (Irawati, 2014). The production of TNF-α (pro-inflammatory cytokines) by Th-1 cells will be inhibited by IL-10 and TGF-β (*Transforming Growth Factor*) (Drewry & Harty, 2020; Niikura et al., 2011). A high Th-1 cytokine response will stimulate lymphocytes to produce Th-2 cytokines such as IL-10, which have *Immunoregulatory* effects and can suppress the dominance of Th-1 (Saito et al., 2010).

Increased expression of Th-2 cytokines is usually associated with high expression of increased regulation of T cytokines, such as the TGF-β, which serves to eliminate parasites (Rahmah, 2021). TGF-β is a multipotent cytokine that plays an important role in immune regulation. Increasing TGF-β production during malaria infection can reduce the risk of severity of malaria infection (Drewry & Harty, 2020). Efforts to eradicate malaria have been widely carried out, but the prevalence is still high due to the resistance of plasmodium to existing drugs (Silver et al., 2010; Tran et al., 2021).

Antimalarial drugs are one of the keys to malaria eradication and control (Cui et al., 2015). *Artemisinin combination therapy* (ACT) has been recommended by WHO and has been implemented since 2004 for the main treatment of malaria. Currently, antimalarial drugs have undergone resistance, except for artemisinin. If resistance also occurs in artemisinin, then treatment against malaria will not be resolved. Research using a combination of artemisinin with plant extracts containing flavonoid compounds provides more effective results in treating and lowering the number of parasitemia. This proves that plant extracts

containing Flavonoids can be used as the main companion drugs in treating malaria (Ilmiah et al., 2020). This study intends to analyze TGF-β levels in Swiss mice inoculated with *plasmodium berghei* ANKA treated with *Azadirachta indica* leaf extract.

## METHOD

This experimental laboratory study uses a true experimental in vivo with a post-test-only with control group design. Experiments were conducted on swiss strain mice weighing 30-35 grams and as much as 24 heads. The determination of samples in this study was by WHO (*Word Health Organization*) standards, based on a trial of research guidelines for evaluating the safety and efficacy of herbal medicine using 5 (five) Swiss strain mice in each Group. Before the treatment, all groups were Inoculated with *plasmodium berghei* ANKA ($2x10^7$PbA) (Najib et al., 2022). The Swiss strain mice used in the study were 15-16 weeks old.

This study was classified into four groups, namely (K); the negative control group was mice inoculated by PbA without giving *A. indica* leaf extract. (T1), mice inoculated with PbA by administration of *A. indica* leaf extract at a dose of 0.25 mg/grBB. (T2), a group of mice inoculated in PbA by administering *A. indica* leaf extract at a dose of 0.5 mg/grBB. (T3), a group of mice inoculated with PbA by administration of *A. indica* leaf extract at a dose of 1 mg/grBB.

Examination of TGF-β spleen expression was measured using *the Enzyme Linked Immuno Assay* (ELISA) method with pg/ml units using (TGF-β Elisa Kit Mouse, Number of catalog E0296Mo). Data on the results of *A. indica* leaf extract and TGF-β cytokine levels in this study were presented as mean + (standard deviation) with a CI (confidence interval) of 95%. Suppose the data results are valuable or norm-distributed. In that case, it is continued with a bivariate test using paired T-test samples to see the difference in TGF-β cytokine levels in groups K (negative control), T1, T2, and T3 after treatment. In addition, an ANOVA One Way Test was also carried out to see the differences in TGF-β cytokines between groups and continued with post hoc LCD analysis.

## RESULT AND DISCUSSION

Based on the study's results, the average value of TGF-β levels in group K (Negative control) was obtained, namely + 14.37 pg/ml. After administration of *Azadirachta indica* Leaf Extract dose, 0.25 mg / grBB, the average value of TGF-β levels increased to +18.24 pg/ml with p = 0.0006. The T2 group had an average value of +22.13 pg/ml higher than the K group with p=0.0000. Meanwhile, the T3 group had an average of 38.46 pg/ml also increased compared to the K group with p = 0.002. So groups T1, T2, and T3, compared to group K had different TGF-β cadaver measurement results with p=0.002.

