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Original Research Article

The Effect of Propolis Extract from Sumatra, Indonesia on *Escherichia coli* and IL-6 Gene Expression in Male Wistar Rats Fed with a High-Fat Diet

Imam Megantara[1]*, Chrysanti Murad[1], Fauzan Pahlevi[2], Nova Sylviana[3], Hanna Goenawan[3], Ronny Lesmana[3]

[1]*Microbiology Division, Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Jatinangor, Kab. Sumedang, 45363, Indonesia*
[2]*Undergraduate Program, Faculty of Medicine, Universitas Padjadjaran, Jatinangor, Kab. Sumedang, 45363, Indonesia*
[3]*Physiology Division, Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Jatinangor, Kab. Sumedang, 45363, Indonesia*

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ABSTRACT

Propolis is a resinous substance produced by honey bees. It was collected from Sumatera, Indonesia, and its antibacterial properties against *Escherichia coli* and immunomodulatory effect on local intestinal inflammation was evaluated after ingestion of chow and high-fat diets. Male Wistar rats (*Rattus norvegicus*) were divided into four groups: chow diet (NCD), chow diet with propolis supplementation (NCDP), high-fat diet (HFD), and high-fat diet with propolis supplementation (HFDP) groups. The diets and propolis were administered according to the group assignment for 12 weeks. Faecal samples of the mice were collected at weeks 0, 4, and 12 and subjected to *Escherichia coli* quantitative real-time polymerase chain reaction (qRT-PCR). Further, reverse transcription-PCR (RT-PCR) analysis was performed using intestinal samples to analyze the interleukin-6 (IL-6) levels. In the NCDP group, *E. coli* gene expression decreased significantly at week 4 ($p < 0.005$) and remained low at week 12, whereas in the HFDP group, a significant decrease was observed in week 12. Meanwhile, in obese rats treated with propolis, the expression of the IL-6 gene was found to be high, although not statistically significant ($p > 0.005$). This study demonstrated the antibacterial effect of propolis against *E. coli* in normal and obese rats and indicated that propolis supplementation might exert an immunomodulatory effect in obese rats.

***Keywords*:** Propolis, High-fat diet, *Escherichia coli*, Interleukin-6

Introduction

Propolis, or bee glue, is a resinous substance collected by honey bees from plant sources, such as sap or tree buds. The content of propolis extract varies according to the flowers or trees in a particular area and the geographic and environmental conditions, such as altitude, lighting, season, and nutrients taken by bees.[1,2] Generally, propolis contains resins, waxes, essential oils, pollen, aromatic aldehydes, polyphenols, terpenoids, vitamins, and minerals.[1] Propolis has many benefits for the human body, and its extract is used for different purposes, such as cosmetics, food additives, and natural alternative medicine.[3]

Several active components in propolis, such as flavonoids and polyphenols, exert antibacterial activities by disrupting the cell membrane permeability and ATP production in bacteria, which reduces their activity and movement, ultimately killing these bacteria.[1,4]

***Corresponding author. E mail: imam.megantara@unpad.ac.id
Tel: +62-816612839

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In addition to this, propolis components, particularly those related to flavonoids, phenolic acids, phenolic acid esters, terpenoids, steroids, amino acids, and caffeic acid phenethyl ester, exert anti-inflammatory effects by modulating NF-κB regulation, inhibiting prostaglandins, inhibiting nitric oxide (NO), inhibiting cyclooxygenase (COX), reducing proinflammatory cytokines, and suppressing the inflammatory system.[2,4] While the anti-inflammatory properties of propolis are particularly prominent in acute inflammatory conditions, it can also promote immune functions during chronic inflammatory conditions.[5,6] Due to its immune-modulating properties, propolis can act as an immune system regulator.

Unlike the acute phase, chronic inflammation is prolonged, generally accompanied by an increase in lymphocytes, and is characteristic of metabolic syndrome, with the secretion of inflammatory adipokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α.[7] Obesity, a hallmark of metabolic syndrome,[8] results from an imbalance between energy intake and expenditure, indicating a positive energy balance. Obesity refers to the accumulation of visceral and subcutaneous adipose tissue, causing weight gain.[9] In obese individuals, inflammation occurs mainly due to the influence of white adipose tissue (WAP), which modulates the production and activation of IL-6 and TNF-α.[10] Although IL-6 is a well-known cytokine that mediates inflammatory responses, its primary effects occur at a site different from its origin and are a consequence of its concentration in the circulation, unlike other cytokines.[11] In obese patients, IL-6 regulates body mass and fat metabolism. It is secreted to prevent obesity by stimulating lipolysis in the liver and adipose tissues. It can also cause muscle atrophy by regulating protein synthesis and degradation. IL-6 also exhibits anti-inflammatory effects by inhibiting TNF-α, increasing M2 macrophages and insulin resistance.[12]

