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Original Research Article

Chronic Exposure to Various Propolis Extracts from North Sumatra Does Not Trigger Hepatic Inflammation and Autophagy Gene Expression in Wistar Rats

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ABSTRACT

The liver has key roles in metabolism, detoxification, and immunity, making it prone to inflammation and autophagy disruption. Natural bioactive compounds, such as stingless bee propolis, have been studied for their potential protective effects on various organs, including the liver. The purpose of this work was to analyze the phytochemical compounds of different propolis types and their effects on inflammation (IL-6 and TNF-α) and autophagy (LC3 and p62) gene expression in the liver. Propolis was obtained from different regions of North Sumatra, Indonesia (Karo Regency, Langkat Regency, and Tapanuli Tengah Regency), produced by two stingless bee species: *Geniotrigona thoracica* (Propolis A, B, C) and *Tetrigona apicalis* (Propolis D). Twenty-five Wistar rats (12 weeks old) were divided into five groups: a control group and four treatment groups receiving Propolis A, B, C, and D, each at an approximate dose of 100 mg/kg/day. Propolis was administered daily via drinking water for six consecutive months. Phytochemical screenings were conducted, and inflammation and autophagy gene expressions were evaluated using real-time polymerase chain reaction (PCR). Different phytochemical compounds were found in the four different propolis types, with Propolis B showing the most abundant phenolics, tannins, flavonoids, triterpenoids, and saponins. No significant difference in IL-6 and TNF-α gene expression was found between groups. The study highlights that chronic exposure to various propolis types does not induce hepatic inflammation or autophagy-related gene expression in healthy Wistar rats, while also suggesting that bee species and regional origin may influence their phytochemical composition and bioactivity.

Keywords: Autophagy, Inflammation, Liver, Propolis, Sumatra.

Introduction

The liver controls metabolism, neutralizes toxins, and contributes to immune homeostasis with its multifaceted role.[1,2] However, various stressors, including dietary factors, environmental toxins, and metabolic disorders, can disrupt liver function, leading to inflammation and altered gene expression.[3,4] Recent studies have explored the potential of natural compounds in mitigating liver damage, with propolis emerging as a promising hepatoprotective agent due to its diverse bioactive components, including flavonoids, phenolic acids, and terpenoids.[5–7] Propolis is a sticky, resin-like material that bees synthesize from plant secretions, which has demonstrated anti-inflammatory, antioxidant, and metabolic regulatory properties.[8–11]

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North Sumatran propolis, in particular, is of interest due to the region's rich biodiversity, which influences its chemical composition.[12,13] The biological activity of propolis can vary significantly depending on several factors, including the plant sources available in different geographical regions, the bee species collecting the resin, and the environmental conditions at the time of harvesting.[14,15] Differences in altitude, climate, and seasonal variations further contribute to fluctuations in bioactive compound levels.[8,16] These factors may result in distinct hepatoprotective effects, making it essential to evaluate the specific properties of North Sumatran propolis in liver health.

Inflammatory responses mediated by interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) play a crucial role in maintaining liver function, making them key targets in assessing the immunomodulatory effects of North Sumatran propolis.[17–19] By clearing damaged cellular components and restraining inflammation, autophagy plays a vital role in sustaining liver homeostasis.[20,21] Markers such as microtubule-associated protein 1 light chain 3 (LC3) and sequestosome 1 (p62) regulate cellular degradation and turnover mechanisms, making them essential indicators of liver health. The potential of North Sumatran propolis in maintaining liver function by controlling inflammation and autophagy after long-term chronic treatment remains an area of growing interest. This study aims to investigate the phytochemical compounds and comparative effects of North Sumatran propolis on inflammation and autophagy gene expression in the liver of adult Wistar rats.

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Materials and Methods

Collection of propolis

Propolis samples were collected in January 2023 from four different regions: Propolis A from Karo Regency 1 (outer hive), Propolis B from Karo Regency 2 (inner hive), Propolis C from Langkat Regency, and Propolis D from Tapanuli Regency, North Sumatra, Indonesia. The propolis samples were collected from two bee species: *Geniotrigona thoracica*, which produced Propolis A, B, and C, and *Tetrigona apicalis*, which produced Propolis D.

