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Anti-inflammatory activity of propolis extract from the stingless bee, *Tetragonula pagdeni*, in mangosteen orchard

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ABSTRACT

In Thailand, *Tetragonula pagdeni* (TP) Schwarz is one of the most common and abundantly cultured stingless bee species in mangosteen orchards. The propolis extract of TP has been used as a traditional medicine to treat inflammation. TP propolis extract also exhibited antioxidant, antiproliferative, and antimicrobial properties in scientific reports. However, the anti-inflammatory effect of TP propolis extract has been limited. Thus, this study aims to evaluate the anti-inflammatory and antioxidant effects of TP propolis extract through alteration of mRNA expression of genes involved in inflammatory processes, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and cytokines (e.g., tumor necrosis factor-α [TNF-α], interleukin-6 [IL-6], and interleukin-10 [IL-10]). High-performance liquid chromatography (HPLC) analysis was used to quantify the amount of major compounds, α- and γ-mangostin. TP propolis extract suppressed mRNA levels of COX-2, iNOS, TNF-α, and IL-10 in lipopolysaccharide-stimulated RAW 264.7 mouse macrophages and had no effect on IL-6 expression. HPLC quantification of α- and γ-mangostin yielded values of 36 and 34 μg/g extract, respectively. Therefore, we herein demonstrate that TP propolis extract exhibited an anti-inflammatory effect associated with the downregulation of genes involved in the inflammatory cascade. These findings suggest that TP propolis extract is a promising candidate as a nutraceutical or pharmaceutical product to relieve inflammation.

Keywords: Anti-inflammation, mangostin, propolis, stingless bee, *Tetragonula pagdeni*

INTRODUCTION

Inflammation is a crucial physiological response to irritation, injury, or infection, characterized by redness, heat, swelling, loss of function, and pain.[1] Chronic inflammation is important to the etiology of many chronic diseases, including autoimmunity, cancer, atherosclerosis, and Alzheimer’s diseases.[2,3] Several types of immune cells play important roles in inflammation after tissue damage.[4] Mast cells and macrophages are activated, and some circulating immune cells, including neutrophils, are recruited, leading to the release of various chemical mediators. The resulting “inflammatory cocktail” is rich in cytokines (e.g., tumor necrosis factor-α [TNF-α], interleukin-6 [IL-6], and interleukin-10 [IL-10]), growth factors, amines (e.g., histamine and 5-hydroxytryptamine), arachidonic acid metabolites (e.g., prostanoids and leukotrienes), nitric oxide (NO), bradykinin, ATP, and protons.[4]

Natural products, including those derived from higher plants, have greatly contributed to the development of modern therapeutic interventions for inflammatory diseases. Most plant-derived secondary metabolites are known to interfere with multiple molecules and mechanisms directly or indirectly. These include inflammatory mediators, production of second messengers (e.g., cGMP, cAMP and protein kinases), expression of transcription factors (e.g., activator protein 1 and nuclear factor-kappa B [NF-κB]), expression of proto-oncogenes (e.g., c-jun, c-fos, and c-myc), and expression of...
key pro-inflammatory molecules (e.g., inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), cytokines, neuropeptides, and proteases).

Stingless bees (Meliponini), comprising a large tribe of the bee family (Apoida), are distributed worldwide, especially in subtropical and tropical regions, and have been marketed for the production of a range of commercial honey products with valuable nutritional and medicinal properties. The propolis extract of stingless bees has been used as an indigenous medicine to treat gingivitis, skin inflammation, and acne in Thailand and other Asian countries and has been reported to exhibit antioxidant, antiproliferative, antimicrobial properties, and inhibition on α-glucosidase which vary according to the local diversity of plants and bee species. In Thailand, Tetragonula pagdeni (TP) is one of the common and abundantly cultured stingless bee species, especially in mangosteen gardens.

The active constituents of Thai stingless bee propolis from mangosteen orchards include prenylated xanthones, namely, α-, β-, γ-mangostins; mangostatin; 8-deoxygartanin; gartanin; and garcinone B. Among these compounds, α- and γ-mangostins are the most active and important constituents of this plant and exhibit antibacterial, and antioxidant properties. However, the anti-inflammatory effect of TP propolis from mangosteen orchard has been the subject of few scientific studies. Therefore, this study aimed to investigate the effects of propolis extracts from TP on the mRNA expression patterns of genes involved in inflammatory processes and immune responses, including inflammatory cytokines (TNF-α, IL-10), iNOS, and COX-2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 mouse macrophages.