Consumption of a high-fat diet (HFD) that contains high amounts of lipids contributes to obesity. Among the different lipids, including

triglycerides, sterols, fatty acids, and phospholipids, an HFD includes large quantities of saturated or trans fatty acids and cholesterol.[13,14] An HFD can cause intestinal inflammation directly or indirectly by influencing the abundance of pathogenic bacteria, especially Proteobacteria, which increases lipopolysaccharide (LPS) concentrations. LPS is an endotoxin that activates the toll-like receptor (TLR)4, triggering an inflammatory response.[15–17] *Escherichia coli*, a prominent Gram-negative bacterium belonging to Proteobacteria, contains LPS structures in the outer membrane of the cell wall. Inflammation elicited through TLR4 activation initiates the recruitment of neutrophils and macrophages and NF-κB activation, elevating the levels of proinflammatory cytokines, such as IL-6.[15]

Indonesia has abundant natural resources that can be used in traditional medicine, including Trigona bee propolis, which can be obtained by breeding and managing stingless bees (meliponiculture) in North Sumatra. Reports detailing various aspects of the propolis obtained from North Sumatra, including its mechanism of action, bioactive components, antibacterial effects against *E. coli* (as a representative of Proteobacteria) in the digestive tract, and its role in modulating immune responses related to IL-6, especially in obesity, are still limited. Additionally, the number of constituents identified in propolis samples depends on the geographic origin, including bee food availability, the vegetation from which the propolis originates, and environmental factors, such as season and climate.[18] These factors influence the antibacterial and immunomodulatory effects of each propolis extract. Therefore, its antibacterial effects against *E. coli* and its immunomodulatory potential via IL-6 expression should be investigated. Thus, this study aimed to assess the effect of propolis from North Sumatra, Indonesia, on the growth of *E. coli* and IL-6 expression in male Wistar rats fed an HFD to create obesity conditions. Rodents, such as rats, were chosen for this study based on a good understanding of this animal model, relative ability to control environmental factors, and manipulation of obesity by providing an HFD.[19]

Materials and Methods

This research was conducted in the Central Laboratory of Universitas Padjadjaran following the ethical guidelines of the Padjadjaran University Animal Laboratory Guide and Care Protocol. The animal studies were approved by the Padjadjaran University Research Ethics Committee under ethics number 120/UN6.KEP/EC/2023.

Preparation of Propolis Extract

Trigona spp. bee hive was collected as raw propolis in March 2023 from North Sumatra in western Indonesia. After cutting it into smaller pieces and drying it at room temperature for 2 weeks, 500 g of propolis was added to 5 L of pure ethanol in a shaker (MaxQ™ HP Tabletop Orbital Shaker Thermo Scientific, US) at a speed of 130 rpm. After 72 hours, the ethanol extract solution was filtered using filter paper. The solvent was evaporated using a freeze-drier to obtain the ethanol extract of propolis (EEP), which was stored in sterilized microtubes at 4°C until further use.[20]

Analysis of Propolis Extract

The propolis extract was analyzed in the Central Laboratory of Universitas Padjadjaran to identify its natural compounds. After cleaning the harvested propolis to remove dirt, it was processed via glycerate extraction for 12 hours. Based on the initial analysis results, the process was optimized for microbial sterilization and extraction of active propolis ingredients (flavonoids, alkaloids, terpenoids, phenolics, steroids, saponins, and tannins) while maintaining its biochemical activity. Propolis was extracted using 70% ethanol by soaking for 12 hours (maceration), followed by separation of the solvent (ethanol) using a rotary evaporator (Rotavapor® R-300, Buchi, Switzerland). The extract was then added with a diluent/thinner with solvents, such as propylene glycol and glycerin, to manage its consistency. This mixture of propolis extract and diluent was then stored and used as raw material for various purposes.

Animal Preparation

Sixteen male Wistar rats (*Rattus norvegicus*) were divided into four groups that were fed on a normal chow diet (NCD), normal chow diet with propolis supplementation (NCDP), high-fat diet (HFD), and high-fat diet with propolis supplementation (HFDP) at PT. Surya Sains Indonesia, Indonesia. The composition of the NCD given to the NCD and NCDP groups consists of 65.5% carbohydrates, 25% proteins, 7% fats, and 5% micronutrients. The HFD given to the HFD and HFDP groups consists of 99.5% total fats, including 1% palmitic acid. Propolis extract was administered to the NCDP and HFDP groups at a dose of 300 mg/kg via intragastric gavage.[21]

The rats were housed under temperatures ranging between 22 and 24℃, alternating day and night cycles (12:12) in cages based on their groups, and were given food and water *ad libitum*. The rats were acclimatized on their respective diets for a week, and then, they were given their diet along with the propolis treatment for 12 weeks. All animal procedures were performed in the Animal Science Laboratory, Postgraduate Facility, Universitas Padjadjaran. The faecal samples from the rats were obtained weekly. On the last day of the experiment, the rats were euthanized, and their intestinal samples were harvested for analysis. The samples were stored in the refrigerator until further use.

