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Anti-inflammatory Activity of Indonesian Propolis in Zebrafish (*Danio rerio*) Larvae

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Abstract One candidate for anti-inflammatory agents, polyphenol-rich Indonesian propolis, has been rarely studied. This study was conducted to confirm the presence of its anti-inflammatory activity. Zebrafish larvae, as a model, were divided into four groups consisting of a control group, a lipopolysaccharide (LPS)-treated group, an LPS-treated group followed by treatment in propolis solution for 24 hours, and a propolis-treated group. Myeloid leukocytes migrating into the intestine and intestinal goblet cells were counted. The expression level of pro-inflammatory (*tnf-α, il-1β, il-8,* and *il-6*) and anti-inflammatory (*il-10*) cytokine genes were determined using quantitative reverse transcription polymerase chain reaction (RT-PCR). It was shown that Indonesian propolis administration to LPS-induced zebrafish larvae resulted in reduced myeloid leukocytes in the intestine, increased intestinal goblet cells, and decreased the expression level of *tnf-α* ($P<0.05$). Overall, these results suggest that Indonesian propolis could be a potential agent to protect against inflammatory damage**.**

Keywords: *indonesian propolis, inflammation, cytokine, lipopolysaccharide, zebrafish*

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1. Introduction

Inflammation, a part of innate immune system, defends host from pathogen attack as well as tissue damage. Inflammation involves recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through various family of pattern recognition receptors (PRRs). Recognition process triggers synthesis of molecular mediators called cytokines, pro-inflammatory cytokines (such as *il-1β*, *tnf-α*, *il-6*, *il-8*, and *ifn-γ*) that stimulate inflammation and anti-inflammatory cytokines (such as *il-10*) that suppress inflammation by controlling those pro-inflammatory cytokines [1].

Systemic and long-term inflammation could lead to tissue damage, fever, auto-inflammatory disease, and septic shock. These adverse effects can be prevented by means of anti-inflammatory agents, such as aspirin that have been widely used [2]. However, most of these agents exhibit toxicity and side effects, i.e. aspirin can cause hypertension, edema, and heart failure [3]. Therefore, relatively non-toxic anti-inflammatory agents are needed as alternative. Burdock stated that propolis is relative non-toxic, thus has a potency for being used as anti-inflammatory agent [4].

Propolis is a resinous mixture of plant-derived material collected by honey bees and has been widely used as traditional medicine since antiquity [5]. Its chemical components are responsible for its characteristics and biological activities and influenced by geographic location, bee species, plant source, season, and extraction method [6]. Compared to other types of propolis, Indonesian propolis has been rarely studied especially at cellular and molecular level [7]. According to various studies on other types of propolis, polyphenols and terpenoids are allegedly responsible for anti-inflammatory activity [8].

In this research, zebrafish larvae were used as model to confirm the presence of anti-inflammatory activity of Indonesian propolis, because their immune system is highly similar to its mammalian counterpart, at molecular, cellular, and even physiological level [9]. The parameters included were: (1) expression level of pro-inflammatory cytokine genes (*tnf-α, il-1β, il-8,* and *il-6*), (2) expression level of anti-inflammatory cytokine gene (*il-10*), (3) number of myeloid leukocytes migrating into intestine, and (4) number of intestinal goblet cells. Indonesian propolis is expected to suppress the expression level of pro-inflammatory cytokine genes, promote the expression

level of anti-inflammatory cytokine genes, decrease the number of myeloid leukocytes migrating into intestine, and increase the number of intestinal goblet cells.

2. Materials and Methods

2.1. Indonesian Propolis Preparation

Propolis used in this research was obtained from stingless bee (*Trigona* sp.) farmer from Subang, Indonesia. Raw propolis was extracted using ethanol and its chemical constituents were analyzed using gas chromatography-mass spectrometry (GC-MS).

2.2. Zebrafish Maintenance

Zebrafish were maintained in accordance with standard protocol [10]. Zebrafish embryos, obtained from natural spawning, and larvae were kept in Petri dish containing E3 medium (5 mM of NaCl, 0.17 mM of KCl, 0.33 mM of $CaCl_2$, 0.33 mM of $MgSO_4$, without methylene blue) at 26-30°C.

