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In Vitro Anti-Inflammatory Potential of Buton Forest Honey through Nitric Oxide Inhibition in RAW 264.7 Macrophage Cells

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ABSTRACT: Inflammation is a physiological defense response to injury, infection, or stress, mediated by various signaling molecules, including nitric oxide (NO). However, excessive NO production can contribute to tissue damage and the progression of inflammatory conditions. Honey, a natural product derived from floral nectar by bees, contains diverse bioactive compounds with potential pharmacological properties, including anti-inflammatory activity. Buton Regency, located in Southeast Sulawesi, is a major producer of forest honey, making it a promising source of bioactive honey with therapeutic potential. This study aimed to evaluate the in vitro anti-inflammatory activity of Buton forest honey by assessing its ability to inhibit NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. The results demonstrated that Buton forest honey significantly suppressed NO production in a concentration-dependent manner. At concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 mg/L, the percentages of inhibition were 94.69 ± 2.94%, 74.03 ± 2.43%, 67.40 ± 2.34%, 62.72 ± 3.09%, 51.80 ± 4.73%, 44.40 ± 4.73%, 19.06 ± 1.79%, and 12.82 ± 0.68%, respectively. Statistical analysis using one-way ANOVA followed by post hoc testing revealed significant differences among the tested concentrations ($p < 0.05$). In conclusion, Buton forest honey exhibits significant anti-inflammatory activity by inhibiting NO production in macrophage cells, suggesting its potential as a natural source for the development of anti-inflammatory agents.

KEYWORDS: Anti-inflammatory; honey; macrophage cell; nitric oxide; protective.

1. INTRODUCTION

Inflammation is a fundamental physiological defense mechanism that occurs in response to infection, injury, or physiological stress. This process involves a complex interplay of immune cells and chemical mediators that function to restore tissue homeostasis. However, when inflammation becomes excessive or chronic, it can lead to tissue damage and contribute to the pathogenesis of various chronic diseases, including arthritis, diabetes mellitus, atherosclerosis, and cancer (Fristiohady et al., 2019; Fristiohady A, 2020; Yodha et al., 2024; Fawwaz et al., 2024). Among the key mediators involved in inflammation is nitric oxide (NO), a signaling molecule produced by inducible nitric oxide synthase (iNOS) in activated macrophages. While NO plays an important role in host defense, its overproduction has been associated with the amplification of inflammatory responses and cellular damage. Therefore, the inhibition of NO synthesis has emerged as an important therapeutic target in the development of anti-inflammatory agents (Poulsen-Silva et al., 2023; Sukmawati et al., 2023).

In recent years, increasing interest in natural-based therapies has highlighted honey as a promising candidate for anti-inflammatory applications. In addition to its nutritional value, honey contains a wide range of bioactive compounds, including flavonoids, phenolic compounds, terpenoids, and organic acids, which contribute to its diverse biological activities such as antioxidant, antibacterial, and anti-inflammatory effects (Alqarni et al., 2014; Hariana & Fristiohady, 2025; Hulea et al., 2022). Among the various types of honey, forest honey possesses distinctive characteristics due to its production by wild bees that forage on a diverse array of forest flora. This results in a more complex chemical composition and potentially enhanced bioactivity. Furthermore, variations in nectar sources and environmental conditions contribute to the unique pharmacological properties of honey from different geographical regions (Dewantara, 2021; Lawag et al., 2023; Ndungu et al., 2024).

The investigation of anti-inflammatory mechanisms requires appropriate experimental models, and the RAW 264.7 macrophage cell line is widely used as a standard in vitro system for studying inflammatory responses. Upon stimulation with lipopolysaccharide (LPS), these cells produce various inflammatory mediators, including nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (Faris, 2019; Pham et al., 2021). Consequently, the inhibition of NO production in LPS-stimulated RAW 264.7 cells is commonly employed as a primary indicator of in vitro anti-inflammatory activity. Previous studies have demonstrated that honey can suppress the production of these mediators by modulating inflammatory signaling pathways in macrophages (Yuandani et al., 2016).

Southeast Sulawesi Province is one of the regions in Indonesia with significant potential for forest honey production (BPS, 2024; Nurhasanah Sari et al., 2018). In particular, Buton Regency is characterized by well-preserved tropical forests and a high diversity of nectar-producing plants, which contribute to the unique chemical profile and bioactive composition of its forest honey (Uji et al., 2007). Although several studies have reported the anti-inflammatory activity of honey from various regions through the inhibition of inflammatory mediators such as nitric oxide (Romário-Silva et al., 2022), research specifically investigating the anti-inflammatory potential of forest honey from Buton Regency remains limited.

Therefore, this study aims to evaluate the in vitro anti-inflammatory activity of Buton forest honey by examining its ability to inhibit nitric oxide production in LPS-stimulated RAW 264.7 macrophage cells. This study is expected to provide new scientific insights into the pharmacological potential of forest honey from Southeast Sulawesi as a natural anti-inflammatory agent.

