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Sorted by: Impact Factor SORT AGAIN

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*Ranking is based on your journal and sort selections.*

| Mark | Rank | Abbreviated Journal Title (linked to journal information) | ISSN | JCR Data | | | | | | Eigenfactor® Metrics | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total Cites | Impact Factor | 5-Year Impact Factor | Immediacy Index | Articles | Cited Half-life | Eigenfactor® Score | Article Influence® Score |
| ☐ | 141 | BIOCATAL BIOTRANSFOR | 1024-2422 | 769 | 0.892 | 0.939 | 0.080 | 25 | 9.5 | 0.00074 | 0.211 |
| ☐ | 142 | BIOCONTROL SCI TECHN | 0958-3157 | 1632 | 0.848 | 0.978 | 0.239 | 113 | 8.6 | 0.00214 | 0.260 |
| ☐ | 143 | FOOD BIOTECHNOL | 0890-5436 | 373 | 0.814 | 0.894 | 0.222 | 18 | >10.0 | 0.00035 | 0.202 |
| ☐ | 144 | PLANT BIOTECHNOL-NAR | 1342-4580 | 689 | 0.715 | 0.850 | 0.022 | 45 | 6.7 | 0.00112 | 0.214 |
| ☐ | 145 | GENES GENOM | 1976-9571 | 344 | 0.692 | 0.697 | 0.173 | 110 | 3.6 | 0.00102 | 0.159 |
| ☐ | 146 | ANIM BIOTECHNOL | 1049-5398 | 367 | 0.686 | 0.837 | 0.704 | 27 | 8.8 | 0.00053 | 0.272 |
| ☐ | 147 | BIOCONTROL SCI | 1342-4815 | 225 | 0.677 | 0.933 | 0.051 | 39 | 5.1 | 0.00048 | 0.236 |
| ☐ | 148 | CHEM BIOCHEM ENG Q | 0352-9568 | 559 | 0.675 | 0.941 | 0.295 | 61 | 6.1 | 0.00093 | 0.226 |
| ☐ | 149 | APPL BIOCHEM MICRO+ | 0003-6838 | 1242 | 0.671 | 0.788 | 0.169 | 118 | 8.0 | 0.00145 | 0.170 |
| ☐ | 150 | CHIM OGGI | 0392-839X | 427 | 0.538 | 0.481 | 0.071 | 70 | 5.6 | 0.00079 | 0.114 |
| ☐ | 151 | J AM SOC BREW CHEM | 0361-0470 | 563 | 0.492 | 0.669 | 0.100 | 50 | >10.0 | 0.00034 | 0.134 |
| ☐ | 152 | GENET COUNSEL | 1015-8146 | 368 | 0.384 | 0.422 | 0.026 | 39 | >10.0 | 0.00047 | 0.122 |
| ☐ | 153 | ROM BIOTECH LETT | 1224-5984 | 452 | 0.381 | 0.478 | 0.034 | 118 | 5.1 | 0.00090 | 0.087 |
| ☐ | 154 | BIOTECHNOL BIOTEC EQ | 1310-2818 | 596 | 0.373 | 0.365 | 0.220 | 168 | 5.4 | 0.00095 | 0.075 |
| ☐ | 155 | IRAN J BIOTECHNOL | 1728-3043 | 200 | 0.309 | | 0.000 | 35 | 6.2 | 0.00034 | |
| ☐ | 156 | INDIAN J BIOTECHNOL | 0972-5849 | 600 | 0.287 | 0.552 | 0.017 | 60 | 7.5 | 0.00063 | 0.115 |
| ☐ | 157 | MINERVA BIOTECNOL | 1120-4826 | 70 | 0.246 | 0.233 | 0.000 | 31 | | 0.00003 | 0.018 |
| ☐ | 158 | RES J BIOTECHNOL | 2278-4535 | 210 | 0.242 | 0.336 | 0.024 | 168 | 3.8 | 0.00050 | 0.062 |
| ☐ | 159 | AGRO FOOD IND HI TEC | 1722-6996 | 218 | 0.202 | 0.229 | 0.038 | 80 | 6.2 | 0.00032 | 0.048 |
| ☐ | 160 | BIOPHARM INT | 1542-166X | 239 | 0.181 | 0.244 | 0.141 | 71 | 7.0 | 0.00027 | 0.053 |

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# The effects of propolis to anti-inflammatory in tumor necrosis factor-α-stimulated human periodontal ligament fibroblasts