Table 1. The average difference in levels in each group

| No | Sample group | TGF-β levels (pg/ml) Mean+SD | P-value |
|---|---|---|---|
| 1. | Group K (inoculated PbA without therapy) | 14,37 ± 4,77 | 0,022[a] |
| 2. | Group T1 (inoculated PbA and *A. indica* leaf extract therapy at a dose of 0.25 mg/grBB) | 18,24 ± 9,95* | 0,011[a] |
| 3. | Group T2 (inoculated PbA and *A. indica* leaf extract therapy at a dose of 0.5 mg/grBB) | 22,13 ± 8,23* | 0,025[a] |
| 4. | Group T3 (inoculated PbA and *A. indica* leaf extract therapy at a dose of 1 mg/grBB) | 38,46 ± 16,38* | 0,006[a] |
| | P-Value | 0,002[b] | |

**ANOVA*
*[a]Repaited Anova*
*[b]One way Anova presented in average+SD form*

The average level of TGF-β in the T1, T2, and T3 groups has increased compared to the average of group K; this proves that malaria infection by plasmodium berghei ANKA in Swiss mice increased IL-10 spleen levels. TGF-β is one of the important regulators in acute malaria infection or cerebral malaria, which controls the immune response in the experimental model of malaria or malaria in humans (Drewry & Harty, 2020). Data analysis using the Post Hoc test showed a significant difference between groups K and T1, T2, and T3 (p=0.002). Infected erythrocytes containing mature schizonts will undergo lysis and secrete thousands of merozoites, hemozoin, and GPI (*Glycosylphospatidylinositol*). GPI is a toxin compound produced by merozoites and has a role as a PAMP (*Pathogen Associated Molecular Pattern*). The interaction between GPI and TLR (*Toll-like Receptor*) will activate T-Helper (Th) cells which trigger increased secretion from TNF-α. Th-2 cells will also be activated to secrete IL-10 (*Interleukin-10*) and produce TGF-β to offset the production of TNF-α to fight malaria infection (Drewry & Harty, 2020; Sulistyawati et al., 2018).

Th-1 and Th-2 cells contribute to defensive immunity towards malaria infection, but their stability in unique instances determines disorder manifestation. Th-1 cells are responsible for controlling parasitemia at the beginning of infection; subsequently, Th-2 is needed to complete parasite destruction (Rahmah, 2021). Th-1 cells produce proinflammatory cytokines, while Th-2 functions to produce anti-inflammatory cytokines (Wahyuniati & Maulana, 2018). Complex syndrome of malaria is associated with an increase in proinflammatory cytokines. Severe malaria occurs associated with high levels of TNF-α, increased production of other pro-inflammatory cytokines (IFN-γ and IL-1β), and decreased production of anti-inflammatory cytokines, especially IL-10 and TGF-β. Cytokines produced by Th-1 are considered important in controlling Plasmodium infection in both the pre-erythrocytic and erythrocytic phases. However, the increase in excess production also contributes to organ damage (Rahmah, 2021). High TGF-β in malaria infection can reduce

the risk of organ damage severity due to malaria infection (Drewry & Harty, 2020). It is also shown in CBA/J mice infected with *P. berghei* NK65, showing low levels of TGF-β and causing disease severity. Meanwhile, CBA/J mice with high levels of TGF-β can help limit the severity of the disease and help eliminate infections and prolong the survival of mice (Drewry & Harty, 2020).

During the erythrocytic phase, CD4+ cells are critical mediators of anti-parasitic defense by helping to control parasitemia and as TGF-β negative regulatory targets (Harty, 2019; Li, 2013). It is widely believed that the protective function of CD4+ cells during malaria infection derives from the production of IFN-γ (Drewry & Harty, 2020). However, CD4+ cells are not the only subset expressing IFN-γ during malaria infection (Harty, 2019). Despite having anti-parasitic functions, IFN-γ and Th-1 can also cause disease severity since high levels of IFN-γ to correlate with pathologies of malaria infection in humans and mice (Drewry & Harty, 2020; Tongren & Diseases, 2003). TGF-β works by inhibiting the differentiation of Th-1 and Th-2 cells (Li, 2013). Thus, TGF-β inhibition of Th-1 differentiation can suppress production from IFN-γ. Thus, TGF-β can limit inflammatory lesions by modulating Th-1 differentiation and function. TGF-β also acts as a Goldilock in murine malaria experiments, and the right amount of TGF-β at the right time can also control immunopathology. (Drewry & Harty, 2020).

