Research Article

Ethanol Extract of Moringa Propolis Modulates Pro-Inflammatory Cytokines in Mice with Acute Toxoplasmosis

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Abstract | *Toxoplasma gondii* can cause an increase the cytokine, increased cytokine pro-inflamation can cause cell damage. Natural products such as propolis can be choice for treatment toxoplasmosis for reduce cytokine pro-inflamation (TNF-α and IFN-γ). We compare the amount of cytokines in each treatment group. We employed flow cytometry to assess cytokine expression. We used 36 mice were divided into six group, group one mice were not infected and treated as negative control, groups 2 to 6 allmice were infected *T. gondii* with 100 tachyzoites. Group 2 mice were infected but not treated, group 3 mice were infected and treated with orally cotrimoxazole 40 mg/kg body weight, group 4 mice were infected and treated with Ethanol Extract Propolis (EEP) 1 mg/mouse, group 5 mice were infected and treated with EEP 2 mg/mouse, and group 6 mice were infected and treated with EEP 4 mg/mouse one day after infection for three days. We observed cytokines in mice exposed to *Toxoplasma gondii*. Statistical analysis was performed with one-way ANOVA and Duncan Multiple Range Test in SPSS 30 software. Results showed that EEP (1-4 mg) can reduce reduced levels of pro-inflammatory cytokines (TNF-α, IFN-γ) no significant differences caspase-3 in all treatments, TNF-α and IFN-γ from the results we obtained showed lower values when compared to the group of toxoplasmosis mice treated using cotrimoxazole. The best choice for toxoplasmosis therapy using EEP is in group P6 with a dose of 4 mg.

Keywords | Cytokine, Ethanol extract propolis, *Toxoplasma gondii, Trigona* spp., Moringa, West Nusa Tenggara

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INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a parasite that can infect humans and warm-blooded animals causing toxoplasmosis (Pazoki *et al.*, 2020). *T. gondii* can be detected seven to ten days after infection (Sittivarakul *et al.*, 2024). They can be detected in liver cells (Babekir *et al.*, 2022) and spleen (Yahia *et al.*, 2022). The prevalence of

toxoplasmosis is high in tropical areas, most of the disease is asymptomatic and the prognosis is poor in patients with weakened immune systems (Wibawani *et al*., 2019; Zhou *et al*., 2021). During the acute phase of *T. gondii* infection, the liver and spleen will experience damage resulting in hepatomegaly, splenomegaly, multifocal necrosis of the liver and spleen, hepatitis, biliary cirrhosis, and non-alcoholic fatty liver disease (Lu *et al*., 2021; Campbell *et al*., 2022).

Infection *T. gondii* can induce parasite infiltration, causing apoptosis in cells (Du *et al*., 2022). Infiltration of as many as 10 tachyzoites can cause mice to die on the 17th day after infection (Lee *et al*., 2019). During the acute infection phase, immune cells such as NK cells, TNF-α, interferon-γ (IFN-γ), and IL-12 will increase, an increase in these immune cells can be used as a marker of toxoplasmosis infection because in healthy people toxoplasmosis is often asymptomatic (Jafari *et al*., 2023). Current clinical treatment of toxoplasmosis is dominated by the administration of pyrimethamine and sulfadiazine, these treatments have a fairly good effect on *T. gondii* infection (Alday and Dogget, 2017). Currently, existing therapies must be used for one year, if less than one year, the treatment can cause side effects such as liver necrosis, agranulocytosis, quite severe disabilities in infants, and parasite resistance (Bollani *et al*., 2022). Anti-toxoplasmosis drugs must exhibit low toxicity and have tolerable side effects (Hajj *et al*., 2021). Currently available drugs are inadequate, drugs used for toxoplasmosis therapy have shown low bioavailability, limited efficacy, and high toxicity (Jiang *et al*., 2024). Exploring natural products as toxoplasmosis therapies with low toxicity is crucial. Natural products such as propolis have been shown to possess antibacterial (Otreba *et al*., 2022), immunostimulatory (Balica *et al*., 2021), antiviral (Tanuğur-Samanci *et al*., 2023), and antiparasitic activities (de L. Paula *et al*., 2021).