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***Abstract***

*The study investigated whether propolis has inhibitory effects on tumor necrosis factor (TNF)-α-stimulated inflammatory response in human periodontal ligament fibroblasts (hPDLFs). Human periodontal ligament fibroblasts were exposed to TNF-α and treated with propolis. Cytotoxicity was assessed by the MTT assay. mRNA expression levels of proinflammatory mediators including interleukin (IL)-1β, IL-6, TNF-α, and cyclooxygenase (COX)-2 were assessed by quantitative polymerase chain reaction (qPCR). Proinflammatory cytokine production was measured by enzyme-linked immunosorbent assay (ELISA). Nuclear localization of nuclear factor (NF)-κB was examined by western blotting. Exposure to TNF-α resulted in significant elevation of mRNA expression of IL-1β, IL-6, TNF-α, and COX-2 in hPDLFs, increased the production of proinflammatory cytokines, IL-1β and IL-6, and enhanced nuclear translocation of NF-κB.*

*Treatment with propolis downregulated the mRNA levels of IL-1β, IL-6, TNF-α, and COX-2 in TNF-α -stimulated hPDLFs in a dose-dependent manner. TNF-α-induced NF-κB nuclear translocation was reduced in presence of propolis. Propolis treatment inhibited mRNA expression of inflammatory mediators and suppressed NF-κB activation in hPDLFs exposed to TNF-α. Propolis suppresses TNF-α -induced inflammatory responses, including the expression of IL-1β, IL-6, TNF-α and COX-2, suggesting that propolis treatment significantly inhibits the upregulation of inflammatory mediators induced by TNF-α in hPDLFs.*

**Keywords:** Propolis, tumor necrosis factor-α, periodontal ligament fibroblasts, interleukin, anti-inflammatory.

## Introduction

Periodontal disease is an inflammatory disease affecting the periodontium and causing tissue destruction with the ultimate clinical outcome of tooth loss. It is estimated that half of the world's population is affected by some form of periodontal disease.[1,4] Inflammatory periodontal disease is caused by bacteria that adhere to the tooth's surface to form a subgingival dental biofilm.[28] Therapies for periodontitis are mainly focused on mechanical procedures such as scaling and root planning.[5] Although these mechanical therapies may remove subgingival plaque to improve periodontal health, they do not eliminate pathogenic bacteria in inaccessible areas resulting in disease recurrence.[6]

Propolis is a mixture made of resinous materials collected from plants, bee wax, and enzymes. Propolis is used to reinforce the walls of beehives, protect bee colonies against disease, and prevent the decomposition of carcasses of intruders.[31] Propolis is composed of resin, wax, essential aromatic oils, pollen, and various other substances such as vitamins.[25] The biologically active components of propolis vary depending on season, bee species, geographic region, and bearing plants.[2] Bioactive compounds in propolis include polyphenols, phenolic aldehydes, sesquiterpene quinones, coumarins, amino acids, steroids, and inorganic compounds. The biological effects of propolis may be attributed to a single compound or to the synergistic effect of several substances. Propolis has been used as remedy in folk medicine and as a food preservative over several decades.[14]

Several experiments have confirmed that propolis possesses a broad range of biological and pharmacological properties such as antioxidant, antimicrobial, and antiproliferative activities.[14] Moreover, propolis has been demonstrated to exert anti-tumor, anti-ulcer, and immunomodulatory activities *in vitro* and *in vivo*. In dental medicine, propolis is used as an aesthetic and to treat gingivitis, cheilitis, and

stomatitis. However, the effects of propolis on inflammation in the perioral region remain unclear.

On the basis of its long history of use in folk medicine, we hypothesized that propolis exerts anti-inflammatory effects on periodontal fibroblasts under stimulation by inflammatory cytokines. The present study was designed to investigate the effects of propolis on the expression of inflammatory cytokines in human periodontal ligament fibroblasts (hPDLFs) stimulated by tumor necrosis factor (TNF)-α.

## Material and Methods

**Propolis material and preparation:** Propolis was obtained from Yaomei Co., Taiwan and kept in the desiccated state until processing. Propolis was extracted in 95% (v/v) ethyl alcohol in a hermetically closed glass vessel for 4 days at 37°C under occasional shaking. The ethanol extract was then filtered through a Whatmann® grade 4 filter paper and evaporated to dryness under vacuum. The dried residual powder was kept at −20 °C. Immediately prior to use, propolis was weighed, dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO), and diluted with cell culture medium to appropriate concentrations. The final concentration of DMSO was adjusted to 0.1% (v/v). The control cells received the same amount of DMSO.

**Cell culture:** With ethics approval, hPDLFs were obtained from human gingival and periodontal ligaments as described previously.[3] Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, CA) supplemented with 10% fetal bovine serum (Invitrogen, CA) and 1% antibiotics (10000 unit $ml^{-1}$ penicillin, 10 mg $ml^{-1}$ streptomycin, and 0.025 mg $ml^{-1}$ amphotericin) in a humidified atmosphere of 5% $CO_2$ at 37 °C. Culture medium was refreshed and replaced every 2 days. The fibroblasts were used after ten passages in culture.

