Bioactivity-Guided Evaluation of *Tabernaemontana divaricata* Leaf Fractions Reveals Promising Anti-Inflammatory, Antipyretic, and Analgesic Effects in Mice

Chaerul Fadly Mochtar[1]*
[1]Faculty of Sports and Health Sciences, University Negeri Makassar, Makassar, South Sulawesi, Indonesia.

Corresponding author: chaerul.fadly@unm.ac.id

ABSTRACT

Inflammation, pain, and fever are major physiological responses to tissue injury or infection that often require pharmacological intervention. However, prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) may cause gastrointestinal and hepatic toxicity, encouraging the search for safer natural alternatives. *Tabernaemontana divaricata* has been traditionally used to treat inflammatory and febrile conditions, yet limited studies have evaluated the pharmacological properties of its solvent fractions. This study aimed to assess the anti-inflammatory, antipyretic, and analgesic activities of the hexane (HeFrTD) and butanol (BuFrTD) fractions derived from the ethyl acetate extract of *T. divaricata* leaves in male mice. The fractions were obtained through liquid–liquid partitioning and administered orally at doses of 62.5, 125, and 250 mg/kg body weight. Anti-inflammatory, antipyretic, and analgesic effects were evaluated using carrageenan-induced paw edema, peptone-induced pyrexia, and hot plate assays, respectively. Statistical analysis was conducted using one-way ANOVA followed by Bonferroni's post hoc test. Both fractions significantly ($p < 0.05$) reduced inflammation, pyrexia, and pain in a dose-dependent manner. The 250 mg/kg dose produced strong anti-inflammatory and antipyretic responses, with no statistically significant difference from diclofenac and paracetamol within the experimental model. Analgesic activity suggested possible involvement of central pathways commonly associated with opioid receptor modulation. In conclusion, the fractions of *T. divaricata* exhibit notable multi-target pharmacological effects, supporting their traditional use and potential for development as natural anti-inflammatory, antipyretic, and analgesic agents.

Keywords: analgesic, anti-inflammatory, antipyretic, *Tabernaemontana divaricata*

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INTRODUCTION

Inflammation is a complex defensive response triggered by the invasion of pathogens, tissue injury, or other harmful stimuli (Sobeh *et al.*, 2020). It is commonly associated with pain and pyrexia, which often require timely pharmacological intervention (Hossain *et al.*, 2014). Although non-steroidal anti-inflammatory drugs (NSAIDs) remain the mainstay therapy for inflammatory conditions, pain, and fever, their prolonged use can lead to adverse effects such as gastric irritation, bleeding, and hepatic or renal toxicity (Sharma *et al.*, 2020). These drawbacks highlight the need to explore safer and more effective alternative therapies. Medicinal plants are increasingly considered as promising candidates due to their broad pharmacological properties and relatively lower toxicity compared to synthetic drugs (Mochtar *et al.*, 2023a).

Tabernaemontana divaricata, a common ornamental and medicinal shrub widely distributed in tropical regions, has long been used in traditional medicine.

Ethnopharmacological reports indicate that *T. divaricata* possesses therapeutic potential in treating asthma, diarrhea, infections, wounds, and various other ailments (Pratchayasakul *et al.*, 2008; Fadhli *et al.*, 2023). Several previous studies have demonstrated its anti-inflammatory, antipyretic, and analgesic properties using different solvent extracts. For instance, Mochtar *et al.* (2023a) and Ramalingam and Annapurani (2020) reported anti-inflammatory and antipyretic effects of the ethyl acetate leaf extract, while Taesotikul *et al.* (2003) described the analgesic and antipyretic activities of the ethanolic extract. Similarly, Kanthlal *et al.* (2011) examined the antipyretic activity of the methanolic extract; Bintarti (2010) investigated anti-inflammatory and analgesic properties of ethanol, ethyl acetate, and hexane extracts; and Peranginangin (2009) and Anbukkarasi *et al.* (2019) evaluated the anti-inflammatory activity of the ethanolic extract. Moreover, Jain *et al.* (2013) and Mondal *et al.* (2024) confirmed the anti-inflammatory potential of the methanolic extract of *T. divaricata* leaves.