Bee-derived propolis has been confirmed to have significant activity against the inhibition of *Plasmodium falciparum* (*P. Falciparum*) apicomplexa, propolis exhibits stable protein-ligand interactions and has favorable structural properties (Khan *et al*., 2025). Natural compounds such as phenolics, terpenoids, tannins, and alkaloids in propolis that are immunomodulatory significantly increase TNF-α, and IFN-γ (Zulhendri *et al*., 2021). The results of macroscopic liver images of Hegazi *et al*. (2023) study on rabbits infected by Eimeria stidae oocysts and treated with propolis showed low lesions due to infection due to protection from propolis compared to the control group that was not given therapy. Microscopically, the livers of rabbits in the control group without therapy showed proliferation, biliary epithelial hyperplasia, significant enlargement of the bile ducts, bile duct hyperplasia surrounded by a capsule of fibrous connective tissue and edematous. In previous research, we conducted a phytochemical analysis of propolis from *Trigona* spp. bees in Lombok, West Nusa Tenggara, Indonesia, which showed that propolis from moringa resin had an antioxidant activity of 38.4150, which is considered very strong compared to propolis from cashew, jujube, and coffee resins. Antioxidants from natural compounds have beneficial effects on pathological lesions of toxoplasmosis infection caused by the presence of ROS and have the potential to limit parasite growth reactive oxygen species (ROS) (Szewczyk-Golec *et al*., 2021). Extraction of propolis using ethanol produces greater flavonoid and polyphenol antioxidants (Dantas Silva *et al*., 2017; Tirtasari *et al*., 2024), antioxidants are able to improve histological structure and suppress the multiplication of *T. gondii* tachyzoites (Ayad *et al*., 2025). Based on this phytochemical analysis, we wanted to determine the potential of propolis as a treatment for toxoplasmosis infection so that it could be used as a candidate to replace existing drugs.

MATERIALS AND METHODS

Propolis extraction

The propolis used in this study was produced by *Trigona* spp. bees raised in the Moringa vegetation area of Lombok, West Nusa Tenggara, which has been shown to contain high antioxidants (Tirtasari *et al*., 2024). Propolis extraction used 70% ethanol, based on research by Hegazi *et al*. (2017). The propolis was macerated for 7 days in 70% ethanol at a ratio of 1 parts propolis to 10 parts ethanol. Then, it was filtered using paper to separate the pulp and filtrate. The filtrate was evaporated at 75°C–80°C, and this extraction was used for subsequent testing.

Toxoplasma gondii culture

The *T. gondii* parasite used in this study was the RH strain from the Bogor Veterinary Instrument Standard Testing Center (BBPSIV). Maintenance was carried out by passing 100 tachyzoites into the peritoneal cavity, and parasite harvesting was carried out by collecting peritoneal fluid on days 3-4 after infectomy or after the mice showed symptoms (Suwanti and Mufasirin, 2018). The number of tachyzoites obtained was counted using a hemocytometer.

Toxoplasma gondii infection and propolis ethanol extract (EEP) administration in experimental animals

Six groups of mice (Table 1) with a total of 36 mice, each consisting of six mice.

- Group 1: Negative Control (P1): These mice were neither infected nor treated.
- Group 2: Treatment (P2): Infected with *T. gondii* without treatment.
- Group 3: Treatment (P3): Infected with *T. gondii* and treated with Cotrimoxazole 40 mg/kgBW.
- Group 4: Treatment (P4): Infected with *T. gondii* and

treated with EEP 1 mg/ mouse.
- Group 5: Treatment (P5): Infected with *T. gondii* and treated with EEP 2 mg/ mouse.
- Group 6: Treatment (P6): Infected with *T. gondii* and treated with EEP 4 mg/ mouse.