**Cell viability of hPDLFs after treatment:** Cell viability was determined by the 3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells $well^{-1}$ overnight prior to treatment. After treatment, the medium was replaced with 0.5 mg $mL^{-1}$ MTT solution (Sigma-Aldrich, MO). After 3 h of incubation at 37 °C, the supernatant was removed and DMSO was subsequently added to release formazan. The resulting absorbance was measured at a wavelength of 540 nm with background subtraction at 650 nm on an EMax® Endpoint ELISA Microplate Reader (Molecular Devices Inc. CA).

**mRNA expression of proinflammatory mediators by qPCR:** Total RNA was extracted using TRIzol® reagent (Ambion, CA). The amount of extracted RNA was measured using the Qubit™ RNA Assay Kit (Invitrogen, CA). Reverse transcription was performed before qPCR amplification by using high capacity cDNA reverse transcription kits (Applied Biosystems, CA). The relative expression of target genes was assessed using an ABI 7900HT system (Applied Biosystems, CA). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was used as an internal control. The primer sequences are listed in table 1.

**ELISA analysis:** After treatment, the culture medium was collected to estimate TNF-α and interleukin (IL)-6 secretion by using the IL-6 screening set (Thermo, IL). One-hundred microliters of the medium was loaded in the pre-coated plate and incubated for 1 h at room temperature. The plate was washed with phosphate-buffered saline with Tween®-20. Secondary antibody was then added and the plate was incubated for 1 h. Streptavidin-horseradish peroxidase was added, and the plate was incubated for 30 min. Subsequently, tetramethylbenzidine substrate was added to the plate and allowed to develop in the dark for 30 min. The absorption at 650 nm was detected using an ELISA reader.

**Western blotting:** Cells were washed with phosphate-buffered saline. Nuclear and cytoplasmic portions of protein were prepared using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo, IL). The concentration of the extracted protein was measured using the Qubit™ Protein Assay Kit (Invitrogen, CA). Twenty micrograms of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% gel) and transferred to a polyvinylidene fluoride membrane at 200 mA for 1 h. Skim milk (5%) in PBST was used to block the membranes. The membranes were then incubated with rabbit anti-mouse nuclear factor (NF)-κB antibody for 1 h at room temperature. The blots were incubated with secondary antibody for 30 min at room temperature and developed using the ECL system.

**Statistical analysis:** The results of each treatment group are presented as the means ± standard deviation of triplicates. The data were compared by one-way analysis of variance with the Tukey–Kramer multiple comparisons post-test. Differences were considered significant at *p*-values less than 0.05.

## Results

**Effect of propolis on the proliferation of hPDLFs:** We first examined the cytotoxic effect of propolis at various concentrations on hPDLF cell viability by using the MTT assay. Incubation of hPDLFs with various concentrations of propolis (2–25 μg $ml^{-1}$) for 24 h affected cell viability slightly (Fig. 1A). We next evaluated the cytotoxic effect of propolis on TNF-α-stimulated hPDLFs. Cell viability of hPDLFs increased slightly in the presence of TNF-α but propolis exerted no cytotoxic effect on TNF-α-stimulated hPDLFs (Fig. 1B).

**Effect of propolis on the regulation of pro-inflammatory mediator expression in TNF-α-stimulated hPDLFs:** To

investigate whether propolis alleviates inflammatory responses in periodontal tissue, we examined the mRNA expression levels of inflammation -associated genes upon propolis treatment in TNF-α-stimulated hPDLFs by qPCR. The elevated expression level of TNF-α mRNA declined upon treatment with propolis (2–25 μg $ml^{-1}$; Fig. 2A). IL-1β mRNA expression in hPDLFs increased significantly in presence of TNF-α. The elevated level of IL-1β mRNA in TNF-α-stimulated hPDLFs was restored upon exposure to propolis, and the alleviation was dose-dependent in nature (Fig. 2B).

TNF-α induces the activation of cyclooxygenase-2 (COX-2) transcription leading to the release of prostaglandin (PG) E2. TNF-α-stimulated hPDLFs exhibited significantly increased expression of COX-2 mRNA. Treatment of TNF-α -stimulated hPDLFs with propolis resulted in the suppression of TNF-α-induced expression of COX-2 (Fig. 2C). IL-6 is often upregulated in association with TNF- and IL-1β during inflammation. The expression level of IL-6 mRNA in hPDLFs increased in response to exposure to TNF-α. The elevated expression of IL-6 mRNA in hPDLFs was significantly downregulated by propolis treatment at low concentrations (Fig. 2D). The amelioration of upregulated IL-6 expression by propolis was dose-independent in nature whereas the upregulated mRNA expression of the other proinflammatory mediators examined was suppressed by propolis in a concentration-dependent manner.