Analysis of E. coli Gene Expression

The rat faecal samples obtained on weeks 0, 4, and 12 were used to analyze the number of *E. coli* before treatment and one and 3 months into the treatment. qRT-PCR was performed using the MyTaq™ One-Step RT-PCR Kit (Bioline, USA) to analyze the *E. coli* count. The primers used for *E. coli* gene amplification were Forward: 5′-GTTTGTGTGAACAACGAACT-3′ and Reverse: 5′-GACGCACAGTTCATAGAGAT-3′ with a product length of 369 bp. First, *E. coli* DNA was extracted from the faecal samples according to the manufacturer's procedure and optimized. For the RT-PCR, the extracted DNA was mixed with a PCR master mix containing SYBR green mix, forward primer, reverse primer, template, and water. The PCR run consisted of 1 cycle of polymerase activation at 95℃ for 2 minutes, 40 cycles of denaturation at 95℃ for 5 seconds, annealing at 58℃ for 10 seconds, and extension at 72℃ for 20 seconds. From the graphs showing the results, the quantification cycle (Cq) was used to measure the *E. coli* gene expression. The Cq of *E. coli* gene expression was normalized with a universal primer and calculated according to relative quantification using Livak's $2^{-\Delta\Delta CT}$ methods.[22] The relative quantification results of the gene expression were presented as fold changes.

Analysis of IL-6 Gene Expression

For IL-6 analysis, the rat intestine samples were obtained at the end of the treatment and analyzed using RT-PCR to quantify the IL-6 RNA. The primers used for amplifying the IL-6 gene were Forward: 5′-TCCTACCCCAACTTCCAATGCTC and Reverse: 5′-TTGGATGGTCTTGGTCCTTAGCC-3′. After extracting the IL-6 mRNA genes from the intestine samples, they were amplified using reagents, including MyTaq One-Step Mix, forward and reverse primers, reverse transcriptase, RNase inhibitor, RNA template, and nuclease-free water. The PCR run begins with a reverse transcription step at 45℃ for 20 minutes, followed by initial denaturation at 95℃ for 1 minute, denaturation at 95℃ for 30 seconds, 30 cycles of annealing at 59℃ for 30 seconds, extension at 72℃ for 1 minute, and final extension at 72℃ for 10 minutes. The PCR results were visualized using agarose gel electrophoresis (Owl™ EasyCast™ B1A Mini Gel Electrophoresis Systems, Thermo Scientific™, USA), and the bands were quantified using ImageJ. Quantification results of IL-6 mRNA were normalized with GAPDH quantification results. The gene expression results were represented as fold changes.

Statistical Analysis

The data was analyzed using the IBM Statistical Package for the Social Sciences (SPSS) version 26. The normality and homogeneity of the data were evaluated using the Shapiro-Wilk and Levene tests, respectively. A dependent paired t-test was used to analyze *E. coli*

gene expression between the same diet groups at different times. The experimental data was presented as mean ± standard deviation. An independent t-test was also used to compare the *E. coli* gene expression and IL-6 levels between the NCD and NCDP groups and the HFD and HFDP groups on week 12. Statistical significance was set at a p-value < 0.05.

Results and Discussion

Propolis contains bioactive compounds, such as resins, flavonoids, polyphenols, polysaccharides, volatile materials, and other secondary metabolites, which impart many functional benefits, such as antibacterial, antiangiogenic, anti-ulcer, anti-inflammatory, and antioxidant activities.[1–4,23] In this study, the composition of the propolis extract from *Trigona spp*. bee hives in North Sumatra, Western Indonesia, were analyzed, mainly consisting of flavonoids, phenolics, tannins, and triterpenoids (Table 1).

