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Original Research Article

Syzygium cumini Fraction Modulates IFN-γ, Ki67, and IL-10 in Pregnant Murine Model of Malaria

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ABSTRACT

Malaria in pregnancy significantly increases maternal and fetal morbidity and mortality, underscoring the need for effective interventions. This study aimed to evaluate the immunomodulatory effects of *Syzygium cumini* fruit chloroform-methanol fraction, specifically on interferon gamma (IFN-γ), interleukin-10 (IL-10), and antigen Kiel 67 (Ki-67) expressions in a murine model of malaria in pregnancy. Fifty-four (54) healthy pregnant Balb/c mice were divided into six groups of nine mice each: Normal control (NC) – pregnant non-infected mice, Negative control (NegC) – Pregnant infected mice only, Positive control (PosC) – infected mice treated with standard antimalarial (Dihydroartemisinin 40 mg/Piperaquine phosphate 320 mg), and three treatment groups T1, T2, and T3 which consist of infected mice treated with *S. cumini* fraction at 600, 800, and 1200 mg/kg, respectively. Malaria was induced in the mice by intraperitoneal administration of *Plasmodium bergei* (ANKA strain) on gestational day 9, and treatments were administered orally, once daily for 15 days starting from gestational day 11. On completion of the treatment, expression levels of IFN-γ, IL-10, and Ki-67 were assessed using appropriate immunoassays. Administration of *Syzygium cumini* fruit fruit fraction resulted in a significant and a dose-dependent immunomodulatory effect in malaria infected pregnant mice, with the T3 group (1200 mg/kg) exhibiting the most pronounced modulation, with significant reduction in IFN-γ, elevation of IL-10, and restoration of Ki-67 expression compared to infected controls. The effective modulation of IL-10, suppression of IFN-γ, and promotion of Ki-67 by *Syzygium cumini* fraction indicate potential protective immunological and regenerative roles during malaria infection in pregnancy.

Keywords: *Syzygium cumini*, Interleukin-10, Interferon gamma, Ki-67, Malaria in Pregnancy, *Plasmodium berghei*.

Introduction

Malaria remains a major global health burden, with an estimated 263 million cases and 597,000 deaths in 2023. Pregnant women are one of the high risk groups.[1] Malaria infection during pregnancy can cause premature delivery or delivery of a baby with low birth weight. The risk and severity of malaria increases due to a combination of physiological and immunological changes during pregnancy.[2] Malaria is transmitted by Anopheles mosquitoes and caused by Plasmodium parasites, particularly *P. Falciparum*. Prompt treatment of malaria is crucial to prevent severe illness and death.[3,4] Murine models, particularly those using infection with *Plasmodium berghei*, have been instrumental in understanding the pathogenesis of malaria and evaluating novel therapeutic interventions.[3] During exposure to malaria parasites, the imbalance between endogenous antioxidant defences and reactive oxygen species (ROS) is the main cause of oxidative stress.[5] Oxidative stress can result in decreased placental perfusion due to maternal vascular endothelial dysfunction. Endothelial dysfunction causes vasoconstriction in the decidual spiral arterioles, leading to decreased blood flow to the placenta.[6] Although, there are differences between human and mice placentas, there are structural and molecular similarities, especially in the context of vascular function, including similarities in proteomic profiles, transcriptomics, maternal immunity, and mechanisms of host inflammatory response.[7–9] Antioxidants are compounds that inhibit oxidation reactions by neutralizing free radicals and other highly reactive molecules.[10,11] One of the natural antioxidants is *Syzygium cumini*, a well-known medicinal plant with anti-inflammatory potential.[11–16] This study aimed to evaluate the immunomodulatory effect of *Syzygium cumini* chloroform-methanol fraction, specifically on how the fraction modulates the levels of IFN-gamma and IL-10, as well as the expression of Ki67 in a pregnant murine model of malaria infection. The study also seeks to gain a deeper understanding of the systemic and local immunological alterations during acute infection in pregnancy. It is expected that the results obtained will contribute to the development of approaches for the management of malaria cases during pregnancy.