**Effect of propolis on the production of pro-inflammatory cytokines in TNF-α-stimulated hPDLFs:** We next examined the anti-inflammatory activity of propolis, in particular with respect to the production of pro-inflammatory cytokines in TNF-α-stimulated hPDLFs. Production of IL-6 and TNF-α in the culture medium was evaluated by ELISA. IL-6 production decreased significantly upon treatment with propolis at concentrations of 5 and 10 μg $ml^{-1}$ in comparison with treatment with TNF-α alone (Fig. 3). Interestingly, treatment with 25 μg $ml^{-1}$ propolis failed to alleviate TNF-α-induced elevation in IL-6 secretion.

**Effect of propolis on NF-κB regulation:** NF-κB plays a crucial role in regulating the expression of proinflammatory genes such as TNF-α and COX-2. We investigated whether propolis exerts its anti-inflammatory effect through inhibition of NF-κB in TNF-α-stimulated hPDLFs. The mRNA expression and nuclear translocation of NF-κB were examined by qPCR and western blotting. The mRNA expression of NF-κB in hPDLFs was upregulated in the presence of TNF-α. Exposure of TNF-α-stimulated hPDLFs to propolis resulted in minor suppression of elevated NF-κB expression (Fig. 4).

## Discussion

Periodontal disease is one of most prevalent inflammatory diseases in adults. Periodontal disease is initiated by the colonization of tooth-associated pathogenic bacteria leading to aberrant inflammatory responses. Persistent inflammation in the periodontal tissue results in the destruction of soft tissue and bone with irreversible consequences including tooth loss.[27] An ideal treatment for periodontal disease must achieve the following objectives: amelioration of symptoms, abrogation of gum destruction, and safety. In this study, we investigated the *in vitro* response of hPDLFs to TNF-α and the anti-inflammatory effect of propolis in hPDLFs challenged with TNF-α.

Upon colonization by periodontal pathogens, gingival epithelial cells are constantly stimulated with bacterial cell components through pattern recognition receptors such as toll-like receptor, leading to the production of cytokines and chemokines. Recruitment of polymorphonuclear leukocytes to the infection site occurs in response to cytokine release.[8] Monocytes and activated macrophages also migrate to the inflamed tissue and respond to local inflammatory mediators by producing cytokines including TNF-α.[26] Clinical and experimental evidences support the role of TNF-α in the pathogenesis of periodontal diseases.[9,10] Modulation of TNF-α-mediated inflammatory response has been shown to reduce the severity of periodontitis.[24]

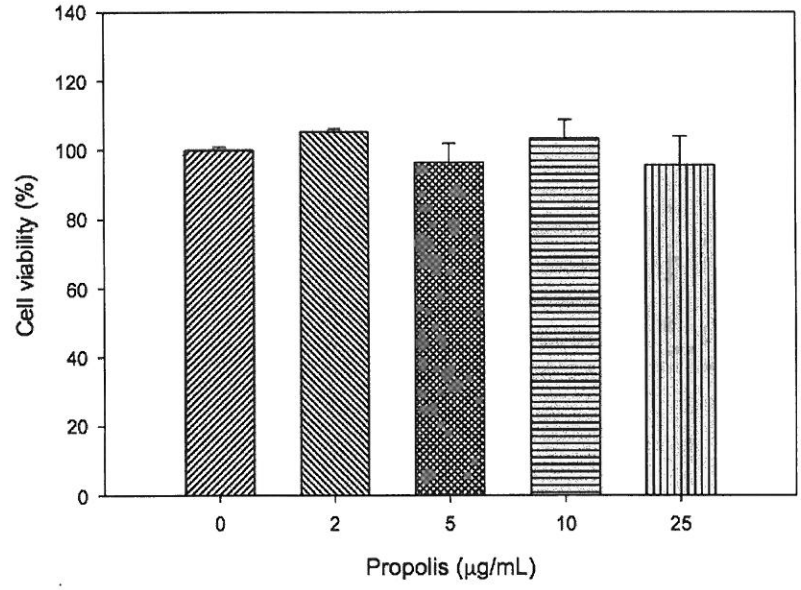
Fibroblasts plays a critical role in maintaining the integrity and homeostasis of periodontal connective tissue.[19] In addition, fibroblasts function as the sentinel cells of the immune system, responding to a broad range of inflammatory stimulants.[12, 30] Fibroblasts have been considered a therapeutic target for the treatment of chronic inflammation.[7] The most commonly used fibroblasts in periodontitis studies include gingival and ligament fibroblasts. Gingival and ligament fibroblasts respond differently to immune stimulants *in vitro*.[17, 18, 29] In addition to fibroblast heterogeneity, the anatomical location of each fibroblast needs to be considered while choosing appropriate immune stimulants.[12] In contrast to gingival fibroblasts, which directly interact with LPS, ligament fibroblasts, which are associated with tooth loss, are likely exposed to TNF-α in inflamed tissue.