MATERIALS AND METHODS

Chemicals

Gallic acid, Folin-Ciocalteu reagent, quercetin, trolox, 2,2’-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich (St. Louis, MO, USA). Aluminum chloride, ascorbic acid, and sodium carbonate were obtained from Ajax Finechem, Taren Point, Australia. Standard α- and γ-mangostins (>98%) were purchased from Chengdu Biopurify Phytochemicals Ltd., Sichuan, China. Dulbecco’s Modified Eagle Medium (DMEM), phosphate buffer saline (PBS), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, fetal bovine serum (FBS), penicillin/streptomycin (P/S) solution, and other tissue culture reagents were purchased from Gibco (Grand Island, NY). All other chemical reagents were of analytical grade and used without further purification.

Propolis Material

Propolis of the stingless bee (T. pagdeni) was obtained from an apiary in a mangosteen orchard in the Markham District of Chanthaburi province, Thailand, in December 2016. Stingless bee was identified by Dr. Chama Inson (Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand). Voucher specimens (No. 121601) have been deposited with the Faculty of Pharmaceutical Sciences, Burapha University, Chonburi, Thailand.

Extraction Method

According to our previous study, acetone was the extracting solvent that produced the highest content of α- and γ-mangostins. Briefly, TP propolis (10 g) was cleaned, cut into small pieces, and extracted with 200 mL of acetone at 40°C for 30 min. The obtained suspension was centrifuged at 4,200 × g for 5 min at 20°C, and the pellet was re-extracted twice. The supernatants were pooled and concentrated in a rotary evaporator, and yield content was calculated. The crude extract was stored in the refrigerator at 4°C until further analysis.

Phytochemical Analysis

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent. TP propolis extract solution (20 μL, 1 mg/mL) and Folin-Ciocalteu reagent (50 μL) (diluted 1:10 with deionized water) were mixed in a 96-well plate for 3 min. Sodium carbonate (80 μL; 75% w/v Na₂CO₃) were added. The mixture was incubated for 2 h in the dark at room temperature. The absorbance at 765 nm was analyzed using a microplate reader (Tecan, Maennendorf, Switzerland). Each sample was analyzed in triplicate. The total phenolic content was expressed as mg gallic acid equivalent per gram of extract (mg GAE/g extract).

Determination of total flavonoid content

The total flavonoid content was determined according to the study by Stankovic with some modifications. TP propolis extract solution (100 μL) at a concentration of 1 mg/mL in methanol was mixed with 2% w/v AlCl₃ solution (100 μL). The mixture was incubated in a 96-well plate for 10 min at room temperature. The absorbance at 415 nm was analyzed using a microplate reader (Tecan, Maennendorf, Switzerland). The sample was analyzed in triplicate for each analysis. The total flavonoid content was expressed as mg quercetin equivalent per gram of extract (mg QE/g extract).

Quantitative analysis of the bioactive compounds α- and γ-mangostins using high-performance liquid chromatography (HPLC)

HPLC analysis was performed according to our previous study. The HPLC system ( Shimadzu, Kyoto, Japan) consisted of a quaternary HPLC pump (LC-10AD) with a degasser (DGU-14A3), a UV-visible detector (SPD-10AV), an autosampler (SIL-10AD), and a system controller (SCL-10A) Separation was conducted on a BDS Hypersil C18 (4.6 × 150 mm; i.d., 5 μm) with a C18 guard column. The mobile phases contained 0.2% formic acid (A) and methanol (B) using gradient elution: 75% B in A to 90% B in 10 min, and 100% B for 10 min. This column was re-equilibrated with 75% B in A for 10 min before each analysis, and the flow rate was set at 1.0 mL/min at 25°C. The UV detector was set at a wavelength of 245 nm.

Determination of Antioxidant Activity

DPPH radical scavenging assay

The DPPH scavenging activity was analyzed as previously described. DPPH radical was freshly prepared in methanol at a final concentration 152 μM. Each TP propolis extract was
diluted in methanol to varying concentrations. In a 96-well plate, 100 μL of each sample was added to each well, followed by the addition of 100 μL of methanolic DPPH solution. Ascorbic acid was used as a standard and treated under the same conditions as the samples. The mixtures were allowed to incubate at room temperature in the dark for 30 min. The absorbance at 517 nm was recorded using a microplate reader (Tecan, Maennedorf, Switzerland). The scavenging activity was calculated by the formula: % inhibition = [(Ac–As)/Ac] × 100, where Ac and As are the absorbance of the control and sample solution at 517 nm, respectively. The IC_{50} (concentration providing 50% inhibition) value was calculated from a linear regression plotting between % inhibition and TP propolis extract concentrations. The antioxidant activity was expressed as the antioxidant activity index (AAI) which calculated by the formula; AAI = final concentration of DPPH (μg/μL)/IC_{50} (μg/μL).[20]