Propolis extract preparation

A 30% weight/volume hydro-glyceric extract of propolis was prepared by macerating 30 g of finely ground raw propolis in a solvent mixture consisting of 70 mL glycerol and 30 mL distilled water (final glycerol concentration: 70% volume/volume). The mixture was heated at 50°C with continuous agitation for 2 hours, then kept at room temperature overnight under agitation. Thereafter, the extract was filtered to remove insoluble residues. To achieve an approximate dose of 100 mg/kg body weight/day in Wistar rats weighing approximately 300 g and consuming around 30 mL of drinking water daily, 1.67 mL of the propolis extract (equivalent to ~34 drops) was added to 500 mL of drinking water. This yielded a final concentration of 1 mg/mL propolis, providing each rat with approximately 30 mg of propolis per day.

Qualitative phytochemical screening

The phytochemical screening of the different propolis extracts (A, B, C, and D) was conducted to identify the presence of phenolics, saponins, tannins, flavonoids, and triterpenoids using standard qualitative phytochemical screening tests. The phenolic content was detected using the ferric chloride test, where the addition of 2-3 drops of 5% $FeCl_3$ solution to the extract resulted in a blue-black or green-black coloration, indicating phenolics. The saponin test was performed using the froth/shake test, where vigorous shaking of the extract with distilled water produced a stable froth/foam, confirming the presence of saponins. The presence of tannins was determined using 2-3 drops of 1% $FeCl_3$, which produced a blue-black or green-black colour. For flavonoids, the alkaline reagent test was used, where the addition of 2-3 drops of 10% NaOH to the extract resulted in a yellow coloration that disappeared upon acidification with HCl. The presence of triterpenoids was confirmed using Salkowski's test, where the addition of chloroform and 1 drop of concentrated H_2SO_4 produced a reddish-brown interface. The results of these tests were recorded based on colour intensity or foam stability to confirm the phytochemical constituents in the propolis extract.[22]

Animals

Adult male Wistar rats, 12 weeks old, with average body weight 270 ± 30 g were obtained from PT Bio Farma, Bandung, Indonesia. The animals were divided into 5 groups of five animals per group: a control group, and four propolis groups A, B, C, and D. Rats in the control group were fed with pelletized rodent chow diet, while those in the propolis groups (Propolis A, B, C, D) were fed with propolis extracts each at an approximate dose of 100 mg/kg BW/day for six consecutive months. Propolis was administered daily via drinking water. The rats were housed in well-ventilated cages at room temperature with 12-hour light and dark cycles every day.

Ethical approval

All procedures were based on the use and care of laboratory animal guidelines. The study was approved by the Research Ethics Committee, Universitas Kristen Maranatha Bandung, Jawa Barat, Indonesia with approval reference number 079/KEP/IV/2023.

RNA extractions and real-time PCR

After the experimental period, the rats were sacrificed under deep anesthesia using isoflurane, in accordance with the institutional animal ethics guidelines. The liver was harvested, and was immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. RNA extraction was performed from frozen liver tissues using Genezol reagent (Geneaid, Taiwan) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the spectrophotometric absorbance at 268/280 nm (Multiscan Go). To conduct real-time PCR, the One Step Real-time PCR Kit (Bioline, United Kingdom) was used. This study employed GAPDH as the internal control gene. The list of primer sequences is provided in Table 1.

Table 1: Primers used for real-time PCR analysis

Gene symbol	Primer sequence (5' to 3') Upper strand: sense Lower strand: antisense	Product size (bp)	Annealing (°C)	Cycle
IL-6	GAAGTTAGAGTCACAGAAGGAGTG GTTTGCCGAGTAGACCTCATAG	105	58	35
TNF-α	GTCGTAGCAAACCACCAAGC TGTGGGTGAGGAGCACATAG	187	58	35
LC3	GGTCCAGTTGTGCCTTTATTGA GTGTGTGGGTTGTGTACGTCG	153	59.5	35
p62	CTAGGCATCGAGGTTGACATT CTTGGCTGAGTACCACTCTTATC	116	56	35
GAPDH	GTTACCAGGGCTGCCTTCTC GATGGTGATGGGTTTCCCGT	177	61	35

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were conducted using SPSS software version 27.0. Prior to group comparisons, tests for normality and homogeneity were performed. One-way analysis of variance (ANOVA) was used to assess differences among groups. For datasets meeting the assumption of homogeneity, Tukey's HSD post hoc test was applied, while for non-homogeneous data, the Kruskal-Wallis or Mann-Whitney test was employed.