Considering the multi-pharmacological potential revealed in previous studies, there is still a lack of investigation focusing on the anti-inflammatory, antipyretic, and analgesic activities of solvent fractions with different polarities derived from the ethyl acetate extract of *T. divaricata*. Fractionation allows the concentration of active compounds, facilitating the identification of bioactive constituents and their mechanisms of action. Therefore, this study aimed to evaluate the anti-inflammatory, antipyretic, and analgesic activities of the hexane and butanol fractions of *T. divaricata* leaf extract in male mice (Mus musculus). The findings are expected to strengthen the scientific basis for the traditional use of *T. divaricata* and contribute to the development of safer natural therapeutic agents for managing inflammation, fever, and pain.

METHODS

Ethical approval

This study received ethical clearance from the Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Indonesia (Approval No. 53/40/EC/KEPK-FKIK/11/2023). All experimental procedures were conducted in accordance with institutional ethical guidelines for animal research.

Plant material

Fresh leaves of *Tabernaemontana divaricata* were collected from Tenggarong District, Kutai Kartanegara Regency, Indonesia. The plant material was taxonomically identified and authenticated at the Laboratory of Ecology and Tropical Forest Biodiversity Conservation, Faculty of Forestry, Universitas Mulawarman.

Extraction procedure

The extraction process followed the maceration method as described by Mochtar *et al.* (2023c). Dried and powdered *T. divaricata* leaves (500 g) were soaked in ethyl acetate (1:3, w/v) in a glass container for five days, with gentle stirring on days two and four. The process was repeated twice (remaceration) using fresh solvent. The combined filtrates were concentrated under reduced pressure using a rotary evaporator to yield a viscous ethyl acetate extract.

Fractionation process

A portion of the concentrated ethyl acetate extract (5 g) was subjected to liquid–liquid partitioning using solvents

of increasing polarity: hexane and butanol (Fadlilaturrahmah *et al.*, 2021; Sembiring *et al.*, 2019).. The crude extract was dissolved in distilled water and successively partitioned with hexane in a separatory funnel. The upper (hexane) layer was separated and the extraction repeated until the hexane layer became clear. The remaining aqueous layer was further partitioned with butanol using the same procedure. Both hexane (HeFrTD) and butanol (BuFrTD) fractions were concentrated using a rotary evaporator to obtain dry fractions for pharmacological testing.

Experimental animals

Male Swiss albino mice (Mus musculus), weighing between 20–30 g and approximately eight weeks old, were used in this study. All animals were healthy and acclimatized for seven days before the experiments under standard laboratory conditions (temperature 25 ± 2 °C, 12-hour light/dark cycle) with free access to standard feed and water. The mice were randomly divided into eight groups, each consisting of three animals for every test. Groups 1, 2, and 3 received hexane fractions of *Tabernaemontana divaricata* (HeFrTD) at doses of 62.5, 125, and 250 mg/kg body weight (p.o.), respectively, while Groups 4, 5, and 6 received butanol fractions (BuFrTD) at the same doses. Group 7 served as the negative control and was given 1% sodium carboxymethyl cellulose (Na-CMC) as the vehicle, whereas Group 8 acted as the positive control, receiving sodium diclofenac (50 mg/kg, p.o.) for the anti-inflammatory and analgesic tests or paracetamol (500 mg/kg, p.o.) for the antipyretic test. The number of animals per group was set at three, calculated using the Federer formula, which meets the minimum sample requirement and adheres to the 3R principle by minimizing unnecessary animal use