In the present study, we demonstrated that TNF-α-stimulation alone led to elevation in the mRNA expression levels of IL-1β, IL-6, TNF-α, and COX-2 in hPDLFs. These results are not only consistent with those reported in previous studies, where IL-6 production was enhanced upon TNF-α exposure,[13, 22] but also provide insights into the inflammatory profile of periodontal ligament fibroblasts. We also found that TNF-α enhanced cell proliferation and activated nuclear localization of NF-κB in fibroblasts. These findings support the hypothesis that TNF-α induces the activation of NF-κB thereby promoting cell survival.[13]

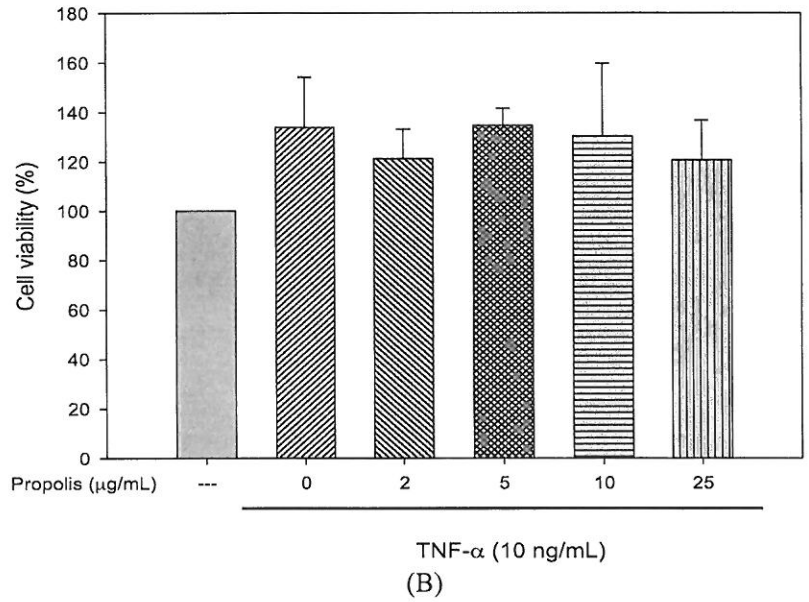
Therefore, we adopted fibroblasts as an *in vitro* model to investigate the effect of propolis in TNF-α-induced

inflammation. Propolis has been demonstrated to possess a broad spectrum of biological and pharmacological properties.[14,31] In the immunopharmacological aspect, administration of propolis enhances the expression of TLR-2 and -4 and promotes the production of proinflammatory cytokines in mice.[20] On the other hand, propolis administration inhibits proinflammatory cytokines and enhances anti-inflammatory cytokine production in experimental animal models.[11,15]

Similar results have been reported for cell culture models as well. Propolis enhances the bactericidal activity of macrophages and increases the production of proinflammatory cytokines in murine macrophages.[21] In LPS-treated macrophages, propolis inhibits cytokine production and nitric oxide release.[32] In this study, we observed that propolis significantly inhibited TNF-α-induced elevation of IL-1β, IL-6 and TNF-α levels in the fibroblasts in a dose-dependent manner. This indicates that propolis is an efficient inhibitor of inflammation in fibroblasts. TNF-α triggers the signaling cascades involved in the phosphorylation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) and the nuclear localization of NF-κB.

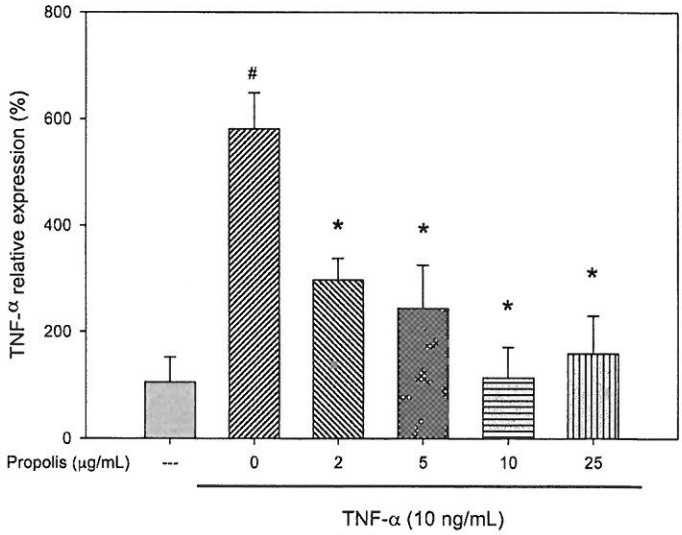



(A)