2.3. Treatment of Zebrafish Larva

Treatment of zebrafish larvae was performed by immersion in 6-well plate for 2 × 24 hours. Thirty 5-dpf-larvae per group were placed in well containing 6 mL of E3 medium. After the first 24 hours, E3 medium was changed with fresh medium. Larvae were divided into four groups: control/CON (untreated), LPS group (exposed to 50-ppm-LPS for 24 hours), LPS-PRO group (treated with 50 ppm of LPS for 24 hours followed by 14 ppm of Indonesian propolis for 24 hours), and PRO group (treated only with 14 ppm of Indonesian propolis for 24 hours). LPS was obtained from phenol-extraction of *Escherichia coli* serotype O111:B4 (Sigma-Aldrich), and prepared according to Bates *et al. [11]*. Subsequent to treatment, larvae were sacrificed using an overdose of tricaine methane-sulfonate [12].

2.4. Expression Level Analysis Using Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from euthanized larvae (30 larvae per sample) and the gene-specific primer used were mentioned in Table 1 (obtained from Genetika Science Jakarta, Indonesia). Relative gene expression analysis was performed using method proposed by Livak and Schmittgen [13].

2.5. Myeloperoxidase (MPO) Staining

First, pigmentation was prevented to facilitate visualization, by using a tyrosinase inhibitor *N*-Phenylthiourea (PTU) [14]. MPO staining was performed in accordance with Cordero-Maldonado *et al*. in five larvae per group [15]. Quantification of black-brown-stained myeloid leukocytes at mid and posterior portion of larval gut were carried out manually under stereomicroscope at 90 × magnification.

2.6. Histology Analysis

Euthanized larvae were fixed in freshly prepared Bouin's solution at room temperature for at least 24 hours, dehydrated in alcohol series, embedded in molten paraffin, and sagittally cut at 5 μm of width. Larval sections were stained using alcian blue and counterstained with hematoxylin-eosin (HE) and enhanced using Scott's tap water substitute [16], according to Hedrera *et al*. with modifications [17]. Quantification of goblet cell at mid and hind gut was carried out manually by light microscopy (AO American Optical MicroStar One-Ten) at 200 × magnification.

2.7. Statistical Analysis

Data were analyzed using either IBM SPSS Statistics 19 (IBM) or R-3.5.1 (The R Foundation) with significance level (α) of 0.05.

Table 1. Oligonucleotide Primer Sequence Used

Name	Sequence	Accession Number
tnf-α	forward 5'-CGTCTGCTTCACGCTCCAT-3' reverse 5'-CTGGTCCTGGTCATCTCTCC-3'	BC124141
il-1β	forward 5'-TGTAAGACGGCACTGAATCC-3' reverse 5'-CGCAGCACAAAATGAAGCAG-3'	AM941672
il-8	forward 5'-GTCGCTGCATTGAAACAGAA-3' reverse 5'-CTTAACCCATGGAGCAGAGG-3'	EH458432.1
il-6	forward 5'-CCGCTGCCTGTCTAAAAACT-3' reverse 5'-TCCATCTCTCCGTCTCTCAC-3'	JN698962
il-10	forward 5'-CACTGAACGAAAGTTTGCCTTAAC-3' reverse 5'-TGGAAATGCATCTGGCTTT-3'	AY887900
sep15 (reference gene)	forward 5'-TATTGTTGATTGTTGCTGAGGG-3' reverse 5'-ACGCTGAGAGATGTACACAGGA-3'	EH535677

3. Results and Discussion

3.1. Chemical Analysis

The chemical analysis by CG-MS revealed 25 distinct compounds in chemical composition of Indonesian propolis. Most of these compounds are phenolic compounds with recognized therapeutic properties. The most abundant chemical compounds are 3-methoxy- (CAS) m-guaiacol (phenol) and phenyl ester (CAS) phenyl carbamate (carbamic acid) guaiacol, respectively 14.07% and 10.15%.

3.2. Indonesian Propolis Reduces Relative Expression Level of *tnf-α*

As shown in Figure 1, in general, expression level of pro-inflammatory cytokines genes examined in this study (*tnf-α, il-1β, il-8*, and *il-6*) shows similar pattern. Larvae from the LPS group has a higher expression level of pro-inflammatory cytokine genes compared to control (CON) group. It is consistent with the result of a research conducted by Yang *et al*. that showed increases of *il-1β*, *il-6*, and *tnf-α* expression levels in the LPS-injected larvae [18]. Non-significant differences in expression level of *tnf-α, il-1β,* and *il-6* between larvae from the control and from the LPS group ($P>0.05$) could be due to LPS tolerance to the LPS concentration used, as teleost fishes, including zebrafish, have a high tolerance to LPS [11].