Results and Discussion

Phytochemical constituents of propolis from different regions of Sumatra

The analysis of four propolis samples (Propolis A, B, C, and D) revealed variations in phenolic, tannin, flavonoid, saponin, and triterpenoid contents (Figure 1). Propolis from Karo Regency (Inner hive) (Propolis B) exhibited the highest phytochemical content across all categories, particularly in phenolics (++), tannins (++), flavonoids (++), saponins (+++), and triterpenoids (++). In contrast, propolis from Central Tapanuli Regency (Propolis D) had the lowest phytochemical content, with no detectable phenolics, tannins, or flavonoids, and only low levels of saponins (+) and triterpenoids (+). Among all phytochemicals, saponins were consistently the most abundant phytochemical in the propolis extracts, with the highest concentration (+++) in Propolis A,

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B, and C. In this study, the propolis samples were produced by two different bee species: *Geniotrigona thoracica* (Propolis A, B, and C) and *Tetrigona apicalis* (Propolis D). Propolis from *Geniotrigona thoracica* contained higher levels of phytochemicals compared to *Tetrigona apicalis*. Research has shown that propolis from different bee species contains unique bioactive compounds, affecting its antimicrobial, antioxidant, and anti-inflammatory properties.[8,23,24] Bee species and botanical sources play a critical role in determining the composition and biochemical content of propolis due to differences in resin collection behavior and hive environments.[24,25] Due to their non-aggressive behaviour, environmental adaptability, and unique honey composition, stingless bees are often preferred over common honeybees for medicinal purposes.[26] *Geniotrigona thoracica* and *Tetrigona apicalis* are stingless bees that are commercially raised in Southeast Asia for meliponiculture, as for rearing stingless bees (cultivating and managing bee colonies for their honey, propolis, and pollen).[27]

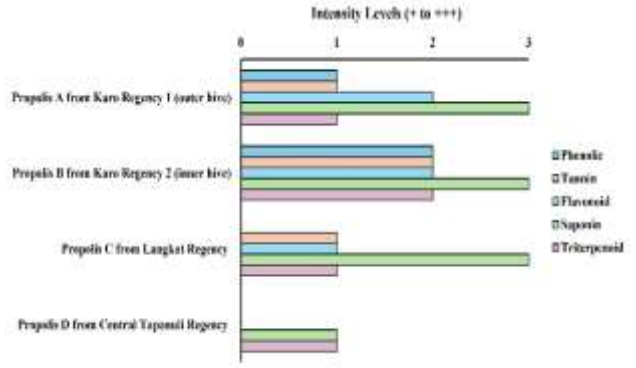

Figure 1: Phytochemical constituents of propolis from different regions of North Sumatra, Indonesia

Research in Malaysia regarding *Geniotrigona thoracica* propolis extracts has found that the presence of flavonoids, terpenoids, saponins, tannins, steroids, and cardiac glycosides might contribute to its antioxidant activity.[28] The phytochemical analysis revealed that Propolis B had the highest levels of phenolic compounds, tannins, flavonoids, and triterpenoids, which have been linked to antioxidant, anti-inflammatory, and autophagy-modulating properties.[8,26,29] Propolis A has less phenolic, tannin, and triterpenoid content than Propolis B. This might be caused by the source of the hive or the outer hive used for harvesting. Propolis C also has less tannins, flavonoids, triterpenoids, and no phenolic contents compared to Propolis B, which was hypothesized that the low altitude of Langkat Regency might contribute to its contents. Research in Indonesia regarding *Tetrigona apicalis* propolis extract has found that alkaloids, flavonoids, triterpenoids, saponins, and tannins might contribute to its anti-inflammatory activity.[30] In contrast, Propolis D had the lowest concentration of these bioactive compounds, possibly explaining its relatively weaker effects on autophagy and inflammation.

Overall, the regional variation in phytochemical profiles suggests that both geographical region (environmental factors and floral sources) and bee species play significant roles in determining the phytochemical profile and bioactivity of propolis.[8,14,16] These differences might be influenced by various factors such as altitude, climate, soil type, and vegetation.[16,31] Karo Regency, located in North Sumatra Province, Indonesia, is home to the Karo people, a distinct sub-ethnic group of the Batak.[32] With its highland topography, Karo Regency provides cooler temperatures and diverse montane vegetation, which might be correlated with higher phytochemical compounds in Propolis A and B. Lowland and coastal terrain of Langkat Regency, and moderate land of Central Tapanuli, provide low and moderate land vegetation, affecting the resin type that the bee collected.[33,34] Propolis from inner hive contains more phytochemical compounds such as phenols, flavonoids, and terpenoids, compared to outer hive/hard propolis.[29,35] Inner hives provide a more stable and controlled environment that is crucial for brood development, food storage, and colony health, contributing to propolis's antimicrobial, antioxidant, and anti-inflammatory properties.[8,36,37] Outer hives act as the first line of defense and are exposed to environmental stressors; thus, they have a lower concentration of phenolics and flavonoids but a higher content of terpenes, volatile oils, and wax.[8,37]