Table 1: Experimental design. A fixed per-mouse dose was applied following established protocols. Body weights were controlled within a narrow range to ensure that effective mg/kg exposure remained consistent across animals.

Group	Treatment	Dose
P1	Negative control, uninfected and untreated	-
P2	*T. gondii*-infected, untreated	-
P3	*T. gondii* -infected, treated with cotrimoxazole	40 mg/Kg body weight
P4	*T. gondii* -infected, treated with EEP	1 mg/mouse
P5	*T. gondii* -infected, treated with EEP	2 mg/mouse
P6	*T. gondii* -infected, treated with EEP	4 mg/mouse

Treatment doses were calculated as fixed amounts per mouse (e.g., 1 mg/mouse). All experimental mice were age- and weight-matched, with initial body weights ranging from 20–22 g, to reduce variability in dose relative to body weight. While minor differences in mg/kg could exist, these are considered negligible given the narrow weight range, and the fixed-dose approach was chosen for practical consistency in administration. Treatment began one day after infection and lasted for three days. On the fifth day post-infection, the mice were euthanized and then the liver and spleen were removed for histopathological examination and to check the amount of TNF-α, IFN-γ, and caspase-3.

Flow cytometry liver and spleen

The livers and spleens of 36 mice were used for flow cytometry, their spleen and liver examine the population of TNF-α, IFN-γ, and caspase-3 within splenocytes and all liver cells. The examination began with sample preparation by grinding the organs and mixing them with phosphate-buffered saline (PBS), then filtering them. The supernatant was transferred to a propylene tube and centrifuged at 2,500 rpm for 5 minutes at 10°C. The supernatant was discarded. The resulting pellet was resuspended in 1 ml of PBS. The suspension was aliquoted into 1.5 ml microtubes, adding 50 µl of PBS. The mixture was centrifuged again at 2,500 rpm for 5 minutes at 10°C. The supernatant was discarded, and staining was continued.

Spleen and liver then labeled with flourescent antibodies, monoclonal antibodies used include anti-TNF-α (PE, clone 52B83) (Novus Biological), anti-TNF-α (FITC, clone ESBA 105) (Novus Biological), anti IFN-γ (FITC-clone XMG1.2) (Sigma-Aldrich), anti IFN-γ (PE, clone XMG 1.2) (Sigma-Aldrich), anti-caspase-3 (PE, clone 31A1067) (Novus Biological), and anti-caspase-3 (FITC, clone 31A1067) (Novus Biological). The initial stage of extracellular staining was carried out by adding 50 µl of specific antibody to the pellet obtained and then incubating it at 40°C for 20 minutes in a dark room. After 20 minutes, 400 µl of PBS was added to the pellet and transferred into a cuvette for flow cytometry analysis. The second stage was intracellular staining, which was carried out by adding 50 µl of fixation buffer solution to the pellet and then incubating it at 40°C for 20 minutes. After 20 minutes, 500 µl of permeability solution and paint were added to the pellet and then homogenized by centrifuging it for 5 minutes at 2,500 rpm at 10°C.

Data analysis

The results of histopathology and flow cytometry examinations were presented as percentages of the cell population TNF-α, IFN-γ, and caspase-3 within all of the cells population (splenocyte and all liver cells), which were then analyzed using one-way ANOVA with a Post Hoc Duncan Multiple Range Test in SPSS 30 software.