Although TGF-β is referred to and discussed as an anti-inflammatory cytokine, it turns out that under certain circumstances, TGF-β can also function as a pro-inflammatory. In vitro research show that at low concentrations (0.1-10pg/ml), TGF-β serves as a chemotactic ligand for human monocytes. Mature monocytes and macrophages can contribute to malaria control at the erythrocytic stage by phagocytes infecting erythrocytes and secreting pro-inflammatory cytokines quickly (Drewry & Harty, 2020)

In this way, TGF-β can dampen the response of IFN-γ and enable effective control of parasite replication from the beginning of infection (Drewry & Harty, 2020). TGF-β may also promote the expansion of Treg cells during malaria infection, It aims/serves to decrease excessive inflammation induced by uncontrolled CD4+ and IFN-γ cells responses (Drewry & Harty, 2020). Tregs play a key role in the activation of effector T cells. Tregs are recognized by the expression of the signature transcription factor Foxp3, which is generated in the thymus (thymus derivative, tTregs) and peripheral blood (peripheral, pTregs) (Dhamne et al., 2013). TGF-β promotes the development of Tregs derived from the thymus and peripherals. In studies in neonatal murine malaria mice, TGF-β signaling disorders via TGF-BRI signaling (TGF-β receptor type I) did not permanently cancel the formation of tTreg, but could eliminate the pronounced Foxp3+ thymocytes in the first week of life (Drewry & Harty, 2020; Liu et al., 2008). However, T cell studies in primary cultures show that TGF- β encourages CD4+ T cells to differentiate into Foxp3-expressing Tregs in the case of pTreg. (Drewry & Harty, 2020; Selvaraj & Geiger, 2007), especially the absence of signaling from strong pro-inflammatory cytokines (Wei et al., 2007).

Preliminary studies on murine malaria, Provide contradictory evidence regarding whether Treg promotes or controls parasitemia and pathology during murine malaria. (Hansen & Schofield, 2010). The heterogeneity in experimental systems, particularly the

infection models utilized, as well as the timing and techniques of Treg depletion, may be responsible for most of the misunderstanding. A popular first strategy that used anti-CD25 therapy to drain Treg was hampered by the preservation of many CD25 thymocytes-Foxp3+ (Drewry & Harty, 2020). Another study employed a *P. yoelli* 17 XNL infection model to demonstrate that CD4 + Foxp3 + Treg develop throughout the erythrocytic stage. During this temporal window, Treg depletion using the Foxp3-diphtheria toxin receptor (DTR) system leads to dramatically accelerated control of parasitemia. Conversely, the depletion of previous Tregs at the beginning of infection resulted in death from this normally non-lethal infection (Kurup et al., 2017). Treg expansion is related to increased parasitemia and constriction of activated CD4 T cells (Kurup et al., 2017). This suggests that the expanded Treg allows parasitic replication by disrupting CD4+ Th-1 cells, and the TFH response is critical to controlling erythrocytic stage infections. Konsisten dengan teori ini, netralisasi Treg oleh penipisan Foxp3 menyebabkan peningkatan jumlah sel CD4+ teraktivasi dan pengendalian parasitemia secara cepat (Drewry & Harty, 2020; Kurup et al., 2017).

If TGF-β signaling promotes Treg cell proliferation during malaria, this may assist in regulating excessive inflammation generated by uncontrolled CD4+ Th-1 and IFN-γ cells. According to one study, neutralization of TGF- may reduce the amount of Foxp3+ Treg cells observed during *P. berghei ANKA* infection (Kurup et al., 2017). The significance of this drop in Treg numbers is unknown because the function of these Tregs in inhibiting effector T cells has not been evaluated. The very early TGF-β neutralization strategy (before the commencement of infection and continuing for the first week of infection) can likewise significantly change parasite multiplication (Drewry & Harty, 2020; Kurup et al., 2017). An in vitro research in which co-cultures of human peripheral blood mononuclear cells and P. falciparum-infected red blood cells induced the development of FoxP3 to express Treg, with TGF-β required for FoxP3 hi Treg production provides independent support for TGF- promoting the development of Treg during malaria (Drewry & Harty, 2020). TGF-β bursts associated with the development of erythrocytic stage illness, as well as Treg identification, have been found in human experimental malaria infections. In vitro investigations show that Plasmodium proteases generated during the rupture of infected erythrocyte cells can activate TGF-β (Drewry & Harty, 2020).