Anti-inflammatory activity

Anti-inflammatory activity was evaluated using the carrageenan-induced paw edema model (Kustiawan *et al.*, 2023; Mochtar *et al.*, 2023b). Thirty minutes after oral administration of the test samples, each mouse received a subplantar injection of 0.05 mL of 1% carrageenan solution into the right hind paw. Paw volume was measured using a plethysmometer at 0 (baseline), 30, 60, 90, 120, 150, and 180 minutes post-injection (Muhammad *et al.*, 2012). The percentage of paw edema and inhibition of inflammation were calculated using the following equations (Mochtar *et al.*, 2023a; Muhammad *et al.*, 2012) :

$$\%Edema = \frac{V_t - V_0}{V_t} x100\%$$

$$Inhibiton\ of\ Inflammation\ (\%) = \frac{a-b}{a} x100\%$$

Where V_0 is the paw volume before carrageenan injection, V□ is the paw volume at time t, a is the mean % edema of the negative control, and b is the mean % edema of the treated group.

Antipyretic activity

Antipyretic activity was assessed using the peptone-induced pyrexia model (Rahmi *et al.*, 2021). Basal rectal temperature of each mouse was recorded using a digital thermometer, followed by induction of pyrexia with 5% peptone solution administered orally. After 30 minutes, the rectal temperature was measured again, and treatment was administered according to the group allocation. Temperatures were recorded at 30, 60, 90, 120, 150, and 180 minutes post-treatment. The percentage of pyrexia inhibition was calculated using the following formula (Herdaningsih *et al.*, 2019):

$$Pyrexia\ inhibition\ (\%) = \frac{t_0 - t_n}{t_0 - t(first)} x100\%$$

Where t(first) is the baseline temperature before induction, t_0 is the temperature after induction, and t□ is the temperature at each observation time.

Analgesic activity

Analgesic activity was evaluated using the hot plate test, as described by Ishola *et al* (2014). Thirty minutes after oral administration, mice were placed on a hot plate maintained at 55 ± 0.1°C. The latency time (in seconds) was recorded as the time until the mouse either licked its paw or jumped. A cutoff time of 10 seconds was used to prevent tissue damage. Latency times were recorded at 30, 60, 90, 120, 150, and 180 minutes. The percentage of analgesic activity was calculated as:

$$Analgesic\ (\%) = \frac{t_l - c_l}{c_{ot} - c_l} x100\%$$

Where t_l is the latency time of the treated group, c_l is the latency time of the negative control, and c_{ot} (10 s) is the cutoff time.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. Differences were considered statistically significant at $p < 0.05$.

RESULT AND DISCUSSION

Anti-inflammatory activity

The anti-inflammatory activity of the hexane (HeFrTD) and butanol (BuFrTD) fractions of *Tabernaemontana divaricata* leaves is presented in Figure 1**.** Complete numerical values supporting Figure 1 are provided in Supplementary Table 1. Treatment of carrageenan-induced mice resulted in a significant reduction of paw edema ($P < 0.05$). BuFrTD at doses of 250 and 125 mg/kg BW, as well as HeFrTD at 250 mg/kg BW, exhibited significant anti-inflammatory activity compared to other treatment groups. Statistical analysis revealed that both BuFrTD and HeFrTD at 250 mg/kg BW showed no significant difference from the positive control (sodium diclofenac, $P > 0.05$), indicating that these fractions possess anti-inflammatory potential comparable to that of a standard non-steroidal anti-inflammatory drug (NSAID).

This finding aligns with the biphasic mechanism of carrageenan-induced inflammation, which involves an early phase (0–2.5 h) mediated primarily by histamine, serotonin, and bradykinin release, followed by a late phase dominated by prostaglandin synthesis through cyclooxygenase (COX) activity (Afsar *et al.*, 2015). The carrageenan-induced paw edema model is well-established for evaluating anti-inflammatory agents, as it mimics the acute inflammatory response characterized by increased vascular permeability and leukocyte infiltration (Jin *et al.*, 2016; Chouikh *et al.*, 2024).

