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Anti-inflammatory effect of Faloak (*Sterculia quadrifida* R. Br) stem bark on TNF-α, IL-1β, and IL-6 in DENV-3-infected Wistar rats

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Abstract

Background: Dengue infection can trigger an immunological response that results in an inflammatory reaction, which acts as a defensive mechanism to protect the host. Dengue infection leads to an elevation in the release of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These three cytokines have been shown to correlate with the development of thrombocytopenia and plasma leakage, which is related to the severity of the disease.

Aim: This study aims to investigate the effect of faloak (*Sterculia quadrifida* R. Br) stem bark on TNF-α, IL-1β, and IL-6 levels in Wistar rats infected with dengue, specifically DENV-3.

Methods: A group of 27 male Wistar rats (*Rattus norvegicus*) aged 2–3 months and weighting 200–300 g were divided into three distinct groups: healthy, dengue, and treatment (dengue infection and extract) groups. The rats in both the dengue and treatment groups were administered an injection of DENV-3 with a titer of 105 pfu at a dosage of 0.8 cc via the intraperitoneal route. The propagation of DENV-3 was initiated using C6/36 cells, and it underwent four passages. The extract was administered orally via a nasogastric tube at a dosage of 1,500 mg/kg body weight once daily for 7 days. The healthy group underwent blood sampling on the first day, whereas the dengue and therapy groups underwent blood sampling on the fifth and eighth, respectively.

Results: Compared with the healthy group, TNF-α levels in the dengue and treatment groups showed significant differences on day 5 post-infection. The *post hoc* analysis revealed a statistically significant difference between the dengue-treatment and dengue-healthy groups. The IL-1β levels in the dengue and healthy groups significantly differed on days 5 and 8 post-infection compared to the healthy group. The treatment group had less of a decrease in IL-6 levels on days 5 and 8 than the dengue group. However, no statistically significant differences were observed.

Conclusion: The stem bark of *S. quadrifida* shows potential as an anti-inflammatory agent in dengue infections, particularly in its ability to decrease levels of TNF-α and IL-1β.

Keywords: Anti-inflammation, Dengue, Pro-inflammatory cytokine, *Sterculia quadrifida* R. Br, Wistar rats.

Introduction

Dengue is an arbovirus infection caused by one of four dengue virus serotypes (DENV1-DENV4), leading to a range of clinical symptoms varying from mild to severe. The worldwide incidence of this disease has seen a significant global escalation in recent decades. Most dengue infections are asymptomatic or exhibit minor symptoms that might resolve without medical intervention. In addition, many cases often go undiagnosed and are misclassified as other febrile illnesses, leading to an incomplete recording of the actual prevalence rate of severe dengue (Iyer and Thangam, 2022). The incidence rate, as reported to the World Health Organization (WHO), has exhibited a significant increase of up to eightfold during the past two decades. The number of instances observed in 2,000 amounted to 505,430, which then increased to 2.4 million cases in 2010 and escalated to 5.2 million cases in 2019. Based on prevalence surveys, the estimated number of individuals at risk of DENV infection is as high as 3.9 billion. Dengue has achieved endemic status in over 100 countries, with the Asian area contributing to over 70% of reported cases (WHO, 2023).

DENV is transmitted to humans via the bite of the *Aedes aegypti* or *Aedes albopictus* mosquito. The virus undergoes replication within skin cells, specifically keratinocytes, and Langerhans cells, thus triggering the activation of the innate immune response to protect the host. DENV can stimulate the innate immune response,

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leading to the release of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These cytokines are crucial in attracting and activating various immune cells involved in inflammatory reactions and initiating adaptive immune responses (Imad *et al.*, 2020). The elevation of these three cytokines has been observed in cases of dengue infection and has been linked to the occurrence of plasma leakage, thrombocytopenia, and tissue damage in dengue infection (Butthep *et al.*, 2012; Masood *et al.*, 2018; Pan *et al.*, 2019; Varghese *et al.*, 2019;).

Currently, targeted therapeutic interventions are absent for effectively managing dengue. The therapeutic approach employed for dengue fever patients is primarily supportive and symptomatic, aiming to alleviate the clinical manifestations of the disease (WHO, 2023). The utilization of herbal medicines as a therapeutic approach for various diseases is experiencing a significant increase among people living in tropical and subtropical areas. In addition, around 80% of the population in developing countries, including Asia and Africa, rely on herbal medicines as their primary source of medical therapy (Saleh and Kamisah, 2021). Hence, it is imperative to investigate the potential of utilizing traditional medicinal herbs as a viable resource for dengue therapy.