The effects of propolis on IL-6 and TNF-α gene expression

The expression levels of IL-6 and TNF-*α*, key inflammatory cytokines, were analyzed from liver tissue to determine the potential effects of propolis on inflammation. IL-6 and TNF-*α* expression levels were not significantly different between groups, with $p = 0.746$ for IL-6 and $p = 0.554$ for TNF-*α*. Although no significant differences were found, Propolis B groups showed the lowest levels of IL-6 and TNF-*α* gene expression compared to other Propolis groups (Figure 2).

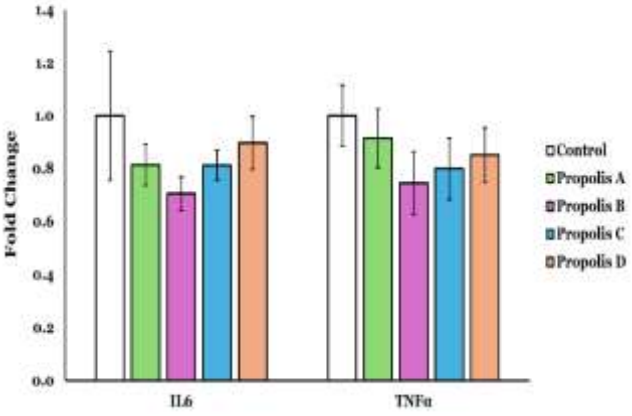

Figure 2: The effects of propolis on IL-6 and TNF-*α* gene expression. No significant differences were observed between groups

Although the study was conducted in healthy Wistar rats, the animals were 6 months old at the beginning of the experiment and received propolis supplementation for 6 months, reaching approximately 12 months of age by the end of the study. At this stage, rats may begin to experience early age-related physiological changes, including low-grade chronic inflammation (inflammaging). Therefore, evaluation of inflammatory cytokines such as IL-6 and TNF-*α* remains relevant, even in the absence of an induced inflammatory or disease model. In this study, we found the IL-6 and TNF-*α* gene expression was the lowest in the Propolis B group, indicating its potential property as a pro-inflammatory reducer compared to other Propolis groups. Propolis, rich in phenolic compounds like caffeic acid, quercetin, and caffeic acid phenethyl ester (CAPE), has been shown to exert anti-inflammatory effects by downregulating key inflammatory signaling components and significantly reducing pro-inflammatory cytokines such as TNF-*α*, IL-1*β*, IL-6, and NLRP3, as demonstrated by Li *et al*. in MDA-MB-231 breast cancer cells.[38] The non-significant results observed in this study showed that administration of various propolis derived from North Sumatra did not induce liver inflammation following six months of treatment. The absence of significant elevation in the expression of IL-6 and TNF-*α* suggests that the different propolis types, regardless of their hive origin or bee species, do not provoke inflammatory responses in hepatic tissue under long-term use. While this finding does not directly demonstrate anti-inflammatory effects, it indicates that prolonged administration of propolis is not associated with hepatic inflammation in a non-disease model. These results support the general findings of previous studies reporting the anti-inflammatory and hepatoprotective effects of propolis, largely attributed to its bioactive constituents such as flavonoids, phenolic acids, and terpenoids.[5–7,30] Notably, the propolis samples used in this study did not demonstrate any hepatotoxic effects, further supporting their safety for long-term use.

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The effects of propolis on LC3 and p62 gene expression

The expression levels of LC3 and p62, key markers in autophagy regulation, were analyzed to evaluate the potential effects of propolis. The effects of propolis on LC3 and p62 gene expression are shown in Figure 3. The results demonstrated no significant difference in LC3 gene expression in all groups, while p62 gene expression was significantly lower in Propolis B, C, and D compared to Propolis A. For the Propolis B group, the LC3 gene expression was the highest, with the p62 gene expression being the lowest, although not significant compared to the control group. As for Propolis A, C, and D, non-significant differences in LC3 and p62 gene expression were found compared to the control group. These results suggest that long-term administration of North Sumatran propolis does not disrupt hepatic autophagic processes.