Therefore, the observed inhibition of edema by HeFrTD and BuFrTD may be attributed to their ability to suppress COX enzyme activity and reduce prostaglandin synthesis. The significant reduction of carrageenan-induced paw edema by HeFrTD and BuFrTD, particularly at 250 mg/kg BW, demonstrates a strong anti-inflammatory potential. These findings are consistent with Jain *et al.* (2013), who reported notable edema inhibition by the hexane fraction of *T. divaricata*, suggesting that nonpolar constituents in

this species may significantly contribute to anti-inflammatory activity.

Earlier phytochemical studies on this species have identified the presence of flavonoids, alkaloids, and saponins, which have been associated with anti-inflammatory activity in related plant models (Peranginangin, 2009). Moreover, several flavonoid classes reported in *T. divaricata* possess dual inhibitory actions on COX-2 and 5-lipoxygenase (5-LOX), suggesting a potential multi-target mechanism that may contribute to the reduction of carrageenan-induced edema (Md Idris *et al.*, 2022). Although the present study did not characterize the chemical constituents of the tested fractions, the anti-inflammatory effects observed here may reflect the activity of such metabolites commonly reported in this species.

Furthermore, Anbukkarasi *et al.* (2019) reported that *T. divaricata* extract reduces the production of pro-inflammatory cytokines such as TNF-α and IL-1β, further supporting its inflammation-suppressing properties. The dose-dependent anti-inflammatory response observed in this study suggests a classical pharmacological relationship, wherein higher concentrations of bioactive compounds provide more complete inhibition of inflammatory mediators. The comparable efficacy of 250 mg/kg BW doses of both fractions to diclofenac indicates sufficient bioactive compound concentrations to achieve maximum therapeutic effect through saturation of target enzymes and receptors (Yuan and Smith, 2015).

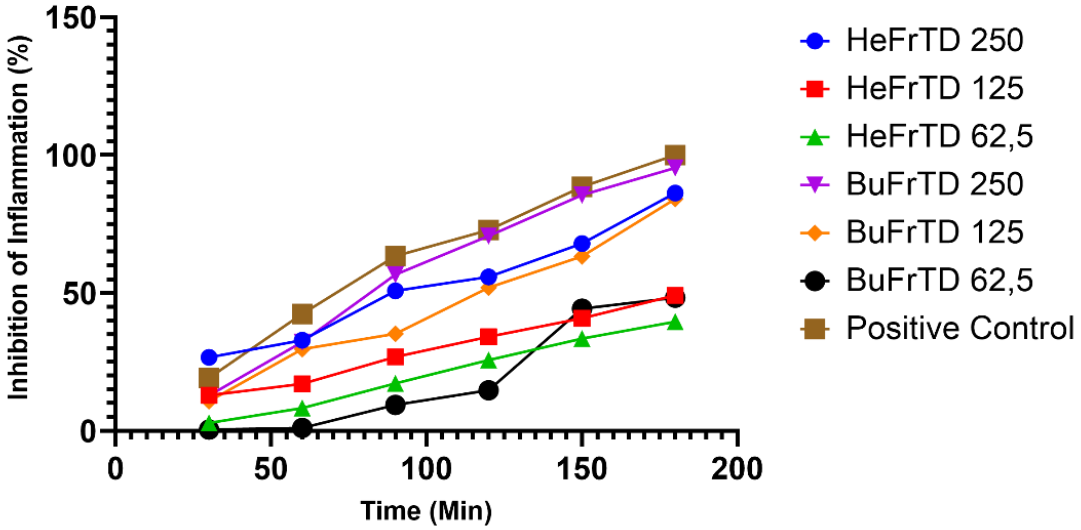

Figure 1. Inhibition of inflammation effect of hexane and butanol fractions in mice.