Sterculia quadrifida R. Br is a traditional medicinal plant commonly used by Aboriginal People in North Queensland, Australia, and the people on Timor Island, Indonesia, for treating various health conditions (Saragih and Siswadi, 2019; Australia Tropical Rainforest Plants, 2020). The people of Timor Island commonly utilize the stem bark of *S. quadrifida* or Faloak (local name in Indonesia) to treat several medical medical conditions, such as hepatitis, kidney disease, rheumatism, lower back pain, and anemia (Saragih and Siswadi, 2019). The stem bark of *S. quadrifida* has been found to contain flavonoids, alkaloids, terpenoids, phenolic compounds, and saponins (Siswadi and Saragih, 2019). In addition, specific compounds such as (+)-catechin (Riwu *et al.*, 2023), epicatechin (Dean *et al.*, 2019), scopoletin (Munawaroh *et al.*, 2020), and β-sitosterol (Lulan, 2020) have been identified. Prior research has established that the stem bark exhibits potent antioxidant activity (Amin *et al.*, 2015; Dillak *et al.*, 2019), and possesses immunomodulatory properties through the regulation of macrophage phagocytic activity, as well as the modulation of nuclear factor-kappaB and TNF-α (specifically in combination with *Phyllanthus urinaria*) (Winanta *et al.*, 2019; Munawaroh *et al.*, 2020; Rollando *et al.*, 2020).

No prior research investigations have been conducted on using *S. quadrifida* stem bark as an anti-inflammatory agent in dengue infections. Based on the background mentioned above, this study aims to investigate the effect of *S. quadrifida* stem bark extract on the levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in Wistar rats infected with DENV-3.

Material and Methods

Plant material

The stem bark of *S. quadrifida* was obtained from Kupang City, located in East Nusa Tenggara, Indonesia. The stem bark is extracted through the infusion method. The powdered simplicia is dissolved in distilled water and boiled to a temperature of 90°C for 15 minutes (Santosa *et al.*, 2024). Afterward, the filtrate went through freeze-drying to get a crude extract.

Virus propagation

DENV-3 was propagated in C6/36 cells. C6/36 cells were cultured in a T-25 culture flask and incubated at 28°C. The cells were maintained in Minimum Essential Medium (MEM) (Sigma Aldrich) media supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich), 2% penicillin-streptomycin (Sigma Aldrich), and 0.5% amphotericin B (Gibco). Once confluent to 90%–95%, the cells were infected with 300 µl of the virus for 1 hour with agitation every 15 minutes. Following incubation, the inoculum was removed, and 5 ml of 2% MEM complete (2% FBS, 2% Penicillin-streptomycin, 0.5% Amphotericin B) was added and cultured for 5–7 days in a 28°C incubator while monitoring the Cytopathic Effect (Chen *et al.*, 2012; Hitakarun *et al.*, 2020). The virus was subjected to four rounds of passages on C6/36 cells, with each passage having an incubation period of 5–7 days.

Plaque assay for virus titrating

DENV titer was determined by plaque assay. C6/36 cells with a density of 1.5×10^5 were cultured on 24-well plates in 10% MEM (Sigma Aldrich) and incubated at 28°C for 1–2 days until confluent. Afterward, the medium was removed, and a six-fold dilution of the virus was added to the cells, with each well consisting of 200 µl. Then, the plates were incubated for 2 hours at 28°C and added with 1% CMC overlay media. Plates were incubated for 5–7 days, fixed in 3.7% formaldehyde, and stained with 1% crystal violet for 15–30 minutes. Plaques were counted manually, and titers were expressed as plaque form unit (pfu)/ml (Alkaff *et al.*, 2019).

Experimental unit

Thirty male Wistar rats (*Rattus norvegicus*) weighing between 200 and 300 g and aged 2–3 months were acquired from the Animal House, Faculty of Medicine-Public Health and Nursing, University of Gadjah Mada Yogyakarta. Three rats were utilized in the initial phase to confirm the infected model, while 27 were employed in the primary study. The 27 rats were divided into three groups: a healthy group (n = 9), a dengue group (n = 9), and a treatment group with dengue infection and administration of the extract (n = 9). The extract was orally administered with a 1,500 mg/kg BW dose dissolved in 3 cc of distilled water.