RESULTS AND DISCUSSIONS

TNF-α, IFN-γ, and caspase-3 cell populations from flow cytometry liver and spleen

The results of TNF-α levels in mouse spleen cells showed a significant difference between the P2 (IFN-infected mice without drug administration) and P6 (4 mg EEP administration) treatments compared to P3 (the cotrimoxazole-treated group). The P2 and P6 treatment groups showed a decrease in TNF-α levels compared to the P3 treatment group, which showed a very high number of TNF-α cells. The mean and SD values for the P2 group were 3.37 ± 1.19 and the P6 group 3.18 ± 1.67, significantly different ($p < 0.05$) from the P3 group, which had a mean and SD of 7.19 ± 3.39. The P1, P4, and P5 treatment groups showed no significant difference ($p > 0.05$). Cotrimoxazole can increase the levels of CXCL8 and TNF-α secretion by macrophages as an initial response to infection to kill infectious agents (Chen *et al*., 2022), increased expression of proinflammatory cytokines, including iNOS, plays an important role in the mechanism of resistance response to infectious agents, proinflammatory cytokines maintain the dormancy of *T. gondii*, but excessive production is often associated with disruption of the antioxidant system and excessive pathological inflammatory reactions (Aboukamar *et al*., 2022). *Toxoplasma gondii* infection results in increased oxidative stress (OS), oxidative stress is often accompanied by nitrosative stress resulting from the production of nitric oxide (NO) and reactive nitrogen species, which are key motivators of inflammation and pro-inflammatory signals. Therefore, antioxidants function as anti-inflammatory

molecules (Salama *et al.*, 2024). In several studies, the antioxidant content of propolis can inhibit inflammation (Sahran *et al.*, 2022; Rebouças-Silva *et al.*, 2023).

IFN-γ cells treated with P2 and P3 showed significantly different results, but treatments P1, P4, P5, and P6 did not differ significantly. The mean and SD values of IFN-γ cells in P2 with a value of 4.10 ± 1.34 were significantly different compared to the mean and SD values of IFN-γ cells treated with P3 9.29 ± 2.35 ($p < 0.05$). The results of Caspase-3 cells showed no significant differences in all treatments.

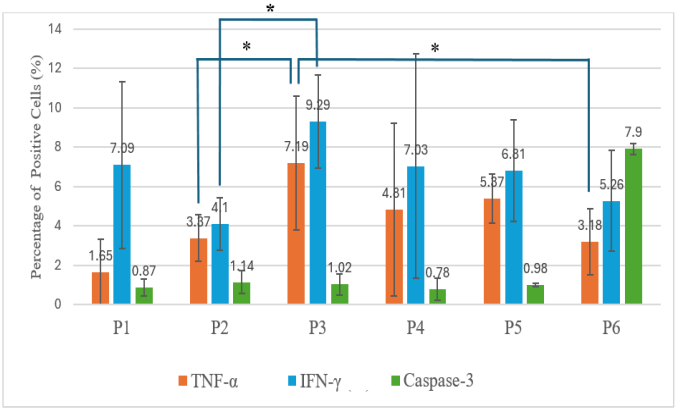

Figure 1: Spleen cytokine expression. Percentage of positive cells (%) for TNF-α, IFN-γ, and Caspase-3 within total splenocyte of mice infected with Toxoplasma gondii and treated with different doses of EEP. Data are presented as mean ± standard deviation (n = 6 per group). Asterisks indicate statistically significant differences between groups ($P < 0.05$) as determined by one-way ANOVA followed by post hoc analysis.

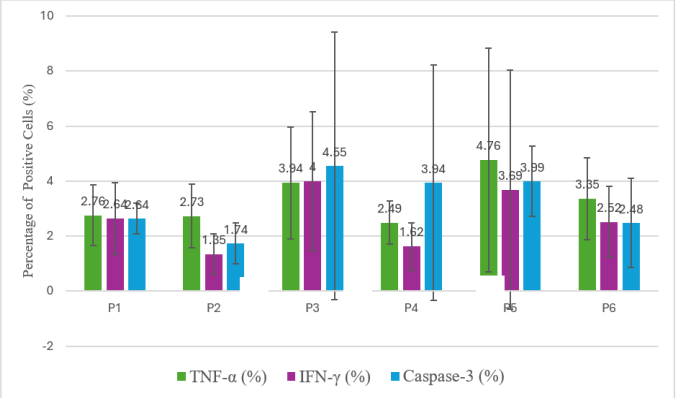

Figure 2: Liver cytokine expression. Percentage of positive cells (%) for TNF-α, IFN-γ, and caspase-3 within all liver cells of mice infected with Toxoplasma gondii and treated with different doses of EEP. Data are presented as mean ± standard deviation (n = 6 per group). No significant difference was observed in the liver at day 5 post-infection.