**ABTS radical scavenging assay**

The procedure for the ABTS assay followed the method of Thaipong et al. with some modifications.[21] Stock solutions included 7 mM ABTS solution and 2.45 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12–16 h in the dark at room temperature. The solution was then diluted by mixing 1 mL of ABTS solution with 25 mL of methanol to obtain an absorbance of 1.100 ± 0.020 units at 734 nm using a microplate reader (Tecan, Maennedorf, Switzerland). Fresh ABTS + solution was prepared for each assay by mixing 10 μL of TP propolis extract (1 mg/mL) with 200 μL of ABTS+ radical cation solution in a 96-well plate. Then, the absorbance of the mixture at 734 nm was determined after 6 min using a microplate reader. All determinations were conducted in triplicate. Results were expressed as mg trolox equivalent antioxidant capacity per gram of extract (mg TEAC/g extract).

**Ferric reducing power (FRAP)**

To detect the antioxidant capacities of TP propolis extracts, FRAP assay was conducted.[22] TP propolis extract solution (500 μL, 1 mg/mL) was mixed with 500 μL of potassium phosphate buffer (0.2 M, pH 6.6) and 500 μL of 1% w/v potassium ferricyanide solution. The mixture was incubated at 50°C for 20 min. Then, 2 mL of trichloroacetic acid was added to stop the reaction. The supernatant (100 μL) was mixed with 100 μL of deionized water before the addition of 20 μL of 0.1% w/v ferric chloride solution. Reactions were conducted in triplicate and allowed to incubate for 30 min before measuring absorbance at 700 nm using a microplate reader. FeSO\textsubscript{4} was used to construct a standard curve. Results were expressed as mmol FeSO\textsubscript{4} equivalents per gram of extract (mmol FeSO\textsubscript{4}/g extract).

**Determination of Anti-Inflammatory Activity**

**Cell culture**

RAW 264.7 mouse macrophages were cultured and maintained in DMEM supplemented with 10% FBS and 1% P/S at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}.[23] After the RAW 264.7 cells reached 80% confluence, the cells were passaged by treatment with 0.25% trypsin-EDTA to maintain exponential cell growth. The medium was changed to serum-free DMEM, and the cells were incubated for at least 30 min before initiating experiments.

**Cell viability test**

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as previously described.[24] Briefly, RAW 264.7 cells were seeded onto 96-well plates at a density of 1 × 10\textsuperscript{4} cells/well in 200 μL of DMEM supplemented with 1% FBS and 1% P/S and incubated overnight. The cells were divided into control and treatment groups. The control group was exposed to various concentrations of vehicle (dimethyl sulfoxide; DMSO), while the treatment groups were treated with various concentrations of TP extract (0.01–100 μg/μL) for 24 h. The TP propolis extract was dissolved in DMSO and further diluted with a complete medium at a final concentration of DMSO 0.1%. After incubation, the culture medium was replaced with 100 μL MTT solution (1 mg/mL dissolved in DMEM), and the RAW 264.7 cells were incubated for an additional 4 h at 37°C. Then, the MTT solution was removed by aspiration, and 100 μL of DMSO was added to dissolve the insoluble formazan product. The samples were mixed, and the absorbance of each well at 570 nm was measured using a Clariostar microplate reader (BMG Labtech, NC, USA). The number of viable cells was calculated as a percentage of the number of control cells. All assays were performed in triplicate and repeated 4 times.

**Induction of inflammatory processes in RAW 264.7 cells**

The anti-inflammatory effect of TP propolis extract was investigated in a model of LPS-induced pro-inflammatory response using RAW 264.7 mouse macrophages. Inflammatory cascades were induced in RAW 264.7 cells by the addition of LPS from Salmonella enterica serotype Typhimurium as previously described.[25] Briefly, RAW 264.7 cells were seeded onto 6-well plates at a density of 1 × 10\textsuperscript{4} cells/well in DMEM supplemented with 10% FBS and 1% P/S and maintained for 24 h in a humidified incubator at 37°C in a 5% CO\textsubscript{2} atmosphere. Then, the culture medium was replaced with DMEM supplemented with 10% FBS and 1% P/S. To ensure that all compounds were completely distributed over the cells, the cells were pretreated with either vehicle, diluted TP propolis extract (10 μg/mL), or dexamethasone (0.1 μM) as a positive control (dissolved in DMSO and further diluted with complete medium at a final concentration of DMSO 0.1%) for 3 h before stimulation with 1 μg/mL LPS for 12 h. The culture medium was then removed, and the cells were washed with ice-cold PBS.

