Protective effects of standardized propolis extract (EEP BBbK100) against oxidative stress, inflammation and periodontal pathogens

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Abstract. Periodontitis is a chronic inflammatory disease driven by microbial dysbiosis, oxidative stress and host immune dysregulation, often leading to progressive periodontal tissue destruction. Propolis is a natural bee-derived resin with recognized antioxidant, anti-inflammatory and antimicrobial properties. However, the chemical variability of propolis across different geographical origins limits its reproducibility and clinical use. The present study investigated the effects of ethanol-extracted propolis (EEP) BBbK100, a standardized mixture of Brazilian green, Brazilian brown and Korean propolis adjusted to 1% total flavonoids. High-performance liquid chromatography (HPLC) analysis was performed to determine its chemical composition. The antioxidant and anti-inflammatory effects were evaluated in human periodontal ligament fibroblasts (HPLFs) exposed to oxidative stress [hydrogen peroxide (H_2O_2)] or inflammatory stimulation [lipopolysaccharide (LPS)]. Antibacterial activity was assessed against six key periodontal pathogens. Furthermore, the therapeutic efficacy of BBbK100 was tested in an LPS-induced periodontitis rat model by analyzing gingival gene expression, plasma antioxidant enzymes and pro-inflammatory cytokines. HPLC profiling revealed that BBbK100 contained bioactive flavonoids and phenolic compounds, including artepillin C, caffeic acid phenethyl ester and pinocembrin. *In vitro*, BBbK100 protected HPLFs against H_2O_2-induced cytotoxicity in a dose-dependent manner, restored antioxidant enzyme expression and attenuated LPS-induced IL-6 expression. BBbK100 also inhibited the planktonic growth of six periodontal pathogens. *In vivo*, oral administration of BBbK100 increased the gingival expression of antioxidant enzyme genes, reduced gingival IL-6 expression and partially restored plasma superoxide dismutase activity and IL-6 levels in periodontitis rats. The present findings suggest that BBbK100, a standardized ethanol extract of propolis, exerts antioxidant and anti-inflammatory effects in cellular and animal models of periodontitis and inhibits the planktonic growth of periodontal pathogens. By combining propolis from multiple origins with a defined total flavonoid content, BBbK100 may help reduce the compositional variability of raw propolis and represents a potential natural therapeutic candidate for the prevention and treatment of periodontitis.

Introduction

Periodontitis is a chronic inflammatory disease of the supporting structures of the teeth, characterized by microbial dysbiosis, excessive host inflammatory responses, and progressive destruction of periodontal tissues (1). The condition is strongly associated with oxidative stress, which contributes to tissue damage by generating reactive oxygen species (ROS) and amplifying inflammatory cascades, such as NF-κB and MAPK signaling, as well as the subsequent production of pro-inflammatory cytokines (2). Oxidative stress not only causes direct cellular damage through excessive ROS generation but also activates the NF-κB and NLR family pyrin domain containing 3 inflammasome pathways, amplifying the release of pro-inflammatory cytokines such as IL-1β and TNF-α that contribute to alveolar bone resorption (3). Beyond its local effects, periodontitis has been implicated as a risk factor for systemic disorders such as cardiovascular disease (4), diabetes (5) and adverse pregnancy outcomes (6), highlighting the importance of effective therapeutic strategies that target both microbial and host-mediated pathways (7,8).

Propolis, a resinous substance collected by honeybees from plant exudates (9), has been traditionally used as a natural remedy due to its broad spectrum of biological activities, including antioxidant, anti-inflammatory, antimicrobial and immunomodulatory properties (10-12). Numerous studies have demonstrated that bioactive constituents of propolis, such

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as flavonoids and phenolic acids, serve key roles in modulating oxidative stress and inflammation (3,11). These bioactivities have been associated with the regulation of NF-κB, MAPK and nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathways, which are central mediators of oxidative and inflammatory responses (2). However, the composition and efficacy of propolis vary widely depending on its botanical and geographical origin, which limits its reproducibility and clinical application (10). To overcome this limitation, standardized propolis extracts have been developed by combining multiple origins to balance and optimize the content of key bioactive compounds. In particular, Brazilian green propolis is rich in artepillin C and is known for its anti-inflammatory and immunomodulatory activity, while Brazilian brown and Korean propolis contain high levels of flavonoids such as pinocembrin and chrysin, which exert strong antioxidant and antimicrobial effects (13-15). A rationally standardized mixture of these extracts, ethanol-extracted propolis (EEP) BBbK100, was formulated to achieve reproducible biological activity by ensuring a defined total flavonoid content (16).

Despite the antioxidant, anti-inflammatory and antimicrobial activities of single-origin propolis having been widely reported (10,14,15), the chemical variability of propolis remains a key obstacle to reproducible biological effects and clinical translation. In addition, it remains unclear whether a chemically characterized, multi-origin propolis formulation can simultaneously target the three key pathogenic components of periodontitis, namely oxidative stress, excessive host inflammation and periodontal pathogens. Therefore, the aim of the present study was to evaluate a standardized multi-origin EEP (namely, EEP BBbK100) by: i) Characterizing its chemical composition through high-performance liquid chromatography (HPLC); ii) assessing its antioxidant and anti-inflammatory effects in human periodontal ligament fibroblasts (HPLFs) exposed to hydrogen peroxide (H_2O_2) or *Porphyromonas gingivalis* lipopolysaccharide (LPS); iii) determining its antibacterial activity against six periodontal pathogens; and iv) evaluating its effects on oxidative and inflammatory markers in a rat model of LPS-induced periodontitis.

Materials and methods

Preparation of propolis extract (EEP BBbK100). For each origin, 1 kg raw propolis (Seoul Propolis Co., Ltd.) was mixed with 3-5 kg of 95% ethanol and stirred at 60˚C for 4-5 h, followed by standing for >12 h. The supernatant was filtered through No. 2 filter paper (5 μm) and the filtrate was used for subsequent experiments. Ethanol extraction is commonly used to obtain propolis extracts rich in phenolic compounds; because the composition of propolis varies with its botanical and geographical origin, the final extract was chemically characterized as described (17). The total flavonoid content was then determined. EEP BBbK100 was prepared as an ethanol extract mixture standardized to 1% total flavonoids by combining Brazilian green ethanol extract, Brazilian brown ethanol extract and Korean ethanol extract (all supplied by Seoul Propolis Co., Ltd.) at a defined ratio (5:3:2).