The antimicrobial activity of propolis tends to be higher against Gram-positive bacteria than Gram-negative bacteria due to the species-specific structure of the outer membrane of Gram-negative bacteria. Although hydrolytic enzymes might break down the active ingredients of propolis[24], ethanolic propolis extract (EEP) has been shown to suppress the growth of *E. coli* by causing cell membrane lysis.[25] Furthermore, propolis can also limit the growth of *E. coli* by hindering anaerobic nitrate respiration by reducing the synthesis of nitric oxide (NO), which can be produced during intestinal inflammation.[4]

In our study, the RT-PCR analysis indicates a significant increase in *E. coli* gene expression at weeks 4 and 12 ($p < 0.05$) in the NCD and HFD rats without propolis supplementation (Figure 1). Meanwhile, the *E. coli* gene expression levels in the NCD and HFD groups that received propolis supplements (NCDP and HFDP) were significantly decreased at week 4 ($p < 0.05$) and remained low till week 12, confirming the antibacterial effect of propolis. The decline in *E. coli* growth was maintained till week 12, indicated by the low *E. coli* gene expression level. However, the average decrease in *E. coli* gene expression in the NCDP group was statistically significant ($p < 0.05$) in mice only from weeks 0 to 4 but not from weeks 4 to 12. Contrastingly, in HFDP mice, the decrease in mean *E. coli* gene expression was statistically significant ($p < 0.05$) from week 4 to week 12. Meanwhile, *E. coli* gene expression remained consistently high in the untreated groups.

Since the physiological characteristics of obese rats fed an HFD harbour are different from those of normal rats fed a chow diet, we further compared the effect of propolis administration between the normal and obese groups. As shown in Figure 1F, the average *E. coli* gene expression in the NCDP rats was lower than that in the NCD rats after propolis supplementation for 4 weeks. Meanwhile, in obese rats, the average *E. coli* gene expression in rats treated with propolis (HFDP group) was lower than that in the HFD rats. At week 12, the mean gene expression was lower in the NCDP and the HFDP groups than in the NCD and HFD groups, respectively ($p < 0.05$). These results show that in normal mice, the optimal antibacterial effect of propolis against *E. coli* was achieved after 4 weeks of administration. However, propolis supplementation could suppress the growth of *E. coli* in the obese rats (HFDP group) until week 12, suggesting that the supplementation with *Trigona* spp. bee propolis extract exhibits optimal antibacterial effects for 12 weeks against *E. coli*.

Several studies have indicated that propolis exhibits a unique antibacterial mechanism against pathogenic or opportunistic bacteria, such as *E. coli*, and can promote the proliferation of the so-called beneficial microbes.[26–29] Therefore, continuous and prolonged propolis supplementation can maintain the health of beneficial bacteria. Furthermore, propolis supplementation can reduce the chances of antibiotic resistance in pathogenic bacteria due to the overconsumption of antibiotics. Therefore, propolis can be considered a natural medicine with complex and synergistic active compounds, which can help prevent the emergence of resistant bacteria.

The anti-inflammatory effect of propolis is achieved by controlling NF-κB, which affects inflammatory cytokines. However, propolis can also function as an immunomodulator, especially to maintain a functional immune response in obese conditions to control low-grade chronic inflammation and impaired insulin resistance. IL-6 is a proinflammatory cytokine that has been shown to be involved in this process, suggesting its dual function as a pro- and anti-inflammatory cytokine.[30,31]

The results of IL-6 PCR gene expression quantification and analysis indicate that the NCDP group showed higher IL-6 gene expression than the NCD group (Figure 2). Similarly, IL-6 gene expression in the HFDP group was higher than that in the HFD group. However, the differences between the NCD and NCDP groups and the HFD and HFDP groups were not statistically significant ($p > 0.05$). Despite the lack of a statistically significant difference, the increase in IL-6 gene expression in the HFDP rats indicated the anti-inflammatory and immunomodulatory effects of propolis, especially on the local inflammatory response. This is consistent with a study by Gao *et al*., who showed that supplementation with Chinese propolis in patients with type 2 diabetes mellitus causes high IL-6 levels.[30]

. Several theories suggest that IL-6 can stimulate two different pathways, a classic pathway and a trans-signaling pathway, which initiate the anti-inflammatory and[31,32] proinflammatory properties of IL-6, respectively. This is likely the reason for the lack of a significant difference in the mean IL-6 gene expression between the NCD and NCDP groups and the HFD and HFDP groups in this study. Since IL-6 is essential for regulating inflammation, propolis might help balance the anti-inflammatory properties of IL-6 without increasing its activity and making the proinflammatory properties dominant.[32] IL-6 can also be activated without the NF-κB stimulation via other cis-regulatory elements, such as Specificity Protein 1 (SP1) and Nuclear Factor IL-6 (NF-IL-6), which can be inhibited by proteins, such as PPARα. Further, the IL-6 mRNA is stabilized with proteins such as Arid5a and Regnase.[33]

According to the *E. coli* gene expression results in the propolis-treated group, the number of *E. coli* was decreased by week 4, and this low level was maintained until week 12. This suggests that the effect of propolis might start around week 4 or earlier, and by week 12, the level of *E. coli* might reach homeostasis. This is consistent with the IL-6 analysis in this study, probably because *E. coli* levels may correlate with gut inflammation. During week 4, the intestinal wall environment might still be in the early inflammatory response, making the effect of propolis more pronounced. However, at week 12, the IL-6 level might have reached an optimal level, eliminating inflammation.