(B)

**Fig. 1: Effects of propolis on cell viability by MTT assay. (A) hPDLFs treatment with propolis (approximately 0–25 μg/mL) for 24 h. (B) Cells pre-stimulated with TNF-α; 10 ng/mL for 4 h, followed by incubation with propolis (approximately 0–25 μg/mL) for 24 h. $^{\#}p < 0.05$ compared with the control; $^{*}p < 0.05$ compared with TNF-α alone.**


800
600
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TNF-α relative expression (%)
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*
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Propolis (μg/mL)
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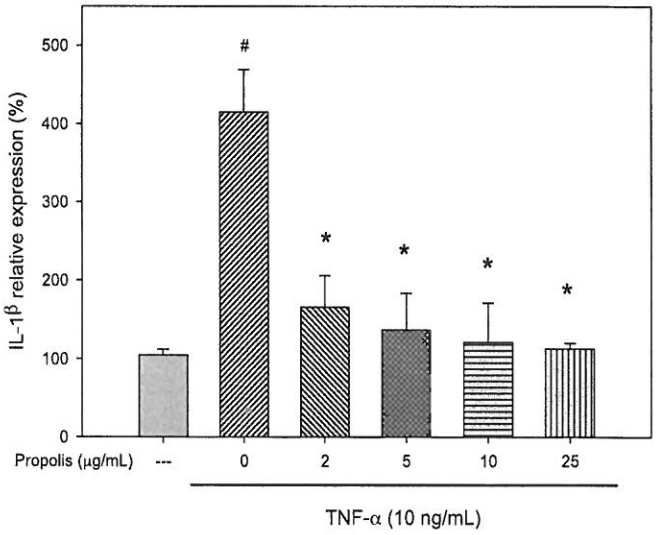
2
5
10
25
TNF-α (10 ng/mL)

(A)


500
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IL-1β relative expression (%)
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Propolis (μg/mL)
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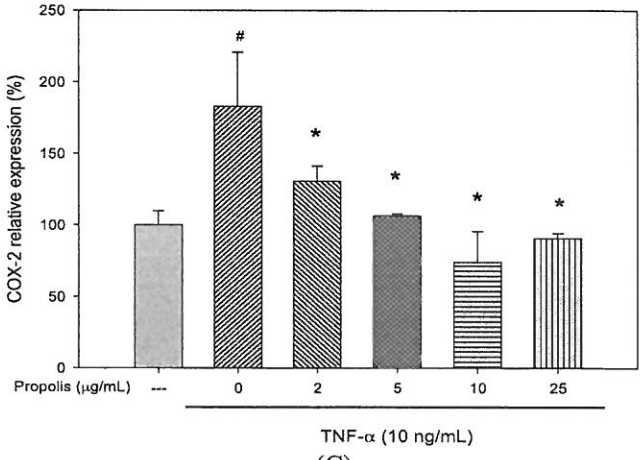
(B)


250
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COX-2 relative expression (%)
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Propolis (μg/mL)
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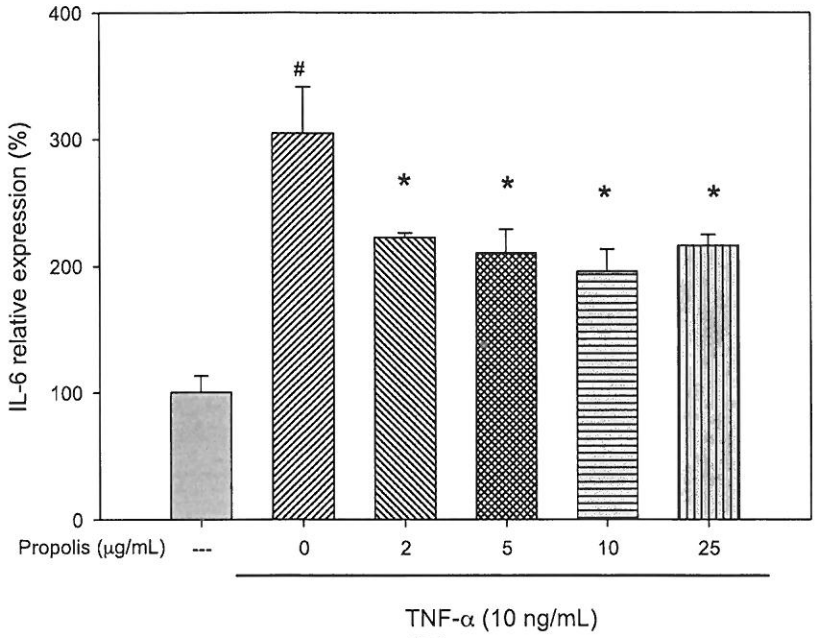
(C)