The chemical profile of BBbK100 was characterized by HPLC through the quantification of ten reference compounds (Table I), including a prenylated phenylpropanoid (artepillin C), caffeic acid phenethyl ester (CAPE), flavonoids (pinocembrin, chrysin, quercetin, rutin, apigenin and kaempferol) and a phenolic acid (chlorogenic acid). HPLC analysis was performed by an external analytical service using an HPLC system equipped with a 2998 photodiode array detector (Waters Corporation) and data were processed using Empower 3 software (Waters Corporation). Separation was achieved on a reversed-phase column with an injection volume of 10 μl. The total run time was 75 min. Chromatographic detection (bandwidth: 4.8 nm) was carried out at multiple wavelengths (203, 254 and 280 nm) to ensure optimal detection of individual compounds, namely 203 nm for triterpenoids and flavonoids (including lupeol, apigenin and kaempferol), 254 nm for phenolic acids (including chlorogenic acid) and 280 nm for flavonoids and phenolic constituents, including rutin, quercetin, chrysin, pinocembrin, CAPE and artepillin C. Individual compounds were identified by comparing their retention times with those of authentic standards. Quantification was performed based on peak areas using external calibration curves of the corresponding standards. Representative HPLC chromatograms and quantitative results of BBbK100 are shown in Fig. 1 and summarized in Table I.

HPLF cell culture. Primary HPLFs were purchased from ScienCell Research Laboratories, Inc (cat. no. 2630). HPLFs were cultured in DMEM (cat. no. 10-013-CVRC; Corning, Inc.) supplemented with 10% FBS (cat. no. 35-015-CV; Corning, Inc.) and 1% penicillin/streptomycin (cat. no. BS14001C; Bandio Co., Ltd.) at 37˚C in a 5% CO_2 incubator. The inflammatory response was induced by treatment with LPS from *Porphyromonas gingivalis* (1 μg/ml) and oxidative damage was induced by treatment with 400 μM H_2O_2. Cultured cells were pretreated with varying concentrations of BBbK100 (0.25, 0.5 or 1 μg/ml for the cell viability assay; 0.5 or 1 μg/ml for RT-qPCR) for 1 h at 37˚C, followed by treatment with LPS or H_2O_2 for 24 h at 37˚C. HPLFs between passages 5 and 9 were used for all experiments. *Porphyromonas gingivalis* LPS-stimulated and H_2O_2-exposed periodontal ligament fibroblasts have been widely used as *in vitro* models of periodontal inflammation and oxidative injury, respectively (18-20).

Cell viability assay. To determine the appropriate concentration of propolis, an MTT assay was performed. Cells were seeded in 96-well plates at a density of $5x10^4$ cells/well and incubated for 24 h. Cells were then treated with varying concentrations (0.25, 0.5, 1, 2.5, 5 and 10 μg/ml) of propolis and incubated at 37˚C for 24 h. MTT solution (0.5 mg/ml) was added and incubated for 4 h at 37˚C. After removing the medium, DMSO was added to dissolve the purple formazan crystals and absorbance was measured at 570 nm using a microplate reader. To evaluate the protective effect of propolis against oxidative cell damage induced by H_2O_2, cells cultured in 96-well plates were pretreated with a non-cytotoxic concentrations (0.25, 0.5 and 1 μg/ml) of BBbK100 at 37˚C. After 1 h, 400 μM H_2O_2 was added to induce cell damage and cell viability was assessed after 24 h using an MTT assay.

RNA extraction and cDNA synthesis. Total RNA was extracted from HPLF cells using the Axen™ Total RNA Kit (Macrogen,

Table I. Quantitative analysis of major bioactive compounds in propolis by high-performance liquid chromatography.

Compound name	Amount, mg/g
Artepillin C	5.51
Chlorogenic acid	0.99
Caffeic acid phenethyl ester	1.04
Chrysin	0.42
Pinocembrin	1.70
Lupeol	0.01
Quercetin	0.02
Rutin	0.10
Apigenin	0.07
Kaempferol	0.23

Inc.). A total of 1 μg total RNA was reverse transcribed into cDNA using the TOPscript™ cDNA Synthesis Kit (Enzynomics Co., Ltd) with random primers, according to the manufacturer's instructions. For reverse transcription, 1 μg RNA was mixed with 1 μl 50 μM random primers and 1 μl of 10 mM dNTP mix and the volume was adjusted to 10 μl with RNase-free water. The mixture was heated at 65˚C for 5 min to denature secondary RNA structures and immediately cooled on ice. Subsequently, 4 μl 5X TOPscript RT buffer, 0.5 μl RNase inhibitor (40 U/μl) and 1 μl TOPscript reverse transcriptase were added and the final volume was adjusted to 20 μl with RNase-free water. The reverse transcription reaction was carried out at 42˚C for 60 min, followed by enzyme inactivation at 85˚C for 5 min. Synthesized cDNA was stored at -20˚C.

Quantitative real-time PCR (qPCR). qPCR was performed using the Axen™ qPCR Master Mix (2X Probe; Macrogen, Inc.) according to the manufacturer's instructions. Each 20 μl reaction mixture contained 10 μl 2X qPCR Master Mix, 1 μl template cDNA, 0.4 μM each of forward and reverse primers, with the remaining volume adjusted using nuclease-free water. Amplification was detected using the SYBR Green dye included in the master mix. Primer information for qPCR are shown in Table II. All reactions were performed in triplicate using the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Inc.). Thermal cycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. The threshold cycle (Cq) values obtained from qPCR were analyzed using the comparative $2^{-\Delta\Delta Cq}$ method (21). The Cq value of the target gene in each sample was normalized to that of the housekeeping gene GAPDH to calculate the ΔCq value. Subsequently, the ΔCq values of the experimental groups were compared with those of the control group to obtain the ΔΔCq values. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and expressed as fold changes. All reactions were performed in triplicate to ensure reproducibility.

Antimicrobial assay. For the antimicrobial activity assay, six oral bacterial strains associated with periodontitis were used, consisting of Gram-negative anaerobic bacteria. Among the bacteria known as the 'red complex' associated with gingival and periodontal diseases: *Porphyromonas gingivalis* [cat. no. 33277; American Type Culture Collection (ATCC)] was cultured in brain heart infusion (BHI; BD Biosciences) broth supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml); *Treponema denticola* (cat. no. 35405; ATCC) was cultured in new oral spirochete (NOS) medium; and *Tannerella forsythia* (cat. no. 43037; ATCC) was cultured in modified NOS medium. All cultures were maintained at 37˚C under anaerobic conditions (5% H_2, 10% CO_2 and 85% N_2). For the 'orange complex' bacteria: *Fusobacterium nucleatum* (cat. no. 25586; ATCC) and *Prevotella intermedia* (cat. no. 25611; ATCC) were cultured in BHI broth supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml) at 37˚C under anaerobic conditions (5% H_2, 10% CO_2 and 85% N_2). For the 'green complex' bacterium: *Aggregatibacter actinomycetemcomitans* (cat. no. 33384; ATCC) was cultured in BHI broth at 37˚C under anaerobic conditions (5% H_2, 10% CO_2 and 85% N_2). The antimicrobial activity assay was performed according to the guidelines recommended by the Clinical Laboratory Standards Institute (22). To assess growth inhibitory concentrations, bacterial suspensions were centrifuged at 4,000 x g for 5 min at room temperature, resuspended in fresh broth and quantified using a bacterial counting chamber. Anaerobic bacterial suspensions were adjusted to $1.5x10^6$ cells/ml using fresh medium. BBbK100 was prepared in DMSO at 4 mg/ml. For broth microdilution, 180 μl fresh broth was added to each well of a 96-well plate. The first column received the prepared BBbK100, which were serially diluted two-fold across the plate. Then, 20 μl bacterial suspension was added to each well. Plates were incubated in an anaerobic chamber for 36 or 48 h. Bacterial growth was analyzed by measuring absorbance at 660 nm using a spectrophotometer (Biotek; Agilent Technologies, Inc.).