Notably, even if TGF-β promotes Treg expansion during malaria infection, the impact of Treg-depletion is adjusted for contractions from CD4+ Th-1 cells during *P. yoelli* 17XNL infection, calling into question the notion that TGF-β responses are optimized to promote optimal outcomes while resolving an infection in murine malaria. The thinning of Treg in this key window, in particular, greatly enhanced parasitemia management without associated fatalities caused by inflammatory diseases. (Kurup et al., 2017), This suggests that TGF-β synthesis was not delayed sufficiently to allow for optimal infection control. Furthermore, the repressor of CTLA-4 antigen-presenting cell co-stimulation is a putative mediator of Treg suppression of CD4+ T cell response. CTLA-4 inhibition not only improves parasitemia

management, but also the quality of the germinal center response and the capacity to build protective immunity to future Plasmodial species challenges (Kurup et al., 2017). Assume CTLA-4 inhibition enhances this outcome by neutralizing the function of Treg cells. In that circumstance, TGF-β-induced Treg regulation will result in a continuous response of CD4+ Th-1 cells, resulting in enhanced functional immunity. These findings were not confined to the *P. yoelli* 17XNL Model since CTLA-4 inhibition during the challenged ANKA *P. berghei* also reduced mortality (Kurup et al., 2017). Combined with prior results, TGF-β neutralization can protect against mortality during *P. berghei* ANKA infection, our data suggest that TGF- signaling is unlikely to induce Treg development during malaria. TGF-β, on the other hand, may only have pleiotropic effects that are exclusive to differentiating the temporal window of infection, as appears to be the case with Treg (Drewry & Harty, 2020)

Although TGF-β is usually considered an anti-inflammatory cytokine, it can also have pro-inflammatory effects in specific conditions. TGF-β acts as a chemotactic ligand for human monocytes at low doses (0.1-10pg/mL), according to in vitro research (Drewry & Harty, 2020). TGF-β has also been proven in human PBMC cells to increase the transcription of pro-inflammatory cytokines IL-1 and TNF-α (Drewry & Harty, 2020). Monocytes, as well as their mature hereditary macrophages and inflammatory dendritic cells, can help control malaria during the erythrocytic stage by phagocytizing infected erythrocyte cells and rapidly secreting pro-inflammatory cytokines (Amaya & Stephen, 2018). Macrophages are also involved in parasitemia with infected erythrocyte cell phagocytes and nitric oxide production. They are thought to be targets of IFN-γ activation, which helps to explain IFN-γ significant involvement in the regulation of blood-stage illnesses (Drewry & Harty, 2020; Harty, 2019).

TGF-β activation of innate immune cells such as monocytes and macrophages may explain the quick identification of increased parasitemia following TGF-β neutralization within two days after commencing blood-stage infection. TGF-β, IL-10, IFN-γ, and TNF-α production kinetics have been linked to varied outcomes in deadly and non-lethal *P. yoelli* infections, which differ significantly in the first few days of infection (Drewry & Harty, 2020). As a result, it is intriguing to suggest that, early in murine malaria, before a strong inflammatory profile emerges, TGF-β stimulates the activation of congenital anti-parasitic effectors such as monocytes and macrophages. TGF- may switch to limiting inflammatory diseases when parasitemia and inflammation grow (Drewry & Harty, 2020; Harty, 2019).

According to other research, Treg enhances protection against erythrocytic stage malaria in mouse models. Furthermore, greater Treg production corresponds with slower parasite development in experimentally infected patients during therapeutic studies (Drewry & Harty, 2020). As a result, Treg might have an anti-parasitic impact at different times of the day. In this setting, increasing TGF-β from Treg differentiation may aid parasite control. TGF-β T-cell derivatives, on the other hand, have been demonstrated to limit tTreg growth (Drewry & Harty, 2020). TGF-β activated during malaria may also inhibit Treg growth, encouraging greater parasite control.

In this study, results were obtained that Azadirachta indica leaf extract in the T1, T2, and T3 groups could increase TGF-β levels in the spleen. This is because *Azadirachta indica* leaf extract contains active substances that are antimalarial such as Azadirachtin, Nimbolide which both substances can also affect the function of pro-inflammatory cytokine immunomodulators and pro-inflammatory immune systems (Agustin et al., 2016).

## CONCLUSION

Based on the results and discussion, it can be concluded that giving neem leaf extract (*Azadirachta indica*) can increase TGF-β cytokine levels in the Swiss mice spleen inoculated by Plasmodium berghei ANKA. Elevated levels of TGF-β were strongly seen in all treatment groups compared to the control group. Further research needs to be conducted to determine the levels of other pro-inflammatory and anti-inflammatory cytokines and unravel the relationship between cytokines and specific immune cells in administering Azadirachta indica leaf extract as a companion drug with ACT.

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