2. EXPERIMENTAL SECTION

2.1. Sample Preparation

Forest honey samples were collected from natural forest areas in Buton Regency, Southeast Sulawesi, Indonesia (geographical coordinates: 5°17′9.53″S, 122°46′38.39″E; −5.285981, 122.777331). The honey was identified as forest honey based on its physicochemical characteristics and confirmation by local apiarists. A 1-gram sample of this forest honey was subjected to sonication using a sonicator (Elmazonik®, India) with 10 mL of 70% ethanol (Onemed®, Indonesia) for 30 minutes at room temperature to ensure thorough mixing. Following homogenization, the mixture was filtered through filter paper, transferred into a 100 mL volumetric flask, and diluted with ethanol up to the calibration mark, resulting in a final concentration of 10,000 mg/L. The prepared sample was then stored at 2–4°C until further analysis for its anti-inflammatory activity (Hulea et al., 2022).

2.2. Anti-inflammatory Activity Assay Using RAW 264.7 Macrophage Cells

RAW 264.7 cells were seeded in 96-well plates at a density of 2×10^5 cells/well (100 µL) and incubated for 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, the culture medium was removed, and the cells were washed twice with PBS. The cells were then treated with DMEM medium containing lipopolysaccharide (LPS, 1 µg/mL) to induce nitric oxide (NO) production, followed by the addition of forest honey samples at various concentrations ranging from 3.91 to 500 µg/mL. After incubation for 18–24 hours, 50 µL of the supernatant was collected and mixed with 50 µL of Griess reagent, consisting of 1% sulfanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediamine. The mixture was incubated for 10 minutes at room temperature, and the absorbance was measured at 540 nm using a microplate reader.

The nitrite concentration was determined based on a standard $NaNO_2$ calibration curve, while the percentage of NO inhibition was calculated using the following formula:

$$\text{Inhibition (Anti} - \text{inflammatory Activity)NO} = \frac{\text{Absorbance of control} - \text{Absorbance of Sample}}{\text{Absorbance of control}} \times 100 \quad [1]$$

The control absorbance was obtained from LPS-treated cells minus the absorbance of untreated cells, whereas the sample absorbance was calculated from LPS + sample-treated cells minus the absorbance of untreated cells (Ruangnoo et al., 2012).

2.5. Data Analysis

Statistical analysis was performed using SPSS software through a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to determine significant differences among groups. A p-value of < 0.05 was considered the threshold for statistical significance.

3. RESULTS AND DISCUSSION

The anti-inflammatory activity of forest honey from Buton was evaluated by assessing its ability to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Macrophages were selected as the experimental model due to their central role in inflammatory responses, as they produce a variety of mediators, including NO, prostaglandin E_2 (PGE_2), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), upon activation. Stimulation with LPS activates Toll-like receptor 4 (TLR4), which subsequently triggers intracellular signaling

pathways such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), leading to increased expression of the inducible nitric oxide synthase (iNOS) gene (Faris, 2019; Sukmawati et al., 2023).

The iNOS enzyme catalyzes the conversion of L-arginine into NO, which is further oxidized into more stable metabolites, namely nitrite (NO_2^-) and nitrate (NO_3^-). In this study, NO production was quantified indirectly by measuring nitrite levels using the Griess assay. Prednisolone, a synthetic corticosteroid with well-established anti-inflammatory properties, was used as a positive control. This compound exerts its effects by suppressing the expression of pro-inflammatory enzymes such as iNOS and cyclooxygenase-2 (COX-2), as well as reducing the production of pro-inflammatory cytokines, including TNF-α, IL-6, and PGE_2, thereby serving as a reference standard for evaluating the efficacy of Buton forest honey (Hussein et al., 2012). The results of NO inhibition, expressed as percentage inhibition of cell-mediated NO production, are presented in **Table 1** and **Figure 1.**

Table 1. Percentage of nitric oxide (NO) inhibition by Buton forest honey and prednisolone in LPS-stimulated RAW 264.7 macrophage cells.