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Materials and Methods

Chemicals and equipment

Chloroform ($CHCl_3$), Methanol (CH_3OH), and Phosphate Buffer Saline (PBS) were products of Merck KgaA, Darmstadt, Germany. The equipment used were Rotary evaporator (IKA RV 10, Germany), microtome (Leica RM2235, Germany), and light microscope (Olympus BX51, Tokyo, Japan).

Plant collection and identification

Syzygium cumini fruits were collected from Lumajang Regency, East

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Java, Indonesia, in May 2023. The plant material was identified and authenticated at UPT Laboratorium Herbal Materia Medica Batu, Indonesia. Herbarium specimen with voucher number 067/1873/102.20/2023 was deposited.

Preparation of plant fraction
Syzygium cumini fruits were dried, powdered, and the powdered fruits (500 g) were extracted by maceration in 70% ethanol (1.5 L) at room temperature for 72 hours. The extract was filtered, and the filtrate was concentrated *in vacuo* using rotary evaporator at 40°C until a thick extract was obtained. The extract (45 g) was fractionated with 200 mL of chloroform:methanol (1:1) by liquid-liquid partitioning. The resulting chloroform-methanol fraction was concentrated *in vacuo* using rotary evaporator, and the concentrated extract was stored at 4°C until needed for use.

Animals
Fifty-four (54) healthy pregnant nulliparous primigravida Balb/c TM III mice (*Mus musculus*) with a body weight of 22-25 grams, 8-10 weeks old were obtained from the Animal Unit, Institut Teknologi Bandung (ITB), Jawa Barat 40132, Indonesia. The animals were kept in well-ventilated cages, and housed in the Laboratory of the Faculty of Medicine, Universitas Brawijaya Malang, Indonesia. The animals were acclimatized to the laboratory conditions for 7 days. They were allowed access to feed and water *ad libitum*.

Ethical approval
Ethical approval with reference number 001-KEP-UB-2024 was obtained from Brawijaya Malang University Ethics Committee. The animals were handled and treated according to international guidelines for use and care of experimental animals.

Impregnation of the mice
One male mouse was placed with two female mice overnight. The vaginal of the female mixe were examined the following morning. The presence of a vaginal plug indicate mating, and the day the plug was observed was regarded as gestational day 0 (GD 0).

Experimental design
This study is a randomized controlled trial (RCT) with a pre and posttest control group design. Fifty-four (54) healthy pregnant nulliparous primigravida Balb/c TM III mice were divided into six groups of nine animals each: Normal control (NC) group which consist of non infected pregnant mice only, the negative control (NegC) group which consist of pregnant mice with malaria infection but without treatment), positive control (PosC) group which consist of pregnant mice with malaria infection treated with antimalaria drug (Dihydroartemisinin 40 mg/Piperaquine phosphate 320 mg) at a dose of 10 mg/kg, T1, T2, and T3 groups which consist of pregnant mice with malaria infection treated with *Syzygium cumini* fraction at doses of 600 mg/kg, 800 mg/kg, and 1200 mg/kg. All the mice except those in the normal control group were infected with *Plasmodium berghei* parasites (ANKA strain bred by the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia). The parasites inoculum (1 x 10^6 cells/mL) at the erythrocytic stage were administered intraperitoneally on day 9 of pregnancy. Treatment with the positive control drug, and the chloroform-methanol fraction of *Syzygium cumini* fruit started on day 11 of pregnancy. Treatment was given orally once daily for 15 days.
After 15 days of treatment, proinflammatory cytokine IFN-γ, anti-inflammatory IL-10, and proliferation marker Ki-67 cells were examined.