**Measurement of mRNA expression of inflammation-related genes using quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

Total RNA from RAW 264.7 cells was extracted using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, MA, USA) as previously described.[25] The expression levels of inflammation-related genes, including TNF-α, iNOS, IL-6, IL-10, and COX-2, were determined using RT-qPCR with KAPA SYBR One-step RT-qPCR kits (KAPA Biosystems, St. Louis, MO, USA) according to the manufacturer’s protocol. The gene-specific primers for RT-qPCR (mouse) are shown in Table 1. RT-qPCR was performed under the following conditions: Reverse transcription at 42°C for 5 min; reverse transcriptase inactivation and DNA polymerase activation at 95°C for
2–5 min; combined annealing, extension, and data acquisition at 95°C for 5 s and 55°C for 30 s (40 cycles); and final extension at 72°C for 1 min, followed by 25°C for 2 min. Relative mRNA expression levels were evaluated using the comparative cycle threshold method and normalized to an endogenous reference, glyceraldehyde-3-phosphate dehydrogenase.

**Statistical Data Analysis**

Data were expressed as means ± SEM. Statistical analyses were performed using one-way analysis of variance, followed by Dunnett’s multiple range tests for post hoc analysis. P < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Phytochemical Analysis**

Our TP propolis extract was brownish-green in color with a sour and astringent taste and characteristic odor. The extraction yield was 11.65 ± 2.24%. The HPLC chromatogram of the TP propolis extract showed prominent peaks at retention times of 14.73 and 11.96 min, which corresponded to the reference standards for α- and γ-mangostins [Figure 1]. The α- and γ-mangostin content in the TP propolis extract was 35.80 ± 0.79 and 33.58 ± 0.78 mg/g, respectively. The total phenolic content of the TP propolis extract was 14.23 ± 0.62 mg GAE/g extract, and the total flavonoid content was 1.42 ± 0.05 mg QE/g extract.

**Antioxidant Activities of TP Propolis Extract**

Table 2 shows the antioxidant activities and total phenolic and flavonoid contents of the TP propolis extract. The DPPH and ABTS radical scavenging activities are reported as AAI values of 0.70 and 242.78 mg TEAC/g extract, respectively. The TP propolis extract exhibited a reducing power value of 115.72 mmol FeSO4/g extract.

**Anti-inflammatory Effect of TP Propolis Extract in RAW 264.7 Cells**

Using MTT colorimetric assays, we found that TP propolis extract was not toxic to RAW 264.7 cells at concentrations between 0.01 and 10 μg/mL [Figure 2]. Therefore, 10 μg/mL TP propolis extract was used for subsequent experiments. The ability of TP propolis extracts to inhibit mRNA expression of LPS-induced inflammatory genes in RAW 264.7 macrophages was investigated using RT-qPCR. The mRNA expression of inflammatory-related genes including COX-2, iNOS, TNF-α, IL-6, and IL-10 was investigated using RT-qPCR. The mRNA expression of inflammatory-related genes including COX-2, iNOS, TNF-α, IL-6, and IL-10 was increased after stimulation with LPS for 12 h [Figure 3a]. Interestingly, treatment with TP propolis extract significantly inhibited LPS-induced COX-2, iNOS, TNF-α, IL-6, and IL-10 mRNA expression compared with that in control-treated cells (P < 0.05) [Figure 3a-d]. Conversely, the mRNA expression of IL-6 was unchanged [Figure 3e]. The effect of TP propolis extract on these inflammation-related genes was comparable to that of dexamethasone.