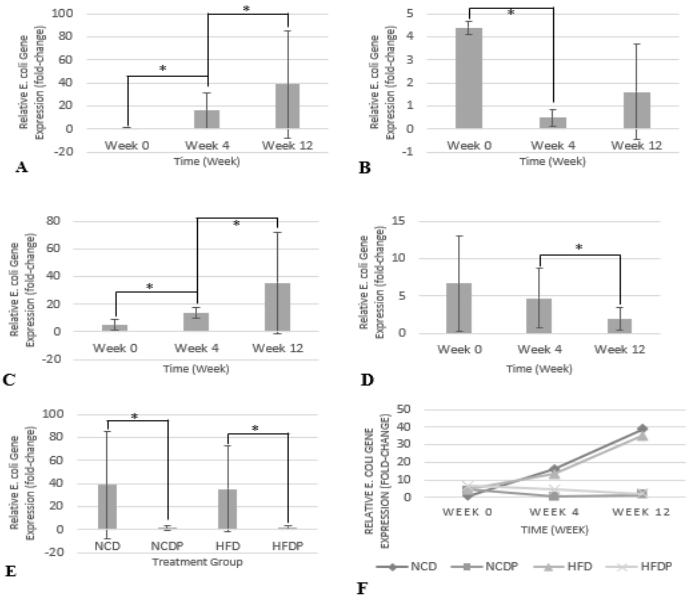

Figure 1. Relative quantification of *E. coli* gene expression in terms of fold changes. *E. coli* gene expression changes in the (A) NCD, (B) NCDP, (C) HFD, and (D) HFDP groups. (E) *E. coli* gene expression on week 12 with or without propolis supplementation. (F) Relative quantification of *E. coli* gene expression in all groups across the weeks. Data are presented in mean ± standard deviation. *$p < 0.05$ (NCD = Normal Chow

Diet; NCDP = Normal Chow Diet + Propolis; HFD = High-Fat Diet; HFDP = High-Fat Diet + Propolis)

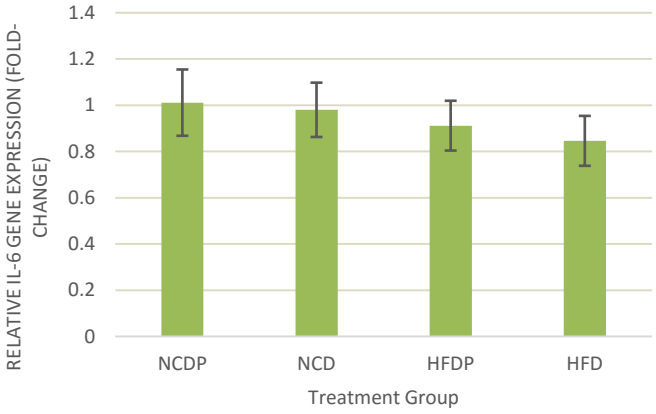

Figure 2. Average IL-6 gene expression normalized by GAPDH. Data are presented as mean ± standard deviation. * $p < 0.05$

Table 1. Results of Phytochemical Screening of *Trigona* spp. Propolis

Secondary metabolites	Results
Phenolics	+
Tannins	+
Flavonoids	+
Saponin	+
Triterpenoid	+
Alkaloid	-

+; presence of component, -: absence of component

Conclusion

This study provides evidence regarding the antibacterial effect of propolis extract obtained from North Sumatra, Indonesia, against *E. coli*. Consuming propolis continuously for 12 weeks can keep the level of *E. coli* bacteria low. Lastly, the findings also showed that propolis can act as an immunomodulator by increasing IL-6 levels in response to chronic intestinal inflammation in HFD-induced obese rats. Therefore, propolis can support the natural immune system of the body and strengthen its ability to fight bacterial infections, making it a suitable candidate for anti-infective therapy. Further studies in humans are required to validate the potential of propolis as a natural agent for supplementation or treatment of *E. coli* infections and chronic inflammatory processes leading to obesity. Further, propolis supplementation can also reduce the dependence on antibiotics and anti-inflammatory drugs, which have several adverse side effects.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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