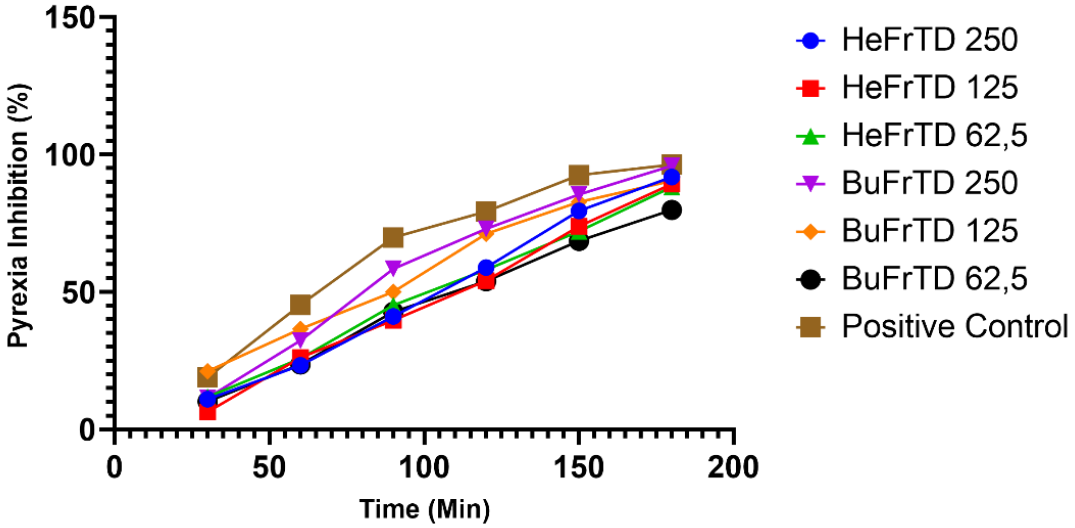

Figure 2. Pyrexia inhibition effect of hexane and butanol fractions in mice

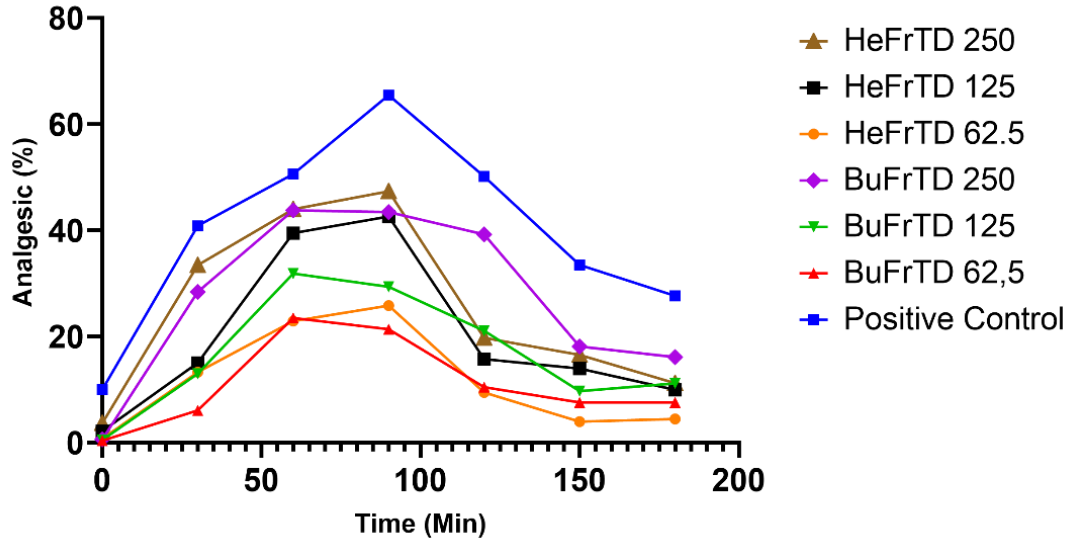

Figure 3. Analgesic effect of hexane and butanol fractions in mice.