Cytokines are mediator cells that are released when there is an infection and are not specific to antigens in the acute infection phase, macrophages will increase followed by an increase in chemokines, interleukin-12 (IL-12), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) (Atmaca and Atmaca, 2022). The results of our study showed that the expression of TNF-α and IFN-γ cells in the spleen of the treatment group given EEP treatment showed no significant increase when compared to control mice. The expression of IFN-γ and TNF-α will increase significantly in patients infected with *T. gondii* compared to the healthy group, the pro-inflammatory cytokines IFN-γ and TNF-α are often associated with protection against acute *T. gondii* infection (Abdeltawab *et al.*, 2024). During the acute phase of IFN-γ and TNF-α are produced by innate immune cells, these cytokines kill free parasites and are assisted by macrophages, while NK and CD8+ cells lyse infected cells (Rico-Torres *et al.*, 2025). In line with our previous research, that EEP at a dose of 4 mg on the sixth day was able to suppress the expression of pro-inflammatory cytokines (IL-2 and IL-17) and increases expression antiinflammatory cytokines cell (IL-10) (Novryantoro *et al.*, 2024).

The values of lymphatic TNF-α cells in the control group, treatment groups with EEP doses of 1 mg and 2 mg were 1.65 ± 1.65, 4.81 ± 4.39, and 5.37 ± 1.24, respectively. In our study, the number of lymphatic TNF-α and IFN-γ cells increased in the treatment treated with cotrimoxazole. TNF-α can play a role in controlling *T. gondii* infection, excessive TNF-α production causes immunopathology and worsens inflammation, TNF-α, IL-6 and IL-1β increased significantly at 6 to 24 hours after *T. gondii* infection (Hameed *et al.*, 2025). The increasing number of TNF-α cells in the acute phase of *T. gondii* infection will increase the response of IFN-γ and IL-12 cells, IL-12 induces T cells and the release of IFN-γ on the lytic activity of NK cells, generally IFN-γ cells depend on the presence of CD8+ and the effectiveness of CD8+ cells depends on the presence of CD4+ cells which are the initiators of cytokine movement in mice, the presence of TNF-α and IFN-γ in toxoplasmosis infection can produce nitric oxide which plays a role in inhibiting pathogens (Sana *et al.*, 2022).

The cytokine IFN-γ is an immune response that prevents parasitemia due to *T. gondii* infection (Mukhopadhyay *et al.*, 2020). In line with research by Althobaiti (2022), treatment using royal jelly can increase IL-1β and IFN-γ levels. The increase in proinflammatory cytokine levels occurs due to acute inflammation due to *T. gondii* infection. Day by day until the end of treatment, proinflammatory cytokine levels will decrease because propolis content such as antioxidants, flavonoids, and phenolics are able to suppress the increase in proinflammatory cytokines (Hegazi *et al.*, 2021).

However, our results differ from the results of Shehata *et al.* (2024) study in which significant differences occurred

in TNF-α and IFN-γ in the liver of the treatment group given anti-parasitic drugs (P3) compared to the control group, while in our study TNF-α and IFN-γ in the liver of all treatment groups were not significantly different. According to the results of the study of Atmaca and Almaca (2022), the levels of pro-inflammatory cytokines in RH strain mice infected with *T. gondii* without treatment increased on the second day and decreased on the sixth day, in line with our study where we saw the levels of TNF-α, IFN-γ, and caspase 3 on the fifth day it is very possible that the number of pro-inflammatory cytokine cells is at a decreasing rate.