LPS-induced periodontitis model. The present experimental protocol was approved by the Dankook University Institutional Animal Care and Use Committee (Cheonan, South Korea; approval no. DKU-25-002). Male Sprague-Dawley rats (7-weeks old; body weight: 250-280 g) were obtained from Raonbio Co., Ltd. Following a 1-week acclimatization period in controlled conditions (temperature: 21±2˚C, humidity: 50±5%), rats were housed individually with *ad libitum* access to standard chow and water. While rats were anesthetized with isoflurane (initiated with 5% and maintained at 2%) in 25% O_2/75% N_2, LPS extracted from *Porphyromonas gingivalis* (Sigma-Aldrich; Merck KGaA) was injected into the gingival tissue around the maxillary first and second molars at a dose of 10 μg each, five times at 3-day intervals. A week after the final injection, the rats were euthanized and blood was collected from the heart to separate plasma, which was subsequently used for the analysis of antioxidant enzyme activity and pro-inflammatory cytokine production. Gingival tissue surrounding the teeth was harvested and RNA was isolated using the Axen™ Total RNA Kit (Macrogen, Inc.), as aforementioned. Propolis (EEP BBbK100) was orally administered daily at doses of 0.5 and 1 mg/kg starting 1 week before periodontitis induction and continued for a total of 4 weeks until the day before sample collection. A total of 40 male

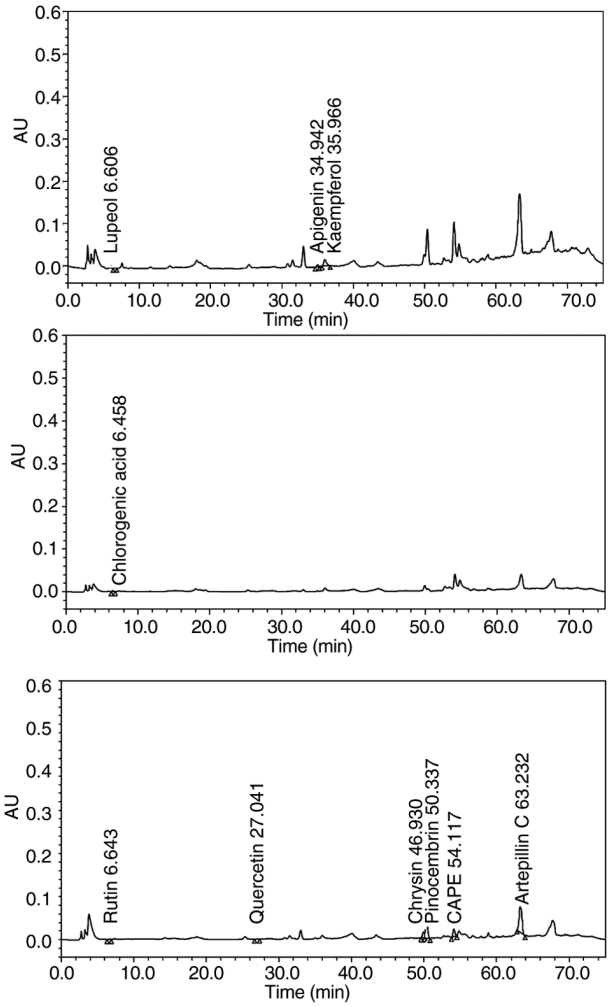


Figure 1. HPLC chromatographic profiles of propolis extract (EEP BBbK100) used for quantitative analysis of major bioactive compounds. Representative HPLC chromatograms of propolis extract (EEP BBbK100) recorded at 203 nm (upper panel), 254 nm (middle panel) and 280 nm (lower panel). Multiple detection wavelengths were selected to enable comprehensive detection and quantification of chemically diverse bioactive constituents in propolis, including phenolic acids, flavonoids and prenylated phenylpropanoids. Detection at 203 nm allowed visualization of a broad range of organic compounds, whereas chromatograms at 254 and 280 nm preferentially highlighted aromatic and phenolic compounds such as CAPE, chrysin, pinocembrin, quercetin and artepillin C. Values after compound names indicate retention time (min). AU, absorbance units; EEP, ethanol-extracted propolis; HPLC, high-performance liquid chromatography; CAPE, caffeic acid phenethyl ester.

SD rats were used in the present study and assigned to four groups (n=10 per group): i) Negative control; ii) LPS-induced periodontitis (LP); iii) LP + BBbK100 0.5 mg/kg; and iv) LP + BBbK100 1 mg/kg. The total duration of the experiment was 5 weeks, including the 1-week acclimatization period.

Animal health and behavior were monitored daily. Humane endpoints were defined as a marked reduction in food intake or apparent signs of severe pain or distress, at which point the experiment was to be terminated for the affected animal. No animals reached the humane endpoints and no mortalities were recorded during the experiment. According to the institutional classification of pain and distress (23), the procedures were classified as category D (moderate pain or distress without expected mortality). To minimize pain and distress, all gingival LPS injections were performed under inhalation anesthesia with isoflurane (5% for induction and 2% for maintenance in 25% O_2/75% N_2). Analgesics were not administered because they could affect the inflammatory outcomes. At the end of the experiment, rats were deeply anesthetized with isoflurane (5%) by inhalation, blood was collected by cardiac puncture and the animals were then euthanized by inhalation of 100% CO_2 at a flow rate displacing 30% of the chamber volume per min. Mortality was confirmed by the absence of heartbeat and respiration. Carcasses were stored in a freezer in the animal facility and disposed of by a licensed waste management company.

Measurement of plasma antioxidant enzyme activities and cytokine levels. Plasma superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were measured using commercial colorimetric assay kits (SOD Assay Kit, cat. no. 706002; CAT Assay Kit, cat. no. 707002; GPx Assay Kit, cat. no. 703102; Cayman Chemical Company) according to the manufacturer's instructions. SOD activity was determined using a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine and absorbance was read at 450 nm; one unit of SOD was defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical. CAT activity was determined based on the peroxidatic function of catalase with methanol in the presence of H_2O_2 and the formaldehyde produced was measured at 540 nm using Purpald (supplied with the CAT Assay Kit) as the chromogen. GPx activity was measured indirectly by a reaction coupled with glutathione reductase, in which the decrease in absorbance at 340 nm resulting from the oxidation of NADPH to $NADP^+$ was monitored. Plasma levels of IL-6, IL-1β and TNF-α were quantified using rat-specific sandwich ELISA kits (cat. nos. ab100772, ab100767 and ab46070, respectively; Abcam) according to the manufacturer's instructions and absorbance was measured at 450 nm using a microplate reader.