The rats were acclimated for a week in a controlled environment with temperatures ranging from 20°C–24°C and humidity levels ranging from 30%–70%. During the adaption phase, food and water were

provided ad libitum. The lighting was controlled with a 12-hour cycle of light and darkness, starting from 06:00 to 18:00.

Dengue infection

The dengue infection model was initially established in three rats to improve the virus dose and injection method. The evaluation of infections includes assessing the NS-1 protein (Right Sign NS-1 Antigen Rapid Test). Rats were infected with DENV-3 by an intraperitoneal injection (Utama *et al*., 2023), with a viral titer of 1×10^5 pfu (Triyono, 2020) at a dosage of 0.8 cc. On days 1 and 3, blood samples were obtained via the retro-orbital vein. On the third day, NS-1 was confirmed to be positive. The viral dosage and injection technique employed in this stage was subsequently adopted as the protocol for the main study.

Blood collection

Before blood collection, all rats were administered an intramuscular injection of ketamine at a dose of 0.5 cc/kg to induce anesthesia. The healthy group was subjected to sampling on the initial day and subsequently euthanized. The dengue and treatment groups underwent blood sampling on the fifth and eighth days. Blood was obtained via the retro-orbital vein on the fifth day using a micro-capillary tube. Cardiac puncture was performed on the first and eighth days using a 23G needle. After blood collection on days 1 and 8, the sedated rats were euthanized by cutting the aorta.

TNF-α, IL-1β, and IL-6 analysis

Serum levels of TNF-α, IL-1β, and IL-6 were analyzed using Enzyme-Linked Immunosorbent Assay using kits from Abbkine EliKine™ (Abbkine Scientific Co., Georgia, USA) catalog numbers KTE9007, KTE9001, and KTE9004, respectively. The concentration of each cytokine is determined using a standard curve.

Statistical analysis

The statistical analysis used one-way ANOVA (normal and homogenous data distribution) or Kruskal-Wallis (abnormal distribution) tests, followed by a post-hoc analysis using the least significant difference test or Pairwise Comparison (Kruskal-Wallis) with the SPSS 26 version. The results are presented as the mean value ± SD. A *p*-value less than 0.05 was determined statistically significant.

Ethical approval

The Ethics Committee of the Faculty of Medicine-Public Health and Nursing, Gadjah Mada University, has approved all treatments and experimental protocols. The approval number was KE/FK/1452/C/2022.

Results

Effect of S. quadrifida stem bark on TNF-α

The levels of TNF-α in the healthy, dengue, and treatment groups can be seen in Figure 1 with the healthy group being used as the reference value. The TNF-α in the dengue and treatment group were increased on day five compared to the healthy group and then decreased on day 8 after infection (Fig. 1). A statistically significant difference with a *p*-value <0.05 was only seen on day 5 after the infection. Table 1 displays the differences within the groups on the fifth day following infection. Significant differences were identified between the healthy-dengue group and the treatment-dengue group.

Effect of S. quadrifida stem bark on IL-1β

IL-1β levels in healthy, dengue, and treatment groups are shown in Figure 2. IL-1β levels in the healthy group were used as a reference value. Statistically significant differences were seen on days 5 and 8 after infection, with *p*-values of 0.028 and 0.038, respectively. Analysis of differences using *post-hoc* tests can be seen in Table 2. Statistically significant differences were seen solely between the healthy group and the dengue group, on both day 5 and day 8 following infection.

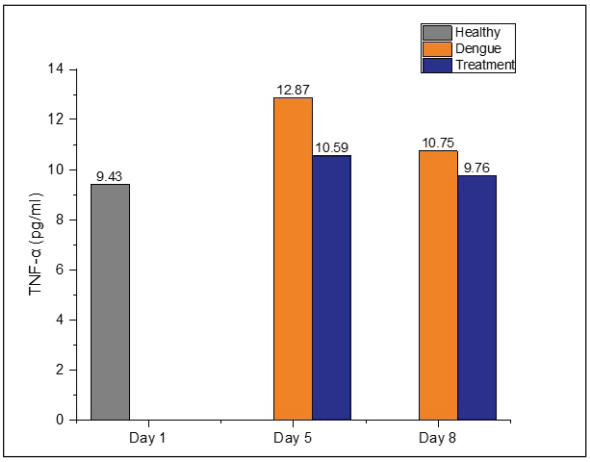

Fig. 1. The levels of TNF-α were measured in three groups: the healthy, dengue, and treatment group (dengue + *S. quadrifida*). TNF-α levels in the healthy group were used as reference values. On day 5 post-infection, there was a statistically significant difference with a *p*-value <0.05 (0.003). However, on day 8, no significant difference was seen [*p*-value > 0.05 (0.529)].