Concentrations (mg/L)	Inhibition (%)	
	Buton forest honey	Prednisolone
500	94.69 ± 2.94	100.03 ± 1.79
250	74.03 ± 2.43	97.42 ± 3.38
125	67.40 ± 2.34	94.30 ± 6.51
62.5	62.72 + 3.09	79.09 ± 2.34
31.25	51.80 ± 4.73	73.25 ± 2.34
15.625	44.40 ± 4.73	66.62 ± 2.94
7.8125	19.05 ± 1.79	44.79 ± 3.76
3.90625	12.82 ± 0.68	29.97 ± 0.68

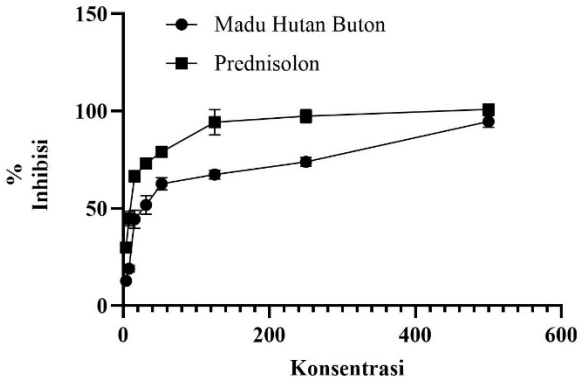

Figure 1. Percentage of NO Production Inhibition by Forest Honey from Konawe in RAW 264.7 Macrophage Cells.

The graph illustrates the percentage of nitric oxide (NO) inhibition in LPS-stimulated RAW 264.7 macrophage cells following treatment with Buton forest honey, compared with prednisolone as a positive control, across a concentration range of 0–500 µg/mL. The horizontal axis represents the sample concentration (µg/mL), while the vertical axis indicates the percentage of NO inhibition. Prednisolone exhibited a rapid and pronounced inhibitory effect, achieving approximately 50–70% inhibition at lower concentrations and approaching near-complete inhibition at higher concentrations (250–500 µg/mL). In contrast, Buton forest honey demonstrated a clear concentration-dependent inhibitory effect, with NO inhibition gradually increasing as the concentration increased. Maximum inhibition, approaching 90–100%, was observed only at the highest concentration (500 µg/mL). The comparatively slower response of Buton forest honey suggests a milder or more gradual mechanism of action, likely associated with the cumulative effects of its bioactive constituents.

Statistical analysis was performed using SPSS software, employing one-way ANOVA followed by Tukey's post hoc test to assess differences among treatment groups. The results revealed statistically significant differences ($p < 0.05$) between the control group and honey-treated groups at concentrations of 250 and 500 µg/mL, indicating effective suppression of NO production in a dose-dependent manner. Prednisolone exhibited significantly greater inhibitory activity

($p < 0.01$) compared to all honey concentrations, consistent with its role as a synthetic corticosteroid with potent and rapid anti-inflammatory effects.

Despite its lower potency relative to prednisolone, Buton forest honey demonstrated a significant and measurable inhibitory effect on NO production, underscoring its potential as a natural anti-inflammatory agent. The observed concentration-dependent inhibition aligns with pharmacological principles, where increased concentrations of active compounds result in enhanced biological effects. This activity is likely attributable to bioactive compounds such as flavonoids and phenolic constituents, which have been reported to suppress inflammatory signaling pathways, including nuclear factor-kappa B (NF-κB) activation and inducible nitric oxide synthase (iNOS) expression in RAW 264.7 macrophages (Faris, 2019; Pham et al., 2021).

In conclusion, although prednisolone demonstrated a faster and more potent inhibitory effect, Buton forest honey exhibited significant, dose-dependent anti-inflammatory activity. These findings support the hypothesis that its anti-inflammatory effects are biologically relevant and concentration-dependent, reinforcing its potential as a natural alternative or complementary agent to synthetic anti-inflammatory drugs.

This study also holds important scientific and practical implications. Excessive nitric oxide production is a key contributor to chronic inflammatory conditions (Fristiohady et al., 2025; Hariana et al., 2025), and the significant inhibition observed in this study highlights the therapeutic potential of Buton forest honey. Furthermore, these findings support the valorization of Indonesian local biological resources not only as economic commodities but also as promising sources of nutraceuticals and complementary therapies. Future studies are warranted to further elucidate the molecular mechanisms underlying the anti-inflammatory effects of forest honey, particularly those involving nitric oxide regulation and iNOS-mediated pathways.

4. CONCLUSION

The findings of this study demonstrate that forest honey from Buton exhibits significant anti-inflammatory activity through the inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells. This inhibitory effect was concentration-dependent, with higher concentrations resulting in greater suppression of NO production. Statistical analysis using one-way ANOVA followed by Tukey's post hoc test confirmed a significant reduction in NO levels at concentrations of 250 and 500 µg/mL ($p < 0.05$), indicating the biological relevance of the observed activity. Although prednisolone, as a synthetic corticosteroid, produced a more rapid and potent inhibitory effect, Buton forest honey demonstrated a substantial and measurable anti-inflammatory response. This activity may be attributed to the presence of bioactive compounds such as flavonoids and phenolic constituents, which are known to modulate key inflammatory pathways, including nuclear factor-kappa B (NF-κB) signaling and inducible nitric oxide synthase (iNOS) expression. Overall, these findings suggest that Buton forest honey holds promise as a natural anti-inflammatory agent and may serve as a complementary alternative to conventional synthetic drugs.

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