Statistical analysis. Data are expressed as the mean ± SEM and were analyzed by one-way ANOVA, followed by Tukey's post-hoc test, using SPSS (version 28.0; IBM Corp.) software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HPLC analysis of EEP BBbK100. HPLC profiling of BBbK100 revealed numerous bioactive compounds, including artepillin C (5.51 mg/g), CAPE (1.04 mg/g), pinocembrin (1.70 mg/g) and chrysin (0.42 mg/g), as well as minor amounts of chlorogenic acid, quercetin, rutin, apigenin and kaempferol. These findings indicate that BBbK100 contains a diverse spectrum of polyphenols and flavonoids with potential antioxidant and anti-inflammatory activities (Fig. 1; Table I).

Effect of BBbK100 on H_2O_2-induced oxidative damage in HPLF cells. To identify the optimal propolis raw material, the present study first evaluated the antioxidant and anti-inflammatory effects of ethanol extract of propolis from different

Table II. Primers used for reverse transcription PCR.

Class	Gene	Forward sequence (5'-3')	Reverse sequences (5'-3')
Rat	CAT	ATTGCCGTCCGATTCTCC	CCAGTTACCATCTTCAGTGTAG
	SOD	ACTTCGAGCAGAAGGCAAGC	GTCTCCAACATGCCTCTCTTCAT
	GPx	CAGTTCGGACATCAGGAGAAT	AGAGCGGGTGAGCCTTCT
	IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
	IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
	TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCCGCTTGGTGGTTTGCTACGAC
	GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA
Human	CAT	CTTCACTGAGGTCCACCCTGACTAC	GTGAGATCCGGACTGCACAAAG
	SOD1	AGTGCAGGGCATCATCAATTTC	CCATGCAGGCCTTCAGTCAG
	GPx1	CTGGTCCTGTTGATCCCAGTCTC	GCTGACACCCGGCACTTTATTAG
	IL-6	CCTTCGGTCCAGTTGCCTTCT	CAGTGCCTCTTTGCTGCTTTC
	IL-1β	CCAGGGACAGGATATGGAGC	TTCAACACGCAGGACAGGTACAG
	TNF-α	CGAGTGACAAGCCTGTAGCC	TGAAGAGGACCTGGGAGTAGAT
	GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

origins, including Australia, Brazilian green, Brazilian brown, Korea and China (data not shown). Subsequently, mixtures of extracts from different origins were tested at defined ratios and the combination of Brazilian green, Brazilian brown and Korean extracts (BBbK100) demonstrated the most potent anti-inflammatory and antioxidant activities. Therefore, BBbK100 was selected as the final material for the present study. To determine the appropriate concentration of the selected BBbK100, HPLF cells were treated with BBbK100 at concentrations ranging from 0.25-10 μg/ml and cell viability was assessed using an MTT assay. The results showed that BBbK100 exhibited significant cytotoxicity at concentrations >2.5 μg/ml. At 2.5 μg/ml, cell viability decreased to 88.9±3.2% compared with the control group, while at 5 and 10 μg/ml, cell viability further decreased to 71.4±5.9 and 52.8±2.0%, respectively, in a dose-dependent manner (Fig. 2A). Therefore, subsequent *in vitro* assays to evaluate the anti-inflammatory and antioxidant effects of propolis were conducted at concentrations of 1 μg/ml or lower.

When the protective effect of BBbK100 against H_2O_2-induced oxidative damage in HPLF cells was evaluated, treatment with 400 μM H_2O_2 significantly reduced cell viability to 54.7±4.4% compared with the control group (Fig. 2B). However, pretreatment with BBbK100 at concentrations of 0.25, 0.5 and 1 μg/ml significantly suppressed the H_2O_2-induced decrease in cell viability in a dose-dependent manner. The cell viability in the groups treated with 0.25, 0.5 and 1 μg/ml of BBbK100 was 66.9±3.1, 70.2±2.3 and 74.8±1.2%, respectively (Fig. 2B). These results suggest that BBbK100 exerts a protective effect against oxidative stress-induced cell death.

Effects of BBbK100 on antioxidant enzyme and inflammatory cytokine gene expression in HPLF cells. RT-qPCR analysis demonstrated that propolis modulated the expression of antioxidant enzymes and inflammatory cytokines in HPLF cells. In HPLF cells, treatment with H_2O_2 alone significantly reduced the expression of the antioxidant enzymes CAT and SOD, whereas this inhibitory effect was effectively reversed by pretreatment with 1 μg/ml BBbK100. For another antioxidant enzyme, GPx, expression was decreased by H_2O_2 and increased following propolis treatment; however, the change was not statistically significant (Fig. 3A). The expression of inflammatory cytokines IL-6, IL-1β and TNF-α was significantly upregulated by LPS alone (1 μg/ml). Pretreatment with 1 μg/ml BBbK100 significantly reduced LPS-induced IL-6 expression, whereas IL-1β and TNF-α expression showed decreasing trends that did not reach statistical significance (Fig. 3B). These results indicate that BBbK100 confers antioxidant protection and partial anti-inflammatory effects at the transcriptional level.

Antibacterial activity of BBbK100 against periodontal pathogens. BBbK100 inhibited the planktonic growth of six key periodontal pathogens, including *P. gingivalis*, *T. denticola*, *T. forsythia*, *F. nucleatum*, *P. intermedia* and *A. actinomycetemcomitans*, in a concentration-dependent manner, as determined by absorbance at 660 nm, with maximal growth inhibition observed at 0.032-0.064 mg/ml (Fig. 4). The apparent increase in absorbance at concentrations >0.125 mg/ml was likely attributable to the intrinsic color of the propolis extract.

Effects of BBbK100 in an LPS-induced periodontitis rat model. In the *P. gingivalis* LPS-induced periodontitis rat model (Fig. 5), LPS injection decreased the gingival expression of antioxidant enzyme genes and increased that of IL-6. Oral administration of BBbK100 significantly increased SOD expression at both doses and CAT and GPx expression at 1 mg/kg and significantly reduced IL-6 expression at 1 mg/kg, whereas IL-1β and TNF-α expression were not significantly altered (Fig. 6). In plasma, BBbK100 significantly