(D)

**Fig. 2: Propolis reduced the mRNA expression levels in (A) TNF-α, (B) interleukin (IL)-1β, (C) cyclooxygenase (COX)-2, and (D) IL-6 of inflammatory mediators in TNF-α-stimulated hPDLFs. $^{\#}p < 0.05$ compared with the control; $^{*}p < 0.05$ compared with TNF-α alone.**

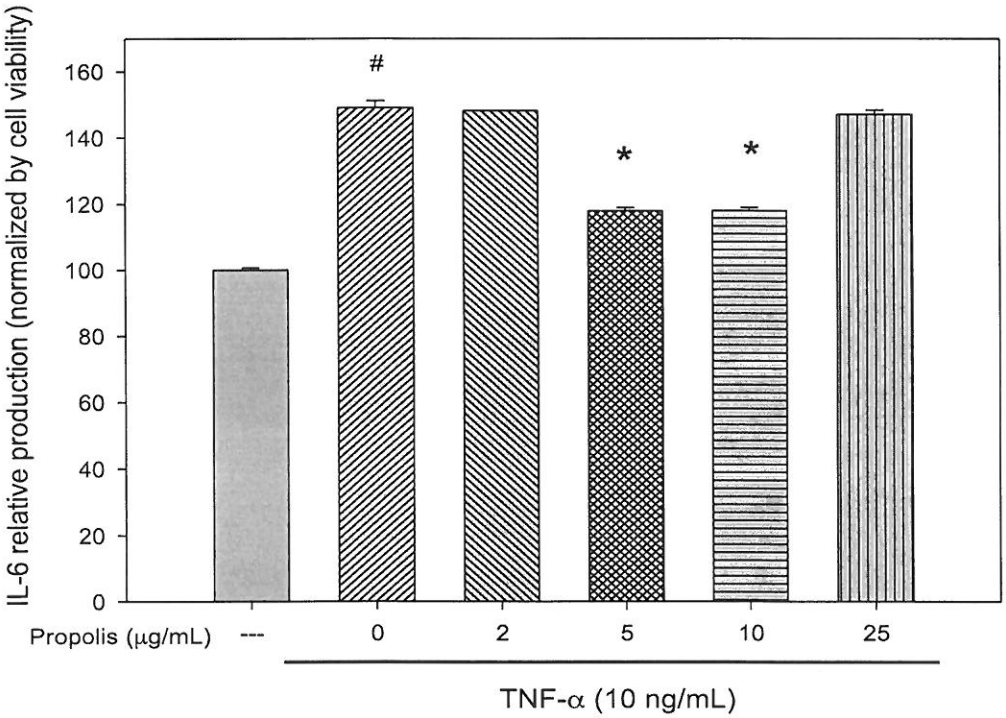



**Fig. 3: Effect of propolis on the production of IL-6 of pro-inflammatory cytokines in TNF-α-stimulated hPDLFs. $^{\#}p < 0.05$ compared with the control; $^{*}p < 0.05$ compared with TNF-α alone.**