Antipyretic activity

The antipyretic activity of HeFrTD and BuFrTD is shown in Figure 2**.** Detailed pyrexia inhibition data corresponding to Figure 2 are presented in Supplementary Table 2. All treatment groups produced a significant ($P < 0.05$) reduction in body temperature in peptone-induced mice. The highest antipyretic effect was observed with BuFrTD at 250 mg/kg BW, which reduced body temperature to 95.83% of the normal level, showing no significant difference from the positive control, paracetamol (96.23%; $P > 0.05$).

The peptone-induced pyrexia model is a standard method for assessing antipyretic effects, as peptone stimulates hypothalamic thermoregulation via prostaglandin synthesis (Rahmi *et al.*, 2021; Alim *et al.*, 2023). Mechanistically, bacterial peptone acts as an exogenous pyrogen that triggers the release of endogenous

pyrogens, particularly IL-1β, IL-6, and TNF-α from immune cells (Zampronio *et al.*, 2015). These pro-inflammatory cytokines cross the blood-brain barrier and stimulate the expression of COX-2 in the hypothalamic preoptic area, leading to increased prostaglandin E2 (PGE2) synthesis (Sluter *et al.*, 2023). PGE2 subsequently alters the thermoregulatory set point in the hypothalamus, resulting in elevated body temperature through increased heat production and conservation mechanisms (Zampronio *et al.*, 2015).

Therefore, the temperature reduction in the treated groups indicates that both fractions may inhibit prostaglandin formation, similar to the mechanism of paracetamol. While the exact mechanism of paracetamol's antipyretic action remains debated, current evidence suggests central COX inhibition, particularly of COX-2 in the hypothalamus, as the primary mode of action (Ayoub, 2021). Additionally, paracetamol may exert effects through multiple pathways, including activation of the endocannabinoid system via its metabolite AM404, and modulation of transient receptor potential ankyrin 1 (TRPA1) channels (Gentry *et al.*, 2015; Sharma *et al.*, 2017).

The consistent antipyretic effects observed across all doses of HeFrTD and BuFrTD may indicate the presence of bioactive constituents with the ability to interfere with pyrogenic signaling pathways, particularly those involving COX-mediated prostaglandin synthesis. Previous phytochemical investigations of Tabernaemontana species have reported indole alkaloids such as coronaridine, ibogamine, and apparicine (Deng *et al.*, 2018; Li *et al.*, 2019; Naidoo *et al.*, 2021), which have demonstrated anti-inflammatory and neuromodulatory activities in various models. These compounds have been shown in earlier studies to modulate cytokine production and influence receptor systems implicated in thermoregulation (Ghosh *et al.*, 2021; Devi *et al.*, 2024). Although the present study did not characterize the alkaloid profile of the tested fractions, it is possible that similar metabolites commonly reported in *T. divaricata* contribute to the observed reduction in fever. The dose-responsive temperature-lowering pattern further supports the likelihood of active constituents capable of attenuating pyretic mediators, although confirmation through targeted phytochemical and mechanistic analyses is required (Ayoub, 2021; Irinmwinuwa *et al.*, 2022).

Analgesic activity

The analgesic effect evaluated by the hot plate method is presented in Figure 3. Additional numerical data supporting Figure 3 are available in Supplementary Table 3. All treatment groups receiving HeFrTD and BuFrTD at doses of 62.5–250 mg/kg BW showed a significant increase in latency time compared with the negative control ($P < 0.05$). The peak analgesic effect occurred between 60 and 90 minutes post-administration, followed by a gradual decline up to 180 minutes. HeFrTD at 250 mg/kg BW demonstrated the highest increase in latency (47.36%), whereas BuFrTD at the same dose produced a more prolonged analgesic effect. However, the positive control (sodium diclofenac) still exhibited the strongest analgesic response, with a latency increase of 65.49% ($P < 0.05$).