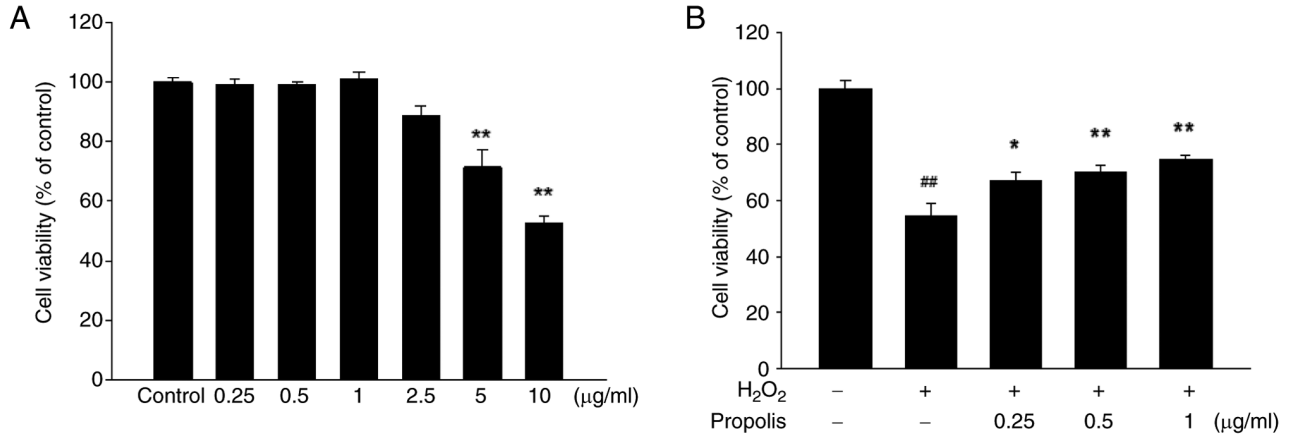


Figure 2. Effects of propolis on cell viability in H_2O_2-treated human periodontal ligament fibroblast cells. (A) Cell viability was determined by an MTT assay. Results are expressed as percentages of control cells. (B) Cells were pretreated with propolis (0.25, 0.5 and 1 μg/ml) for 1 h, then stimulated with H_2O_2 (400 μM), for 24 h. Values are expressed as the mean ± SEM. $^{\#\#}$P<0.01 compared with control; *P<0.05 and **P<0.01 compared with H_2O_2 treated cells. H_2O_2, hydrogen peroxide.

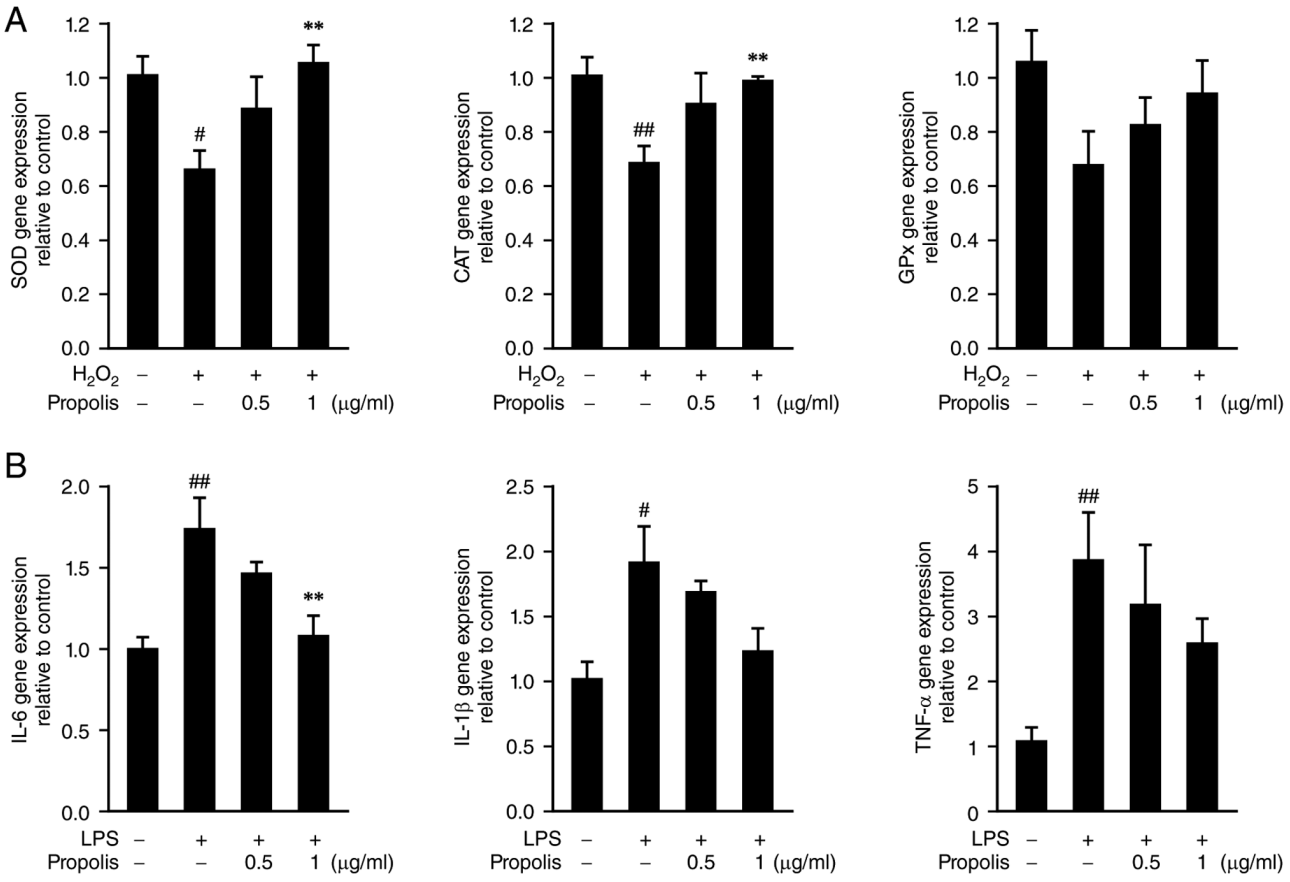


Figure 3. Effects of BBbK100 on the gene expression of antioxidant enzymes and inflammatory cytokines in human periodontal ligament fibroblasts. The gene expression levels of (A) antioxidant enzymes and (B) inflammatory cytokines in human periodontal ligament fibroblast cells, determined by reverse transcription-quantitative PCR. Values are expressed as the mean ± SEM. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with control cells; **P<0.01 compared with H_2O_2- or LPS-treated cells. H_2O_2, hydrogen peroxide; LPS, lipopolysaccharide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

increased SOD activity at 1 mg/kg and reduced IL-6 levels at 0.5 mg/kg, whereas CAT and GSH-Px activities and IL-1β and TNF-α levels showed no significant changes (Figs. 7 and 8). Collectively, these results indicate that BBbK100 partially ameliorates oxidative stress and inflammation in gingival tissues and in the circulation.