**DISCUSSION**

The inflammatory response to irritation, injury, or infection is a highly efficient protective and repair mechanism initiated and perpetuated by an array of interactive chemical mediators and cells.[1] Despite dramatic progress in the understanding of the

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**Figure 1:** High-performance liquid chromatography chromatogram of *Tetragonula pagdeni* propolis extract from mangosteen orchard

**Table 1:** The gene-specific primers for RT-qPCR (mouse)

<table>
<thead>
<tr>
<th>Gene-specific primers</th>
<th>Sequences</th>
</tr>
</thead>
</table>
| COX-2                 | Sense 5′-TGCTGTGCTGGAATGTCA-3′  
Antisense 5′-CAGTAGAGACGGGCTCTCC-3′ |
| iNOS                  | Sense 5′-GTGTTCCACCAGAGATTG-3′  
Antisense 5′-CTCTGCCACCTGAAGTGC-3′ |
| TNF-α                 | Sense 5′-TACTGAACTCGGATTTTG-3′  
Antisense 5′-CAGCTTGTGCCGGTGAAG-3′ |
| IL-6                  | Sense 5′-CCGAGAGAGACCCAGAC-3′  
Antisense 5′-GGAAATTGGGGTGAAG-3′ |
| IL-10                 | Sense 5′-GCTGGACAACTACTGCTAAC-3′  
Antisense 5′-ATTTCGATAAGGCTTGGC-3′ |
| GAPDH                 | Sense 5′-GGCTGTTCCACCATC-3′  
Antisense 5′-GGCTCGAGGACATTC-3′ |

COX-2: Cyclooxygenase-2, iNOS: Inducible nitric oxide synthase, TNF-α: Tumor necrosis factor-α, IL-10: Interleukin-10, RT-qPCR: Quantitative reverse transcription polymerase chain reaction, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
inflammatory process and the myriad of mediators involved, there is no effective and safe disease-modifying treatment for the management of inflammatory diseases thus far. Various plant-derived compounds present anti-inflammatory properties by interfering with targets involved in the inflammatory process. Therefore, this study aims to investigate the effect of TP propolis extract on the production of crucial inflammatory mediators, including cytokines (TNF-α, IL-6, and IL-10) and COX-2, and on the activity of iNOS in vitro.

Variation in the chemical composition of propolis was directly related to plant sources at the collection site, geographical region, climatic environment, and harvest seasons.[26] Our previous HPLC and HPTLC analysis studies, indicated the presence of α- and γ-mangostin as the major active constituents of Thai stingless bee propolis from mangosteen garden.[13,19] α- and γ-mangostin possess a wide range of biological activities including antioxidant, anti-inflammatory, and antimicrobial activities. The antioxidant activity of α- and γ-mangostin has been extensively studied in several in vitro and in vivo model systems.[27] Moreover, propolis extract from TP was reported to have a higher amount of total phenolic content than propolis extracts from other Thai stingless bee species.[15] The considerable amount of phenolic compound in TP propolis extract indicated the possible influences of its high antioxidant activity. In accordance with the studies of propolis produced by Brunei and Indonesian stingless bee, their antioxidant activities were also correlated with the amount of

<p>| Table 2: Total phenolic content, total flavonoid content, and antioxidant activities of (TP) propolis extract |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Assay</th>
<th>TP propolis extract*</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC (mg GAE/g extract)</td>
<td>14.23±0.62</td>
<td>-</td>
</tr>
<tr>
<td>TFC (mg QE/g extract)</td>
<td>1.42±0.05</td>
<td>-</td>
</tr>
<tr>
<td>DPPH (AAI)</td>
<td>0.70</td>
<td>14.22</td>
</tr>
<tr>
<td>ABTS (mg TEAC/g extract)</td>
<td>242.78±3.74</td>
<td>-</td>
</tr>
<tr>
<td>FRAP (mmol FeSO₄/g extract)</td>
<td>115.72±4.24</td>
<td>-</td>
</tr>
</tbody>
</table>

*Data are expressed as mean±SD (n=3), TPC: Total phenolic content, TFC: Total flavonoid content, AAI: Antioxidant activity index, FRAP: Ferric reducing power

![Figure 2: Cytotoxicity of Tetragonula pagdeni propolis extract on RAW 264.7 macrophage cells for 24 h. Cell viability was quantified, expressed as a percentage of untreated cells, and shown as the means ± SEM (n=4). *P<0.05 versus group](image)

![Figure 3: Effects of Tetragonula pagdeni propolis extract (10 μg/mL) on mRNA expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and interleukin-6 (IL-6) in lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells. Relative mRNA levels of COX-2 (a), iNOS (b), TNF-α (c), IL-6 (d), and IL-10 (e) were quantified, normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase level, and expressed as relative to the control. Data are expressed as means ± standard errors of the mean (SEM) (n = 4). *P<0.05 versus control; **P<0.05 versus LPS-stimulated group; Dexa, dexamethasone (0.1 μM)](image)
phenolic compounds in the propolis.\textsuperscript{[28,29]} Chen et al. reported that \(\alpha\)- and \(\gamma\)- mangostin significantly inhibited NO and PGE\(_2\) production from LPS-stimulated RAW 264.7 cells.\textsuperscript{[30]}