The expression level of pro-inflammatory cytokine genes in larvae from the LPS-PRO group was lower than in larvae from LPS group, though significant difference only observed in the expression level of *tnf-α* ($P<0.05$). *tnf-α* is one of the early genes expressed at an early stage of infection in fish and important for regulating inflammation [19]. Based on the similar pattern, Indonesian propolis tends to repress the expression of pro-inflammatory cytokine genes in LPS-induced zebrafish larvae. By comparing to other organism model, such as that conducted by Neiva *et al*., propolis were shown to decrease LPS-induced pro-inflammatory cytokines in human pulp cells and osteoclasts [20].

3.3. Indonesian Propolis Insignificantly Raises Relative Expression Level of *il-10*

As shown in Figure 2, the expression level of *il-10* in zebrafish larvae from the LPS group was significantly higher than in larvae from the control group ($P<0.05$). This result is consistent with previous study using adult zebrafish induced by LPS [21]. *il-10* has a protection capacity from toxic effects of *tnf-α* induced earlier by LPS, by triggering secondary signaling pathways, which modulate the expression of direct LPS target genes.

Indonesian propolis tends to reduce the expression level of *il-10* in LPS-induced zebrafish larvae, but not for the larvae that are not induced by LPS. This is denoted by the result obtained in this study. Zebrafish larvae from the LPS-PRO group and Propolis group are relatively similar in their expression level of *il-10* and also similar with larvae from the control group ($P>0.05$). These results are consistent with the result of a previous study that shows inhibition of the expression of *il-10* in RAW 264.7 cell line after propolis treatment [22].

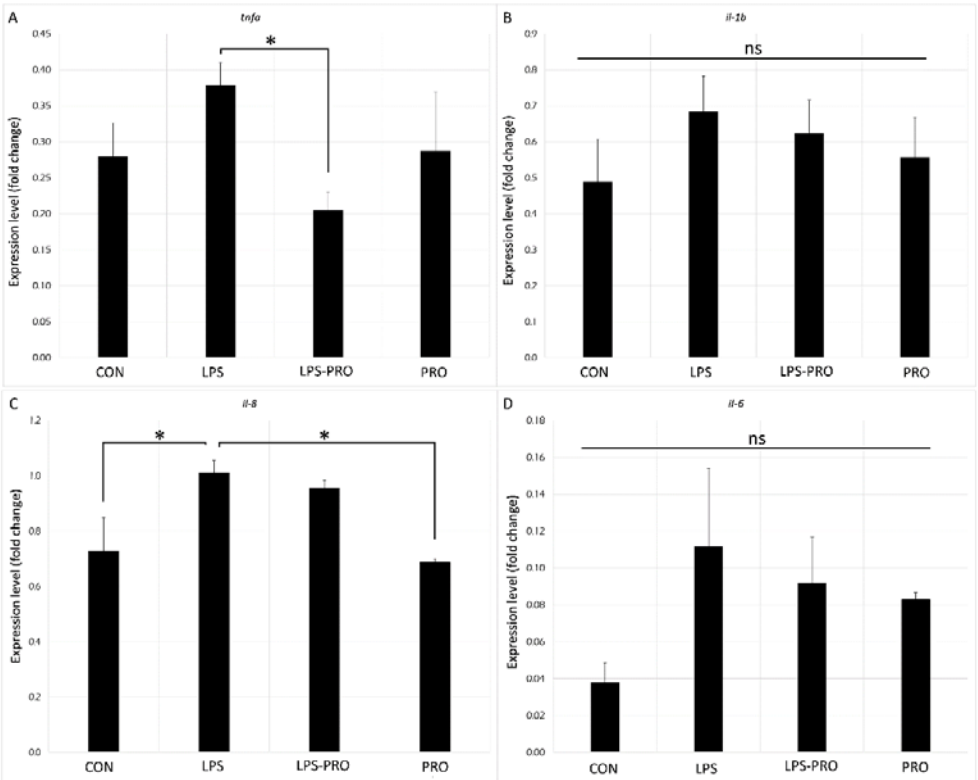


Figure 1. The expression level of pro-inflammatory cytokine genes: (A) *tnf-α*, (B) *il-1b*, (C) *il-8*, and (D) *il-6*. Significant differences are shown by * ($P<0.05$)