Discussion

The present study demonstrated that EEP BBbK100, a standardized mixture of Brazilian green, Brazilian brown and Korean propolis, exerted antioxidant and anti-inflammatory effects *in vitro* and *in vivo* and inhibited the planktonic growth of

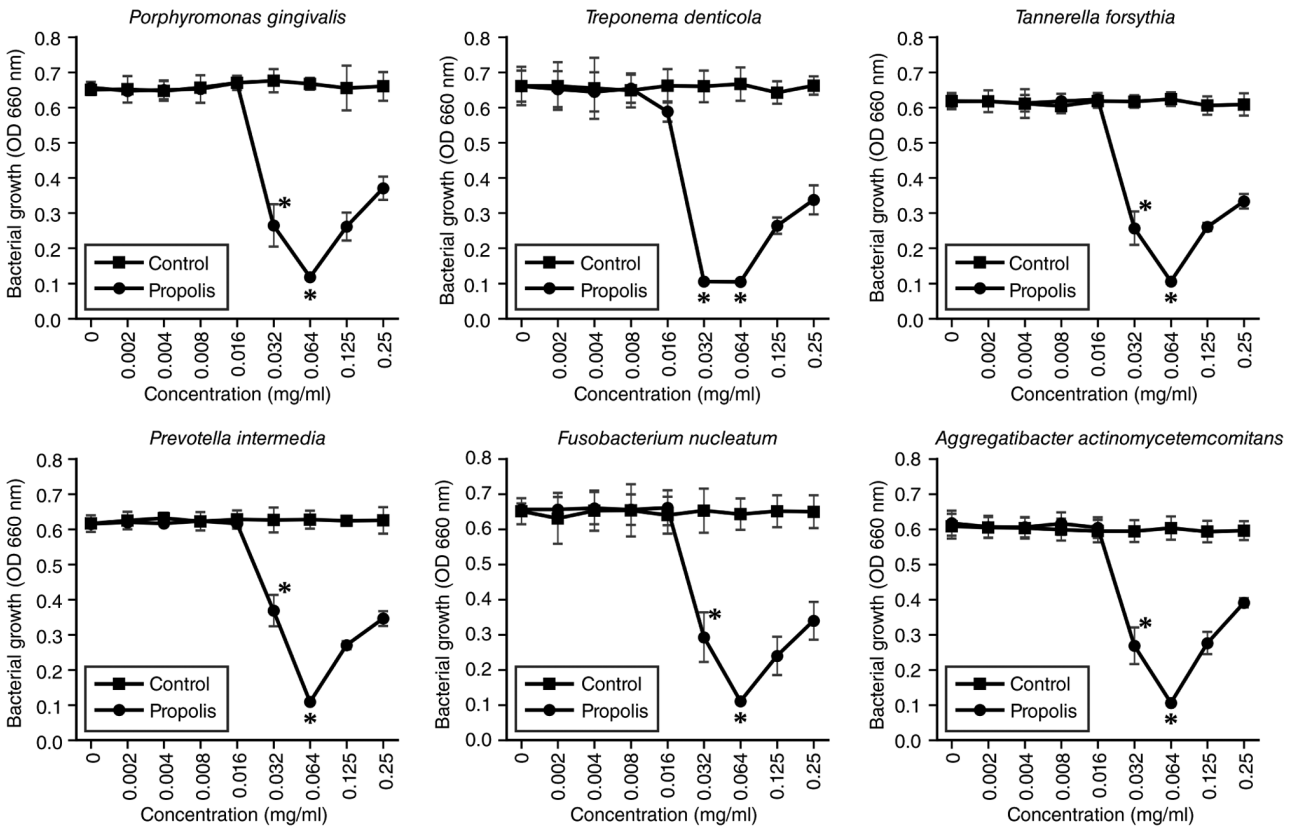


Figure 4. Growth-inhibitory activity of propolis against planktonic periodontal pathogens. The bacteria were incubated with or without propolis extract in the indicated concentrations and the bacterial growth was measured by a microplate reader at 660 nm. The experiments were performed three times in duplicates and data are presented as the mean ± SD. *P<0.05 compared with the untreated control bacteria. OD, optical density.

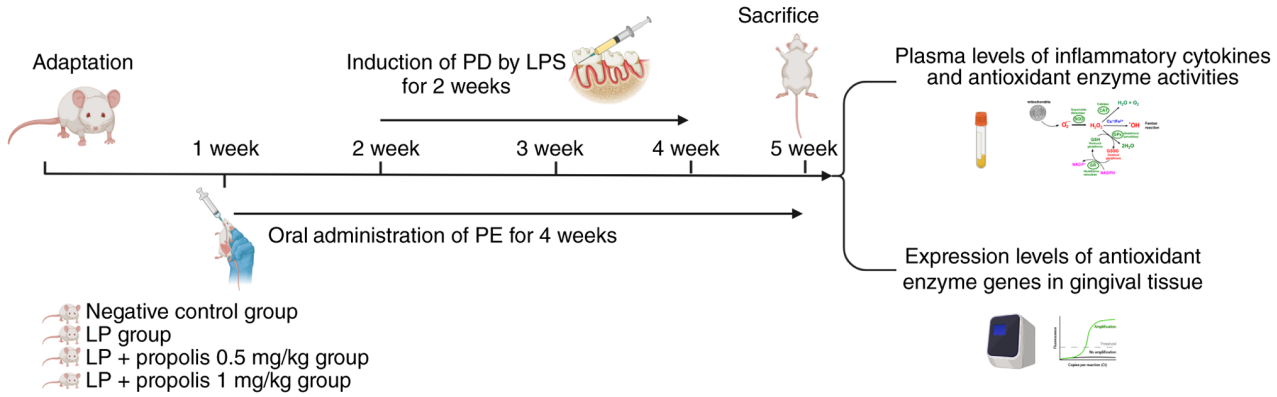


Figure 5. Experimental workflow for evaluating effects of propolis in *Porphyromonas gingivalis* LPS-induced periodontitis rat models. PE, propolis extract; LPS, lipopolysaccharide; LP, LPS-induced periodontitis; PD, periodontitis.

periodontal pathogens. HPLC profiling revealed that BBbK100 contains multiple bioactive compounds, including artepillin C, CAPE, pinocembrin and chrysin, which are well established polyphenols that contribute to the antioxidant, anti-inflammatory and antimicrobial activities of propolis. This chemical diversity may contribute to the biological activities of BBbK100.