LPS from \(S. \) enterica serotype Typhimurium, a constituent of the cell wall of Gram-negative bacteria, has been used to induce inflammatory response and cytokine production in mice\textsuperscript{[31]} and various types of immune cells, including monocytes and dendritic cells.\textsuperscript{[32,33]} From the previous studies, it was found that after 12 h of LPS stimulation, the main mediators of acute inflammatory process, TNF-\(\alpha\) and IL-6, are released to the maximal level and gradually decreased for TNF-\(\alpha\) and remained stable for IL-6 until 24 h of induction.\textsuperscript{[34,35]} In our study, mRNA expression of the pro-inflammatory cytokine TNF-\(\alpha\) and the anti-inflammatory cytokine IL-10 greatly increased after exposure to LPS. Dexamethasone was used as a positive control since it can directly inhibit PLA2 and NF-kB activation, reducing the expression of COX-2 and inflammatory cytokines.\textsuperscript{[36]} Franchimont et al. reported that dexamethasone reduced TNF-\(\alpha\), IL-1\(\beta\), and IL-6 levels in LPS-stimulated human whole blood with an IC\(_{50}\) value of 1.1 \times 10\(^{-9}\) M.\textsuperscript{[37]} In the present study, TP propolis extract (10 \(\mu\)g/mL) exhibited a potent inhibitory effect on the production of TNF-\(\alpha\) and IL-10, while the mRNA expression of IL-6 was unaltered. Nevertheless, our results indicate that TP extracts not only attenuated the expression of pro-inflammatory cytokines but also affected anti-inflammatory cytokines, particularly IL-10. However, the reduction of anti-inflammatory cytokines did not relate to an anti-inflammatory effect of TP propolis extract. This observation has been due to extract component-mediated inhibition of TNF-\(\alpha\) release, which is the first upstream signaling event that initiates a pro-inflammatory cytokine activation cascade and subsequent reduction of other downstream cytokines.\textsuperscript{[38,39]}

Prostanoids derived from arachidonic acid through COX activity are important mediators of inflammation.\textsuperscript{[40]} During inflammation, COX-2 activity can be rapidly upregulated by more than 20-fold.\textsuperscript{[41]} There is extensive evidence based on human and animal studies supporting the role of COX-2 in the development of inflammation. Inflammatory cytokines and endotoxins, including LPS, can induce a 10–80-fold increase in COX-2 expression in monocytes, macrophages, chondrocytes, fibroblasts, and endothelial cells.\textsuperscript{[42,43]} In our study, LPS from \(S. \) enterica serotype Typhimurium was used to stimulate COX-2 activity. The present study clearly shows that TP propolis extract (10 \(\mu\)g/mL) strongly inhibited the mRNA expression of COX-2. The corticosteroid dexamethasone was used as a positive control since the expression of COX-2 and the production of prostaglandins can be inhibited by such glucocorticoids.\textsuperscript{[44]} Interestingly, 10 \(\mu\)g/mL TP propolis extract demonstrated high potency in the inhibition of COX-2 mRNA levels that were similar to that of dexamethasone (0.1 \(\mu\)M).

The mechanism of anti-inflammation by propolis extract from TP may be associated with the sequential release of various cytokines, which are regulated by the release of mediators during inflammation. Constituents of the extract may affect the formation and release of TNF-\(\alpha\) from tissue macrophages. TNF-\(\alpha\) release reduces the release of other cytokines, including IL-6 and IL-1\(\beta\), leading to a decrease in COX-2 expression.\textsuperscript{[45]} Altogether, we provide evidence that TP propolis extract can exhibit anti-inflammatory activity by reducing inflammatory cytokines, which are important mediators of the inflammation processes, and inhibiting COX activity. Constituents of TP propolis may be developed for further use in the prevention or treatment of inflammatory diseases and lead to the development or synthesis of novel anti-inflammatory medicines. The efficacy and safety of these compounds should be confirmed by further study.

**CONCLUSION**

TP propolis extract exhibited anti-inflammatory effects in LPS-stimulated RAW 264.7 mouse macrophages in association with the downregulation of genes involved in the inflammatory cascade, including COX-2, iNOS, TNF-\(\alpha\), and IL-10. Therefore, TP propolis extract from mangosteen orchard is a promising candidate as a natural treatment for inflammatory diseases.

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