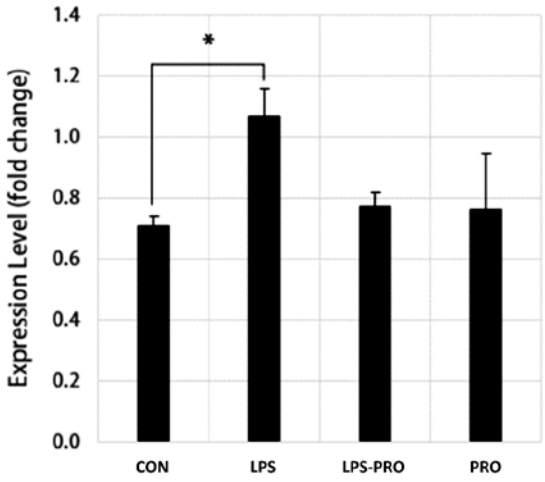
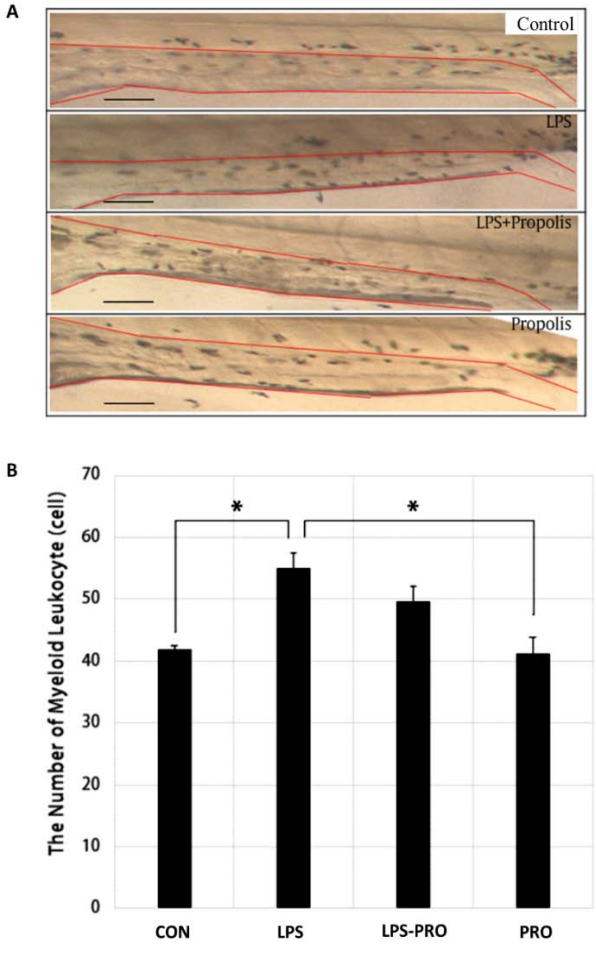


Figure 2. The expression level of anti-inflammatory cytokine gene, *il-10*. Significant differences are shown by * (P<0.05)

3.4. Indonesian Propolis Reduces the Number of Migrating Myeloid Leukocytes



.**Figure 3.** The number of migrating myeloid leukocytes into intestine. (A) Zebrafish larvae intestine. Area confined by the red line was for quantifying the number of myeloid leukocytes (black dots). Horizontal bar = 100 μm; Magnification = 90X. (B) Quantification result. Significant differences are shown by * (P<0.001)

Representative zebrafish larvae stained with myeloperoxidase are shown in Figure 3A. The number of myeloid leukocytes in larvae from the LPS group was significantly higher than in larvae from the control group (Figure 3B). This suggests that LPS increases myeloid leukocyte migration into intestine which is the site of mucosal inflammation. Migration of myeloid leukocytes is mediated by chemokines, such as IL-8, which are secreted by the endothelial cells induced by TNF-α and macrophages [23].

Zebrafish larvae from the LPS-PRO group (P>0.05) and from the PRO group (P<0.001) had a lower number of myeloid leukocytes compared to larvae from the LPS group. This result suggest that Indonesian propolis tends to inhibit myeloid leukocytes migration into intestine in LPS-induced zebrafish larvae. It was consistent with relative expression of *il-8* shown in Figure 1C because *il-8* is a pro-inflammatory chemokine produced by various cell types to recruit leukocytes to sites of infection or tissue injury. Also, the TNF-α protein enhances the phagocytic activity of fish leukocyte, by getting involved in the regulation of leukocyte homing, proliferation, and migration [19]. These results also consistent with the study done by Franchin *et al.* where LPS-induced mouse was exposed to vestitol (compound isolated from propolis) [24].