The present *in vitro* experiments using HPLFs showed that BBbK100 effectively protected against H_2O_2-induced oxidative stress by preserving cell viability in a dose-dependent manner. In addition, BBbK100 restored the expression of antioxidant enzymes (SOD and CAT) suppressed by H_2O_2 and significantly reduced LPS-induced IL-6 expression, with decreasing trends in IL-1β and TNF-α expression. Mechanistically, CAPE has been shown to protect oral cells against oxidative stress and to attenuate inflammatory responses through the induction of HO-1, a key downstream target of the Nrf2 antioxidant pathway (24). Activation of the Nrf2/HO-1 axis may therefore underlie the restoration of SOD and CAT expression in H_2O_2-exposed HPLFs, whereas inhibition of NF-κB signaling by CAPE and artepillin C, may account for the reduction in LPS-induced IL-6 expression. These findings are consistent with previous reports (3,24)

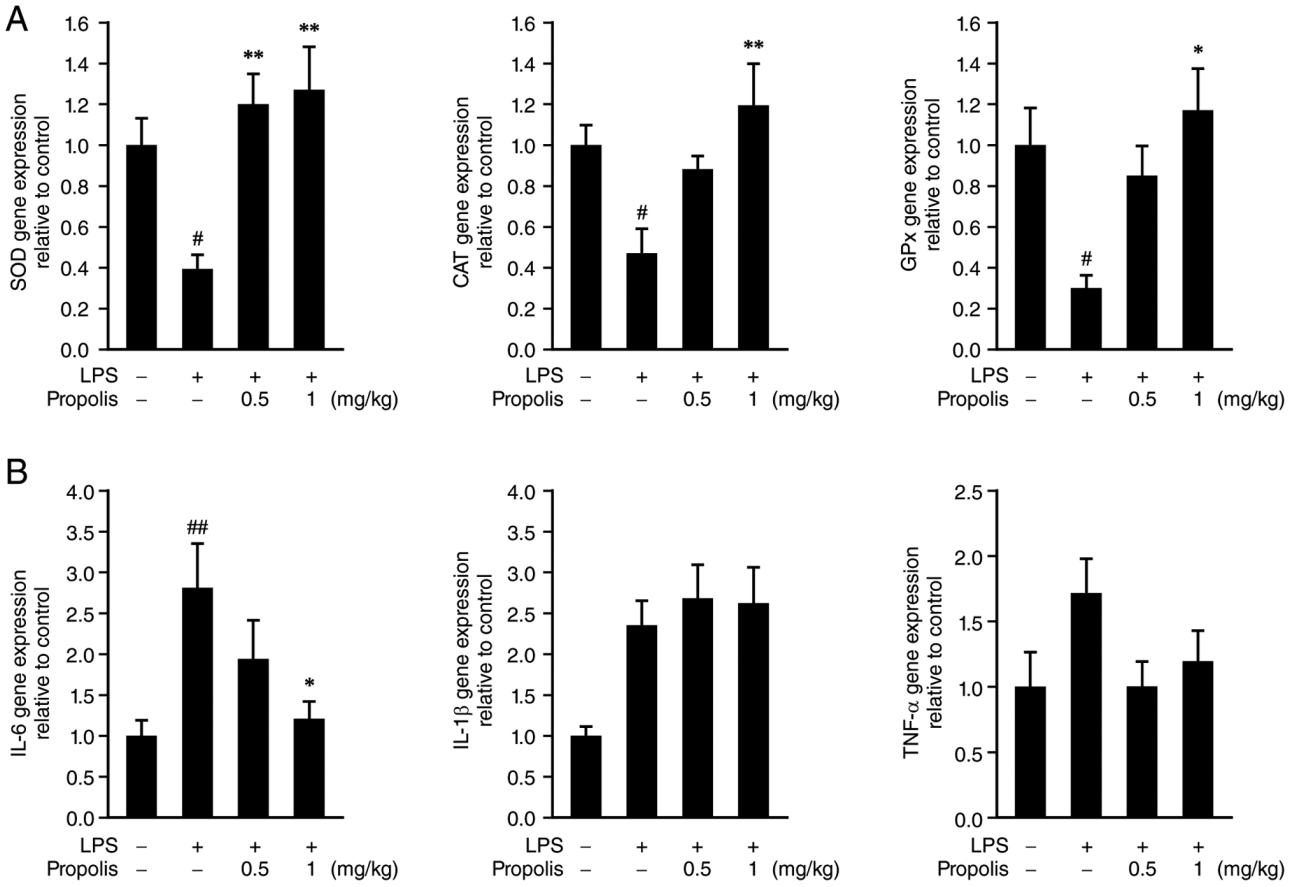


Figure 6. The gene expression levels of antioxidant enzymes and inflammatory cytokines in gingival tissues of LPS-induced periodontitis rat models. (A) Relative mRNA expression levels of antioxidant enzymes, including SOD, CAT and GPx. (B) Relative mRNA expression levels of inflammatory cytokines, including TNF-α, IL-1β and IL-6. These were measured by reverse transcription-quantitative PCR. Values were expressed as the mean ± SEM. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with the negative control group; *P<0.05 and **P<0.01 compared with the LPS-induced periodontitis group. LPS, lipopolysaccharide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

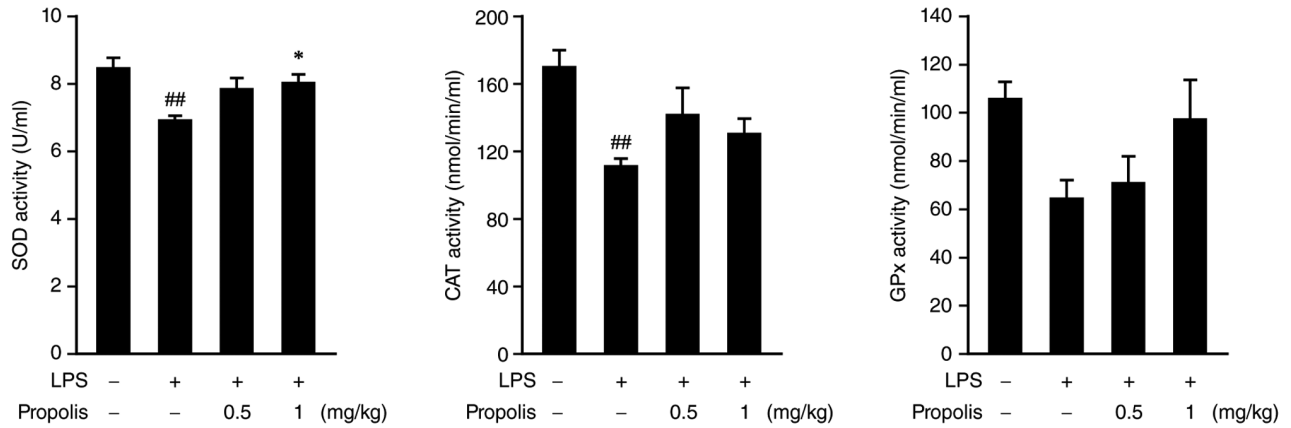


Figure 7. Effects of propolis on the activities of SOD, CAT and GPx in plasma of LPS-induced periodontitis rat models. Values measured using commercial colorimetric activity assay kits. Values are expressed as the mean ± SEM. $^{\#\#}$P<0.01 compared with the negative control group; *P<0.05 compared with the LPS-induced periodontitis group. LPS, lipopolysaccharide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

demonstrating that propolis extracts exert cytoprotective effects through the regulation of redox homeostasis and cytokine signaling pathways. Importantly, the concentrations used in the present study (≤1 μg/ml) were below the cytotoxic threshold, suggesting that BBbK100 can be applied safely within this therapeutic window.