The active component of propolis caffeic acid pohenethyl ester (CAPE) has an anti-inflammatory effect through the NF-*kB* signaling pathway which is one of the inflammatory pathways, propolis is able to reduce inflammation, cell damage, and injury due to its high antioxidant content (Jalali *et al*., 2020). Propolis modulates the trans-differentiation of MQ macrophages thereby suppressing myeloid (MDSC) D11b+ and Gr-1+ in adipose tissue of visceral organs and the peritoneal cavity, propolis administration significantly increases IgG levels and reduces the number of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, and IL-6) (Zulhendri *et al*., 2022). The main mechanism of propolis includes reducing the secretion of inflammatory cytokines through reducing oxidative stress, there is a difference in the reduction of serum TNF-α and IL-6 in Asian and American propolis, Asian propolis significantly reduces serum TNF-α and IL-6 compared to American propolis, this occurs because the levels of phenolic acids and total flavonoids are very different (Gholami *et al*., 2024). In line with previous research, the results of the antioxidant activity test of moringa resin in our previous study were very high so we used it for the current test which showed the effect of reducing TNF-α and IFN-γ levels (Deghbar *et al*., 2019; Gűlpinar *et al*., 2025).

Liver images in mice infected with *T. gondii* showed focal hepatocellular, individual cell apoptosis, lymphocytic cholangitis, lymphoplasmacytic infiltration, and portal biliary reaction (Mustofa *et al*., 2024). Propolis therapy can increase liver protection through antioxidant effects compared to antitoxoplasma drugs, liver images of mice treated with propolis showed moderate to minimal inflammation in the portal tract, moderate vascular dilation, local and minimal inflammation of the hepatic portal, and no capsule edema with barely visible parasites (Hagras *et al*., 2022).

Overall, our findings indicate that *T. gondii* infection treated with EEP can reduce the levels of pro-inflammatory cytokines (TNF-α, IFN-γ, and caspase 3), suggesting that EEP has potential as an agent to mitigate pro-inflammatory responses. However, it is important to note a limitation of our study: We did not assess liver parasite burden or perform histopathological scoring at the examined timepoints. Therefore, while macroscopic and histological observations suggest liver protection, the absence of quantitative measures limits our ability to fully characterize hepatic infection progression and correlate it directly with cytokine changes.

CONCLUSION

Ethanol extract Propolis of *Trigona* spp. bee from moringa plants native to Lombok, West Nusa Tenggara, showed that have protective effects against *T. gondii* infection in mice, as evidenced by reduced levels of pro-inflammatory cytokines (TNF-α, IFN-γ, and caspase 3), particularly at a dose of 4 mg.

These findings indicate that EEP is a promsing anti-toxoplasmosis agent for suppressing the increase in anti-inflammatory cytokines. Further research is needed to explore the cell characteristics of experimental animals infected with *T. gondii* and treated with EEP.

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NOVELTY STATMENT

This study is the first to investigate the immunomodulatory effects of *Moringa*-derived ethanolic extract of propolis (EEP) on early cytokine responses (TNF-α, IFN-γ, and caspase-3) in the liver and spleen of mice infected with *Toxoplasma gondii*, providing organ-specific insights into the potential of a targeted natural immunotherapeutic against toxoplasmosis.

AUTHOR CONTRIBUTION

KT collecting data, LTS concept and design of this study. MM analysis and data curration. LRY analysis and interpretation of the data. ALDA assembled the data. VN and AS collected the sample and laboratory work. SK and HP handled the infection procedures, dissected the mice, and removed the organs. All authors have read, reviewed, and approve the manuscript.

Ethical approval

The treatment in this study was approved by the Airlangga University Animal Care and Use Committe under Decree No. 1.KEH.119.09.2022.

Generative AI and AI-assisted technology statement

Generative AI tools were used only for language editing and clarity. All scientific content, analysis, and conclusions are original and have been reviewed and verified by the author. No AI was used to generate data or research findings.

Conflict of interest

The authors have declared no conflict of interest.

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