3.5. Indonesian Propolis Increases the Number of Intestinal Goblet Cells

Figure 4A shows the sagittal sections of zebrafish larvae from all groups stained with alcian blue-HE. Larvae from the LPS group had the lowest number of intestinal goblet cells (Figure 4B). It differed significantly with the number of intestinal goblet cells from other groups' larvae. This result suggests that LPS decreased the number of intestinal goblet cells in zebrafish larvae. Previous study performed by Zapata *et al.* which utilized LPS-induced weaned pig shows reduction in the number of intestinal goblet cells [25].

The number of intestinal goblet cells in zebrafish larvae from LPS-PRO group was significantly higher than in larvae from the LPS group (Figure 4B). It suggests that Indonesian propolis causes an increase in the number of intestinal goblet cells in LPS-induced zebrafish larvae. It was consistent with the study conducted by Oehlers *et al.* that showed administration of propolis in mice with damaged nasal mucosa and colitis-induced rat with 2,4,6-trinitrobenzensulfonat acid (TNBS) increased the number of goblet cells in nasal and gastrointestinal mucosa [26]. TNF-α and LPS play a role in the regulation of the number of intestinal goblet cells by strengthening the IFN-γ signaling pathways [27,28].

As the main components of propolis that was used in this study, phenolic compounds are presumably responsible for anti-inflammatory activity of Indonesian propolis. Various studies have proved the existence of anti-inflammatory activity in phenolic compounds and its mechanism that involves inhibition of pro-inflammatory enzymes activity (lipoxygenase, cyclooxygenase, and iNOS) and inhibition of transcription factors (PI 3-kinase, NF-κB, c-JUN, and tyrosine kinase) [29]. The effect of Indonesan Propolis suggests its potential as source of new compounds with pharmacological properties and its use in the control of inflammation.

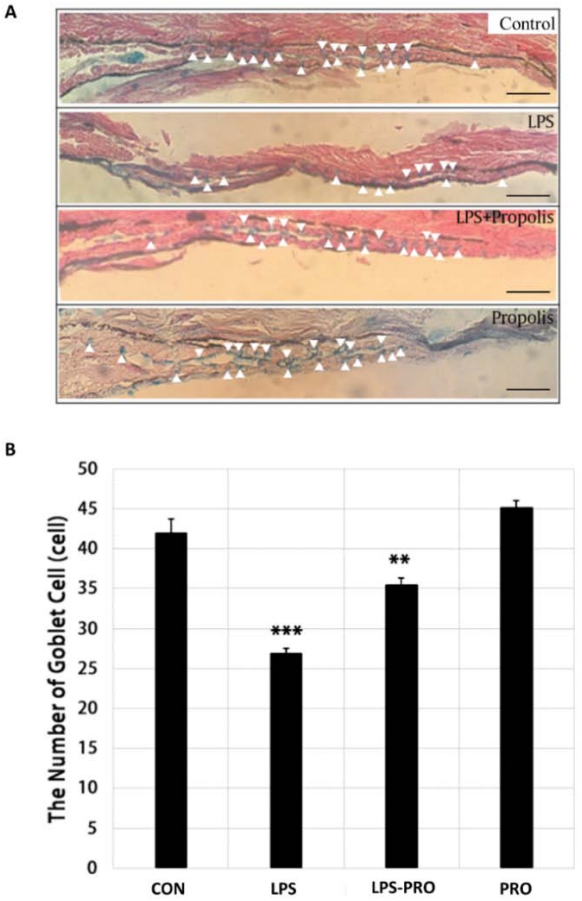


Figure 4. The number of intestinal goblet cells (A) Sagittal section of zebrafish larvae. White triangle pinpoints goblet cell. Horizontal bar = 100 µm; Magnification = 200X. (B) Quantification result. Significant differences are shown by ** (P<0.01 against LPS group; P<0.001 against other groups) and *** (P<0.001 against other groups).

4. Conclusion

Based on the results that have been described, it can be concluded that Indonesian propolis exerts anti-inflammatory activities in the zebrafish larvae stimulated with LPS. The activities include down-regulating pro-inflammatory gene, i.e. *tnf-α* (P<0.05), reducing the number of myeloid leukocytes that migrate into intestine, and increasing the number of intestinal goblet cells, though the other pro-inflammatory genes' expression level did not increase and anti-inflammatory gene's did not decrease in this study. However, Indonesian propolis alone did not show any significant anti-inflammatory activities. Nevertheless, the effect of Indonesan propolis suggests its potential as source of new compounds with pharmacological properties and could be a potential agent to protect against inflammatory damage.

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