Table 1. *Post-hoc* analysis of TNF-α levels on day 5 post-infection

Groups	Mean ± SD (pg/ml)	*p-value*
Treatment	10.75 ± 1.76	0.028*
Dengue	12.87 ± 1.26	
Treatment	10.75 ± 1.76	0.157
Healthy	9.43 ±2.51	
Dengue	12.87 ± 1.26	0.001*
Healthy	9.43 ±2.51	

**p*-value < 0.05: statistically significant difference.

Effect of S. quadrifida stem bark on IL-6

The levels of IL-6 in healthy, dengue, and treatment groups are displayed in Figure 3. On the fifth day after infection, the levels of IL-6 in the dengue group were observed to have increased to 515.05 pg/ml, whereas the healthy group and the treatment group had levels of 399.27 and 448.74 pg/ml, respectively. Nevertheless, no statistically significant difference was identified. On the eighth day post-infection, the levels of IL-6 in both the dengue and treatment groups were similar to the healthy group, indicating no significant.

Discussion

This current study demonstrated that TNF-α, IL-1β, and IL-6 levels were elevated in both the dengue and treatment groups compared to the healthy group on days 5 and 8 following infection. However, the rats in the treatment group administered *S. quadrifida* stem bark extract exhibited comparatively lower cytokine levels than those infected with dengue alone. Notably, significant reductions were observed in the levels of TNF-α on day five and IL-1β on days 5 and 8.

TNF-α is a cytokine with pro-inflammatory properties that exhibits diverse actions and holds significance in the pathogenesis of several inflammatory disorders. This cytokine is primarily synthesized by monocytes/macrophages in significant amounts, as well as by other cell types, including mast cells, neutrophils, natural killer cells, fibroblasts, osteoclasts, T and B lymphocytes, but in smaller levels (Horiuchi, 2010). In dengue infection, TNF-α can stimulate the production of reactive oxygen intermediates and reactive nitrogen intermediates. In addition, it can trigger cell death through apoptosis, resulting in heightened vascular permeability and subsequent bleeding. This is supported by other research findings that indicate a positive correlation between elevated levels of TNF-α

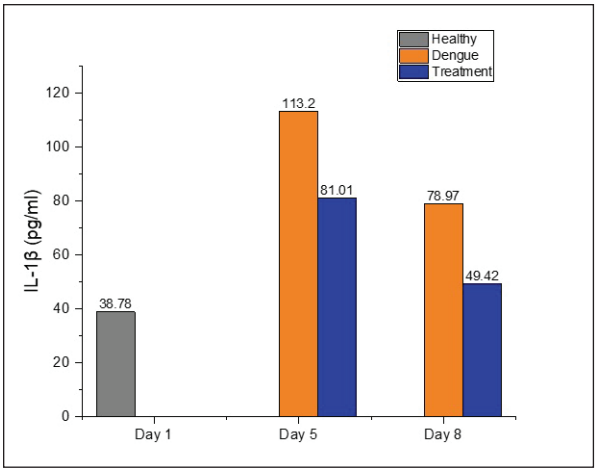

Fig. 2. IL-1β levels in healthy, dengue, and treatment groups (dengue + *S. quadrifida*). IL-1β levels in the healthy group were used as a reference value. Statistically significant differences were seen on days 5 and 8 after infection, with *p*-values of 0.028 and 0.038, respectively.

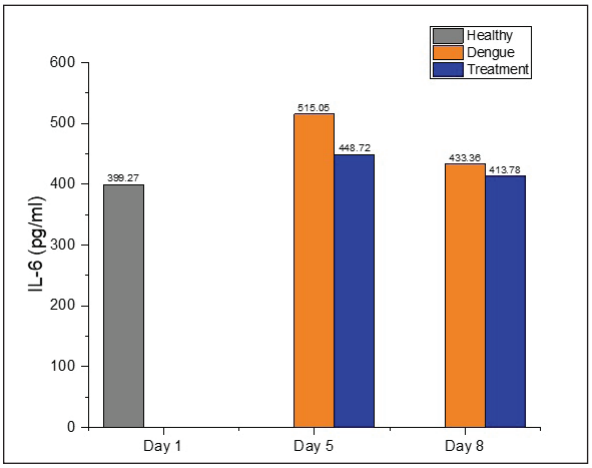

Fig. 3. IL-6 levels on healthy, dengue, and treatment group. The healthy group's IL-6 levels were employed as a standard to determine normal values. No significant differences were seen between the groups on days 5 and 8 following infection (day 5: *p*-value = 0.227; day 8: *p*-value = 0.528).