Immunohistochemistry
Pregnant mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. The vena lateralis was carefully excised and immediately fixed in 10% neutral buffered formalin for 24 hours. Fixed tissues were dehydrated in graded ethanol, cleared with xylene, and embedded in paraffin. Sections of 4 µm thickness were mounted on poly-L-lysine-coated slides. The slides were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval using citrate buffer (pH 6.0) at 95°C for 20 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, followed by blocking with 5% bovine serum albumin (BSA) for 30 minutes. Sections were incubated overnight at 4°C with primary antibodies against IFN-γ, IL-10, and Ki67 (Abcam, dilution 1:100). After washing, slides were incubated with a biotinylated secondary antibody and then treated with the streptavidin–HRP complex. Colour development was achieved using 3,3′-diaminobenzidine (DAB) as the chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted for microscopic examination. Immunopositive cells were quantified in five randomly selected high-power fields per slide.

Statistical analysis
Data were presented as mean ± standard deviation (SD), n = 9. The sata were subjected to normality test using Kolmogorov-Smirnov and Shapiro-Wilk normality test. The differences between means were analysed using One-Way Analysis of Variance (ANOVA), followed by post-hoc analysis using Tukey's Honestly Significant Difference (HSD) test. Significant differences between mean values were set at P-value < 0.05.

Results and Discussion

Effect of Syzygium cumini fraction on *IFN-γ expression levels*
Malaria infection significantly increased IFN-γ levels, especially in the negative control (NegC) group, as shown in Figure 1. IFN-γ is a major proinflammatory cytokine that plays a role in macrophage activation and cellular immune responses against parasites. However, excessively high levels of IFN-γ can trigger tissue damage, systemic inflammation, and placental dysfunction that impairs blood flow to the foetus.[4,17] These findings support earlier studies that reported that excess IFN-γ during pregnancy can aggravate obstetric complications.[4,7,18] The *S. cumini* fraction, especially at T3, was associated with significantly higher IFN-γ levels compared to the infected control (NegC). This suggests a robust Th1-type immune stimulation. This effect suggests that the active compounds in *S. cumini*, such as flavonoids, tannins and anthocyanins, act as anti-inflammatory agents by suppressing the production of pro-inflammatory cytokines. These results support the findings from a previous study that reported that *Syzygium cumini* exhibit anti-inflammatory activity through inhibition of inflammatory signalling pathways.[12]

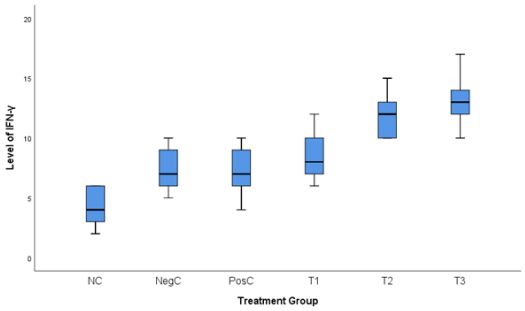

Figure 1: IFN-γ expression levels in pregnant mouse model of malaria after 15 days of treatment. **NC:** Non infected pregnant mice only, **NegC:** Untreated pregnant mice with malaria infection, **PosC:** Pregnant mice with malaria infection treated with antimalaria drug (Dihydroartemisinin 40 mg/Piperaquine phosphate 320 mg), **T1:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 600 mg/kg dose, **T2:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 800 mg/kg dose, **T3:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 1200 mg/kg dose

Effect of Syzygium cumini fraction on *IL-10 expression levels*
IL-10 is an anti-inflammatory cytokine that plays regulatory role in the immune system, especially important in maintaining immune tolerance during pregnancy. The decrease in IL-10 levels in the NegC group

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suggests that malaria infection suppresses natural anti-inflammatory immune mechanisms, which may exacerbate the inflammatory response. *Syzygium cumini* fraction was able to increase IL-10 levels significantly ($P < 0.05$), with the strongest effect seen at a dose of 1200 mg (T3) as illustrated in Figure 2. This increase has implications for restoring immune balance and reducing the risk of overactivation of the immune system that could harm the foetus.[19,20] This suggests that *S. cumini* not only suppresses inflammatory responses, but also actively supports the strengthening of anti-inflammatory pathways that are vital for pregnancy survival.[10,21]