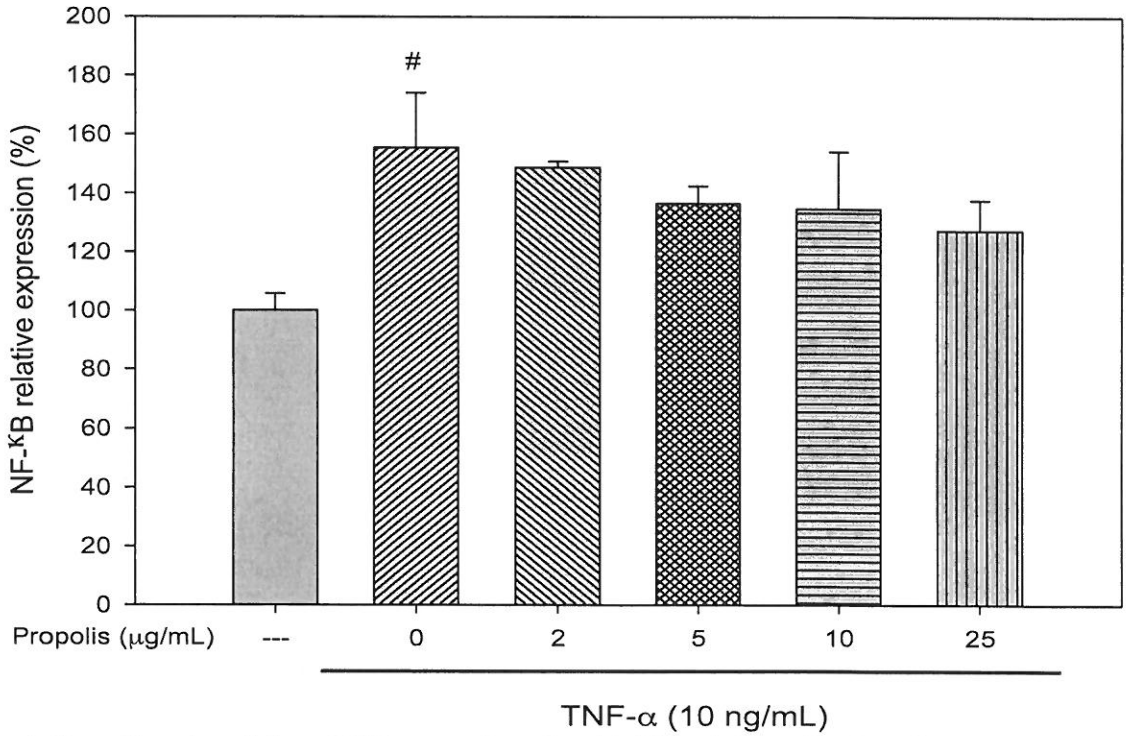



**Fig. 4: Propolis reduced the mRNA expression of and inhibited the activation of nuclear factor (NF)-κB in TNF-α-stimulated hPDLFs.**

**Table 1**
**List of primers for real-time qPCR in the study**

| Target Genes | Forward primer (5'- 3') | Reverse primer (5'- 3') |
|---|---|---|
| COX-2 | TCCCTGAGCATCTACGGTTTG | TCTCCTATCAGTATTAGCCTGCTTGTC |
| IL-1β | TTACAGTGGCAATGAGGATGACTT | AGTGGTGGTCGGAGATTCGT |
| IL-6 | AGCCCTGAGAAAGGAGACATGTA | AGGCAAGTCTCCTCATTGAATCC |
| NF-κB | TGCTGGGATGAAGCATGGA | GATCTTGCTCTGTGGTTTCAATAACT |
| TNF-α | TGTAGCCCATGTTGTAGCAAACC | GATGCGGCTGATGGTGTG |

TNF-α-induced activation of NF-κB in hPDLFs was inhibited by propolis treatment. This result is in agreement with previously reported results.[23] This indicates that the anti-inflammatory effect of propolis on TNF-α-induced hPDLFs is partly through the inhibition of NF-κB activation. Interestingly, treatment with propolis at a concentration of 25μg/ml enhanced the expression of IL-6 and TNF-α indicating that the immunomodulatory effect of propolis is partially dose-specific.

COX converts arachidonic acid to PGs which are mediators of numerous physiological processes. Different from the constitutive enzyme COX-1, COX-2 expression is induced in response to inflammatory stimuli such as LPS and TNF-α. COX-2 expression is induced in periodontitis patients along with the elevation of PGE2.[16] Our data demonstrate that TNF-α upregulates the expression of COX-2 in hPDLFs and that the upregulation is suppressed in response to propolis treatment. Administration of propolis inhibits TNF-α-induced overexpression of COX-2, leading to alleviation of inflammatory symptoms due to PG release. Our result suggests that the suppression of overexpressed COX-2 in TNF-α-stimulated hPDLFs may partly be attributed to the blockade of NF-κB activation. PGE2 contributes to inflammatory pain and treatment with propolis has the additional benefit of pain relief in the treatment of chronic periodontitis.

## Conclusion

Our results demonstrated that propolis suppresses TNF-α - induced inflammatory responses including the expression of IL-1β, IL-6, TNF-α, and COX-2. The results suggest that propolis treatment significantly inhibits the upregulation of inflammatory mediators induced by TNF-α in hPDLFs. The effect of exendin-4, a hormone in saliva, on TNF-α-induced periodontal inflammation is exerted through the inhibition

of NF-κB activation.

It has been postulated that the inhibition of COX-2 expression may contribute to pain management during periodontal therapy. To elucidate the mechanism by which propolis modulates host response, further studies investigating its effects on immune cells during the pathogenesis of periodontal diseases are required. Moreover, clinical trials need to be conducted to confirm the benefits of propolis as an adjunct regimen in periodontal therapy.

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