Table 2. *Post-hoc* on IL-1β levels on days 5 and 8 post-infection.

Groups	Day 5		Day 8	
	Mean ± SD (pg/ml)	*p-value*	Mean ± SD (pg/ml)	*p-value*
Treatment	81.01 ± 49.35	0.387	49.42 ± 32.59	0.089
Dengue	113.20 ± 71.91		78.97 ± 46.32	
Treatment	81.01 ± 49.35	0.078	49.42 ± 32.59	0.420
Healthy	38.78 ± 22.73		38.78 ± 22.73	
Dengue	113.20 ± 71.91	0.009*	78.97 ± 46.32	0.012*
Healthy	38.78 ± 22.73		38.78 ± 22.73	

*****p-value < 0.05: statistically significant difference.

and enhanced vascular permeability in dengue (Masood *et al.*, 2018).

The study observed an elevation in TNF-α levels in both the dengue and treatment groups compared to the healthy group on the fifth day after infection. This finding aligns with the research conducted by Costa *et al.* (2012), which demonstrated an elevation in serum TNF-α levels in C57BL/6 mice infected with DENV-3 on the fifth day. However, the treatment group exhibited slightly lower TNF-α levels than the dengue group on day 5 after infection, which was statistically significant (Table 1). These findings demonstrate that the stem bark extract of *S. quadrifida* can effectively decrease TNF-α levels in Wistar mice infected with DENV-3.

IL-1β is a pro-inflammatory cytokine produced by macrophages using two mechanisms: activation of toll-like receptor, which results in the synthesis of pro-IL-1β, and activation of NLR family pyrin domain containing (NLRP3), which relies on caspase-1 and inflammasomes (Gabay *et al.*, 2010). This cytokine can stimulate the activation of neutrophils and the production of cytokines. It also can activate T and B cells, which leads to the formation of antibodies. In addition, it has a role in angiogenesis by promoting the synthesis of vascular endothelial growth factor in conjunction with TNF-α and IL-6 (Maloney and Gao, 2015). The levels of IL-1β in patients with dengue infection showed a significant rise compared to healthy individuals, and this increase was linked to the severity of the disease. IL-1β has been demonstrated to enhance the permeability of blood vessels primarily through its interaction with TNF-α and interferon-γ, both of which are elevated in individuals with severe dengue (Tuyen *et al.*, 2020).

In the C57BL/6 mouse model infected with dengue, the levels of serum IL-1β were seen to increase on the fourth-day post-infection, along with IL-6 and TNF-α (Marques *et al.*, 2018). Similar to this research, the present study observed a notable rise in IL-1β levels in the dengue and treatment groups on days 5 and 8 following infection compared to the healthy group. Furthermore, the mean IL-1β levels in the extract group were shown to be lower than those in the dengue group. Still, the difference was not statistically significant on either day 5 or day 8 following infection (Table 2). Nevertheless, the extract derived from the stem bark of *S. quadrifida* may possess the capability to reduce the levels of IL-1β.

IL-6 is a pleiotropic cytokine that affects inflammation, immunological response, and hematopoiesis. IL-6 is crucial in immunological responses, as it promotes the generation of antibodies and effector T cells and the proliferation of both immune and non-immune cells (Tanaka *et al.*, 2014). The IL-6 levels in dengue infection showed a comparable increase as TNF-α (Varghese *et al.*, 2019). IL-6 has been identified for its role in regulating the elevation of C-reactive protein and secreted phospholipase A2 (Masood *et al.*, 2018). In addition, IL-6 can produce prostaglandin E2, which leads to enhanced endothelial permeability. The levels of IL-6 exhibited an initial increase during the early phases of the disease and subsequently experienced significant elevation in conditions of shock (Butthep *et al.*, 2012). The research study discovered that the levels of IL-6 in the treatment group were significantly lower than those in the dengue group on days 5 and 8 post-infection. However, the difference was not statistically significant. Nevertheless, there is a possibility that the extract obtained from the stem bark of *S. quadrifida* could decrease IL-6 levels.