The hot plate method is commonly used to assess centrally mediated analgesic effects involving opioid receptor activation (Wang *et al.*, 2022). Unlike peripheral nociceptive tests such as the writhing test, the hot plate assay evaluates supraspinal pain processing mechanisms, as thermal nociception requires integration at the level of the

brain for behavioral responses (Lavich *et al.*, 2005; Magama and Asita, 2017). Our findings suggest that the analgesic properties of HeFrTD and BuFrTD may be associated with central mechanisms, possibly through interaction with μ-opioid receptors (Rezq *et al.*, 2021).

Previous investigations on *T. divaricata* have shown that certain plant parts, particularly the flowers, may modulate opioid receptor pathways, reduce intracellular cAMP levels, and suppress pain-related neurotransmitters such as glutamate and substance P (Ali Khan *et al.*, 2018). Opioid receptors, especially the μ-opioid receptor (MOR), are key targets in centrally mediated analgesia (Wang *et al.*, 2024; Liu *et al.*, 2025). Earlier phytochemical studies have identified flavonoids such as kaempferol and quercetin in this species, and these compounds have demonstrated measurable affinity for MOR and produced dose-dependent analgesic responses in various pain models (Chagas *et al.*, 2022; Iqbal *et al.*, 2025). Although the present study did not analyze the chemical composition of the tested fractions, it is possible that similar metabolites commonly reported in *T. divaricata* contribute to the analgesic effects observed in the hot plate assay.

The lower analgesic potency of both fractions compared with diclofenac suggests that their activity may involve multiple, partially overlapping pathways rather than strong COX inhibition alone. Based on literature, *T. divaricata* metabolites may influence central pain modulation through partial opioid receptor engagement, mild COX suppression, and interactions with descending inhibitory circuits (Bouyahya *et al.*, 2022; Chagas *et al.*, 2022; Iqbal *et al.*, 2025). Such a multi-target mode of action could explain the moderate but sustained analgesic response observed in this study, although direct confirmation of these mechanisms would require phytochemical profiling and receptor-specific assays in future work.

Taken together, these findings suggest that the hexane and butanol fractions of *Tabernaemontana divaricata* leaves exhibit meaningful anti-inflammatory, antipyretic, and analgesic activities. The pharmacological responses observed may reflect the involvement of multiple pathways commonly associated with bioactive constituents reported in this species, including modulation of prostaglandin biosynthesis through COX-related mechanisms and possible engagement of central nociceptive processes such as opioid receptor–mediated signaling. The combined influence of these complementary pathways may underlie the multi-target activity demonstrated in this study and highlights the potential of *T. divaricata* as a promising source of natural agents for managing inflammation, fever, and pain.

CONCLUSION

The present study demonstrates that the hexane (HeFrTD) and butanol (BuFrTD) fractions of *Tabernaemontana divaricata* leaves exhibit notable anti-inflammatory, antipyretic, and analgesic activities in vivo. The BuFrTD fraction at 250 mg/kg BW produced the most pronounced anti-inflammatory and antipyretic effects, while HeFrTD at the same dose showed meaningful analgesic activity. Within the experimental conditions used, these responses showed no statistically significant difference from the respective reference drugs, indicating that the fractions exert substantial biological activity in these models. The pharmacological effects observed may involve pathways commonly associated with bioactive

constituents reported in this species, including modulation of prostaglandin-mediated processes and possible engagement of central nociceptive mechanisms.

Overall, the findings support the traditional use of *T. divaricata* in the management of inflammatory conditions, fever, and pain, and highlight its fractions as promising candidates for further development as natural therapeutic agents. Future studies involving phytochemical characterization and targeted mechanistic approaches are needed to identify the specific active constituents and to better define their pharmacodynamic profiles.

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Author Contribution

Chaerul Fadly Mochtar: Conceptualization, Methodology, Data Curation, Writing-Original Draft Preparation, Writing- Reviewing, Editing Visualization, Investigation, Software, and Validation.

Competing Interest

None.

Ethical Approval

This study received ethical clearance from the Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Indonesia (Approval No. 53/40/EC/KEPK-FKIK/11/2023). All experimental procedures were conducted in accordance with institutional ethical guidelines for animal research.

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