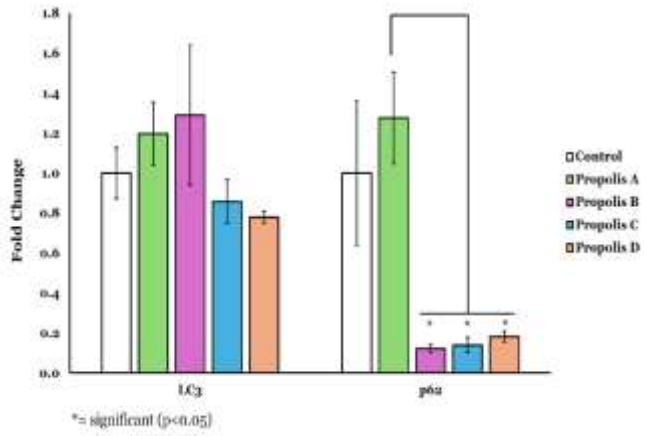

Figure 3: The effects of propolis on LC3 and p62 gene expression. No significant differences were observed between groups. No significant differences in LC3 gene expression were found between groups, while p62 gene expression was different between propolis A and B (p=0.033), propolis A and C (p=0.033), and propolis A and D (p=0.039). LC3 levels were the highest and p62 levels the lowest in propolis B group.

Autophagy is a crucial mechanism for maintaining cellular homeostasis by facilitating the degradation of damaged organelles and proteins, particularly under conditions of metabolic or oxidative stress.[20,21] According to a previous review, propolis and its components act as an autophagy modulator (upregulation or downregulation), correlated with the regulation of redox balance and inflammatory processes. The review further discussed components of propolis that might potentially modulate autophagy, such as kaempferol, galangin, chrysin, pinocembrin, artepillin C, and other components, which might be associated with bee types and geographical sources.[39] The absence of significant changes in autophagy gene expression implies that hepatic cells maintained basal autophagic activity, which was neither upregulated in response to cellular damage nor suppressed to a level that could compromise the clearance of damaged organelles or proteins. This steady-state expression may reflect the ability of propolis to support physiological homeostasis within the liver on prolonged administration, possibly by preserving basal autophagic activity. Rather than inducing stress-related autophagy or suppressing essential autophagic turnover, propolis may help maintain a balanced autophagic state that is important for organelle quality control and cellular integrity in healthy hepatic tissue.

These findings highlight the effects of different origins, regions, and bee species of propolis on their phytochemical compounds, which might finally influence their effects on inflammation and autophagy gene expression in the liver following chronic exposure. The non-significant changes of IL-6, TNF-α, LC3, and p62 indicate the safety of different types of propolis from North Sumatra on liver inflammation and autophagy following 6 months of treatment. The findings of this study are summarized in Figure 4.

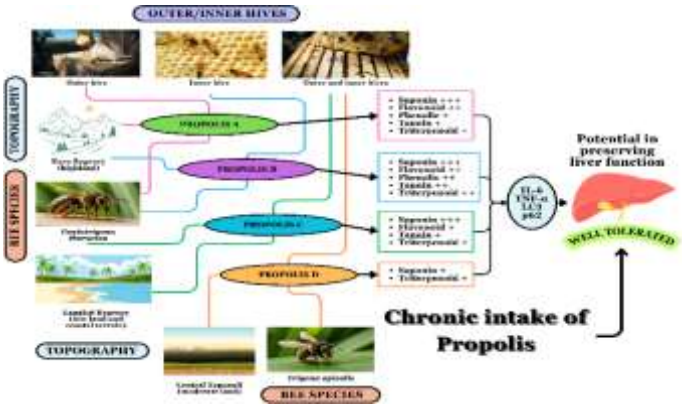

Figure 4: Different types of North Sumatran propolis phytochemical compounds and their effects on liver inflammation and autophagy.[11,40]

The study has several limitations, for example, other inflammation and autophagy markers were not explored, biochemical contents using more advanced technology were not determined, and only one animal (Wistar rat) model was employed. Future research should focus on expanding sample diversity, conducting in-depth analyses on the role of bee species in resin collection and bioactivity, and further standardizing the bioactive content of propolis. In addition, studies using disease-induced animal models are needed to elucidate molecular pathways and validate the long-term hepatoprotective potential of propolis under pathophysiological conditions.

Conclusion

The findings from this study have shown that bee species and geographical sources influence the phytochemical compounds found in each type of propolis from North Sumatra. The propolis type with the most abundant phytochemical content had the highest potential to modulate inflammation and autophagy in the liver.

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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