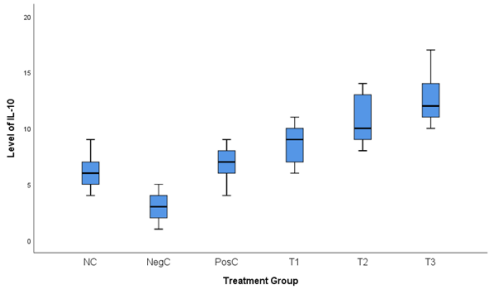

Figure 2: IL-10 expression levels in pregnant mouse model of malaria after 15 days of treatment. **NC:** Non infected pregnant mice only, **NegC:** Untreated pregnant mice with malaria infection, **PosC:** Pregnant mice with malaria infection treated with antimalaria drug (Dihydroartemisinin 40 mg/Piperaquine phosphate 320 mg), **T1:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 600 mg/kg dose, **T2:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 800 mg/kg dose, **T3:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 1200 mg/kg dose

Effect of Syzygium cumini fraction on *Ki-67 expression levels*

Ki-67 is an important marker of cell proliferation. The decrease in Ki-67 expression in the infected group (NegC) observed in Figure 3 suggests that *P. berghei* caused suppression of tissue regeneration, possibly through the effects of oxidative stress and chronic inflammation. This is in line with reports that stated that malaria causes tissue damage, including the placenta through impaired perfusion and vascularisation.[22–24] The increase in Ki-67 expression in the treatment groups (especially T2 and T3) suggests that *Syzygium cumini* fractions do not only have immunomodulatory effect, but also support tissue repair and homeostasis. This effect could be due to the ability of active compounds in *S. cumini* to stimulate proliferative pathways and protect cells from oxidative damage, thereby accelerating cell regeneration in placental and uterine tissues.

Clinical implications and therapeutic potential

Overall, the chloroform-methanol fraction of *Syzygium cumini* fruit showed comprehensive immunomodulatory activity, while associated with an unexpected increase in the proinflammatory marker IFN-γ it also delivered beneficial effects, and stimulation of cell proliferation (Ki-67). This effect is particularly important in the context of malaria in pregnancy, where the immune system must be kept active against infection but must not be overactive to the point of harming the foetus. The 1200 mg/kg dose (T3) proved to be the most effective among all three doses tested, making it an optimal dose for the development of *Syzygium cumini*-based adjuvant therapy. These effects provide opportunities for further exploration of the plant, including in-depth molecular analyses as well as long-term toxicity and safety testing in other experimental animals prior to clinical application.

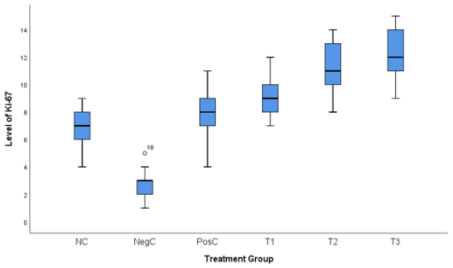

Figure 3: Ki-67 expression levels in pregnant mouse model of malaria after 15 days of treatment. **NC:** Non infected pregnant mice only, **NegC:** Untreated pregnant mice with malaria infection, **PosC:** Pregnant mice with malaria infection treated with antimalaria drug (Dihydroartemisinin 40 mg/Piperaquine phosphate 320 mg), **T1:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 600 mg/kg dose, **T2:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 800 mg/kg dose, **T3:** Pregnant mice with malaria infection treated with *Syzygium cumini* fraction at 1200 mg/kg dose

Conclusion

Administration of chloroform-methanol fraction of *Syzygium cumini* fruit to pregnant mice in the third trimester infected with *Plasmodium berghei* proved to have significant immunomodulatory effects. The plant was able to reduce levels of pro-inflammatory cytokine IFN-γ, increase levels of anti-inflammatory cytokine IL-10, and increase the expression of proliferation marker Ki-67 cells. The 1200 mg/kg dose was found to be the most effective in controlling inflammatory responses and supporting tissue regeneration in malaria in pregnancy. These findings suggest that *Syzygium cumini* fraction has the potential to be developed as a safe and effective natural adjuvant therapy for the management of malaria in pregnant women.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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