Previous studies have shown that the bark of *S. quadrifida* contains a variety of substances, including flavonoids (Saragih and Siswadi, 2019; Munawaroh *et al.*, 2020). Flavonoids are compounds that show anti-inflammatory and antioxidant properties, thereby being important in preventing numerous chronic illnesses, including cancer and cardiovascular disease (Al-Khayri *et al.*, 2022). Several Studies have indicated that flavonoids can inhibit PI3K, JNK, and NF-Kβ signaling pathways, leading to a reduction in the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Wright *et al.*, 2015; Al-Khayri *et al.*, 2022). Furthermore, several flavonoids have shown the ability to decrease the release of TNF-α in macrophages infected with DENV-2 and DENV-3, both in normal infection and antibody-dependent enhancement circumstances (Jasso-Miranda *et al.*, 2019). A study conducted by Munawaroh *et al.* (2020) stated that the total flavonoid content of *S. quadrifida* stem bark extract was positively correlated with macrophage phagocytic activity, with a correlation coefficient (r^2) of 0.61. These findings indicate that flavonoids account for 61% of macrophage phagocytic activity, with the remaining 39% attributed to other compounds, such as terpenoids. In addition, flavonoids exhibit antiviral properties by exerting an effect on the life cycles of various viruses, particularly those belonging to the *Flaviviridae* family, such as hepatitis C virus, ZIKV, and DENV (Badshah *et al.*, 2021; Cateneo *et al.*, 2021).

The stem bark of *S. quadrifida* is also recognized for its presence of catechin derivative chemicals, including epicatechin (Dean *et al.*, 2019) and (+)-catechin (Riwu *et al.*, 2023). Catechin is a compound that can regulate NF-κB, a transcription factor that regulates the production of different inflammatory genes like pro-inflammatory cytokines (such as TNF-α and IL-6). In addition, it stimulates the release of the precursor IL-1β, which is necessary for activating inflammasomes and promoting the maturation of IL-1β (Liu *et al.*, 2017). Meanwhile, catechin can inhibit NF-κB and FOXO3a by activating the AMPK/SIRT1 pathway, reducing the release of pro-inflammatory cytokines (Cheng *et al.*, 2019). Furthermore, a study conducted by Yi *et al.* (2023) using *in vitro* approaches has demonstrated that catechin can inhibit the replication of DENV, particularly in the post-entry stages. In

addition, it has been observed that catechin can reduce the titers of DENV-1, DENV-3, and DENV-4. The study conducted by Munawaroh *et al.* (2020) also mentioned the presence of scopoletin in *S. quadrifida*. Scopoletin is a derivative of coumarin, a class of compounds commonly found in medicinal plants, including *Aster tataricus* and *Foeniculum vulgare.* Scopoletin has been found to decrease proinflammatory cytokines and chemokines by modulating the signaling pathways of MAAPK, STAT-1, and NF-κB (Kim *et al.*, 2004; Bak *et al.*, 2022).

According to the statement above, it may be concluded that *S. quadrifide* exhibits anti-inflammatory properties, probably due to flavonoids, catechin, and scopoletin. Meanwhile, the research findings indicated no significant differences, which could have been due to the method of extraction and solvent used in the current study. The utilization of water as a solvent and heating it to a high temperature of up to 90°C during the extraction process can lead to the degradation of molecules that impact the amounts of certain chemicals and antioxidant activity that may affect the anti-inflammatory effects produced. However, the extraction using the infusion process is considered to be safer, more convenient, and more applicable to the community (Hidalgo and Almajano, 2017; Réblová *et al.*, 2017; Riwu *et al.*, 2023; Xu *et al.*, 2017).

Conclusion

The findings of this study demonstrate that the stem bark of *Sterculia quadrifida* R. Br can exert an anti-inflammatory effect, particularly by decreasing the levels of TNF-α and IL-1β in cases of dengue infections. The presence of flavonoid compounds and secondary metabolite compounds, such as catechin and scopoletin, is thought to be the reason for this phenomenon. Hence, this extract has the potential to be a drug candidate for inhibiting exaggerated inflammatory responses in dengue infections and other infectious diseases.

Acknowledgment

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Conflict of interest

There is no conflict of interest in this study.

Funding

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Author's contribution

All authors conceptualized the idea and design of the study. AGR performed material preparation, data collection, and analysis. JN, EAT, and DAP supervised the entire research process. AGR drafted the manuscript under JN, EAT, and DAP supervision. All the authors read and approved the final manuscript.

Data availability

All data are available in the manuscript.

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