In addition to its host-modulatory effects, BBbK100 inhibited the planktonic growth of six periodontal pathogens belonging to the red, orange and green complexes. This broad-spectrum activity is relevant as periodontitis is characterized by polymicrobial infection and dysbiosis of the oral microbiome. Although the underlying mechanism was not

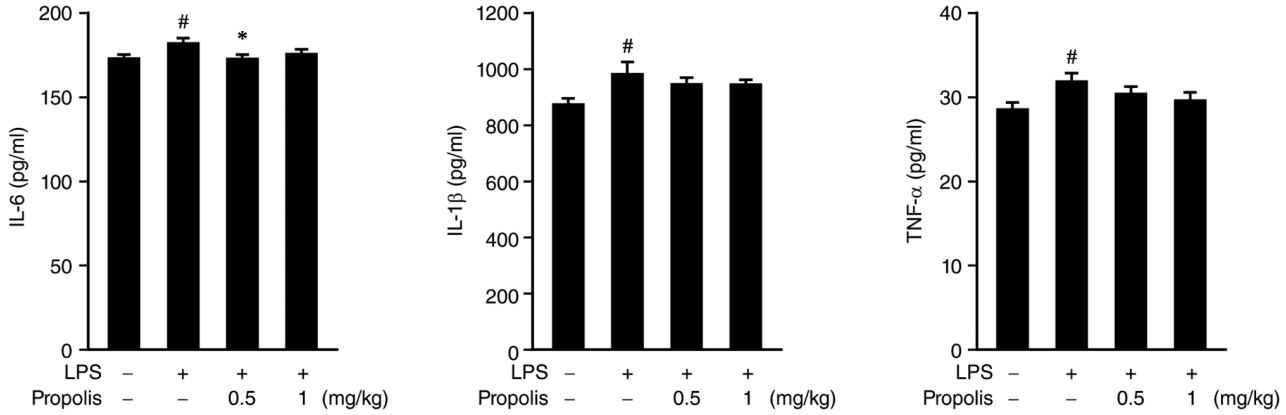


Figure 8. Effects of propolis on the levels of IL-6, IL-1β and TNF-α in plasma of LPS-induced periodontitis rat models. Values measured using ELISA. Values are expressed as the mean ± SEM. #P<0.05 compared with the negative control group; *P<0.05 compared with the LPS-induced periodontitis group. LPS, lipopolysaccharide.

investigated in the present study, previous work has shown that ethanol-extracted propolis rapidly kills *P. gingivalis* by disrupting bacterial membrane integrity, with artepillin C, the most abundant constituent of BBbK100, being identified as one of its active components (25). In addition, prenylated flavonoids have been reported to inhibit gingipain activity as well as the growth and biofilm formation of *P. gingivalis* (26). Propolis-derived flavonoids such as pinocembrin and CAPE have also been reported to interfere with bacterial adhesion and biofilm formation (27-30). However, given that periodontal pathogens reside in structured multispecies biofilms that are markedly more tolerant to antimicrobial agents compared with planktonic cells, the present findings should be regarded as preliminary evidence of antibacterial potential rather than as evidence of efficacy against established subgingival biofilms. Notably, the concentrations at which BBbK100 inhibited bacterial growth (0.032-0.064 mg/ml, ~0.003-0.006% w/v) were markedly higher compared with those effective in HPLFs (≤1 μg/ml) and are therefore unlikely to be reached in periodontal tissues after oral administration. Therefore, the *in vivo* effects observed in the present study are more plausibly explained by host-modulatory actions rather than by direct antibacterial activity. By contrast, these concentrations are ~300- to 800-fold lower compared with those of propolis mouthwashes used in clinical trials, the majority of which contain 2-5% propolis (31), suggesting that inhibitory concentrations could be achieved through local delivery. However, salivary dilution, contact time and substantivity in the oral cavity were not evaluated and no standard antimicrobial agent was included as a comparator. Of note, a recent systematic review reported that chlorhexidine was equally or more effective compared with propolis mouthwashes in some clinical trials (31). Therefore, the relative antibacterial potency and clinical relevance of BBbK100 remain to be established.

In the present *P. gingivalis* LPS-induced periodontitis rat model, oral administration of BBbK100 increased the gingival expression of antioxidant enzyme genes (SOD, CAT and GPx) and reduced that of IL-6, whilst also exhibiting increased plasma SOD activity and reduced plasma IL-6 levels, whereas changes in other plasma antioxidant enzymes and cytokines were not statistically significant. These findings suggested that BBbK100 exerted partial local and systemic antioxidant and anti-inflammatory effects. Given that propolis constituents have low water solubility and may undergo first-pass metabolism, these effects after oral administration imply that at least some constituents or their metabolites reached the systemic circulation and periodontal tissues. However, because their plasma and gingival concentrations were not measured, the constituents responsible for these effects remain to be identified.

The therapeutic potential of propolis has been widely recognized (9,16), yet its clinical application has been hampered by variability in composition due to geographic and botanical origins. By combining extracts from multiple origins at a defined ratio and adjusting the total flavonoid content, BBbK100 was designed to reduce this variability. HPLC analysis showed that BBbK100 contains numerous constituents with established biological activities. Artepillin C, the most abundant quantified compound (5.51 mg/g) and a characteristic marker of Brazilian green propolis, has been shown to exert anti-inflammatory activity and to inhibit NF-κB activation (3,32,33). CAPE (1.04 mg/g) is a well-characterized specific inhibitor of NF-κB activation (34), whereas pinocembrin (1.70 mg/g) and chrysin (0.42 mg/g), which are abundant in Brazilian brown and Korean propolis, are known for their antioxidant and antimicrobial properties (30). Given that NF-κB is a key transcriptional regulator of IL-6, IL-1β and TNF-α, artepillin C and CAPE may have contributed to the attenuation of LPS-induced cytokine expression observed in the present study, whereas the flavonoid constituents may have contributed to the antioxidant effects. In preliminary screening, the 5:3:2 mixture showed greater antioxidant and anti-inflammatory activity than the individual single-origin extracts, suggesting a combined contribution of these constituents. However, because purified compounds were not tested individually or in combination at the ratios present in BBbK100 and no formal interaction analysis was performed, it remains unclear as to whether the effects of BBbK100 are synergistic, additive or driven predominantly by a single component. Furthermore, because different production batches of each source propolis may vary in the relative amounts of minor constituents, standardization based on total flavonoid content alone may not fully ensure batch-to-batch

consistency (17). Multi-marker specifications based on characteristic compounds of each source material, such as artepillin C, CAPE, pinocembrin and chrysin, together with HPLC fingerprint similarity and liquid chromatography-mass spectrometry (LC-MS) analyses, will be required to ensure the reproducibility of BBbK100.

Nevertheless, the present study exhibits a number of limitations. First, while *in vitro* and animal models provide important mechanistic insights, clinical studies are required to determine the efficacy and safety of BBbK100 in human patients with periodontitis. Second, BBbK100 was standardized only on the basis of total flavonoid content, which is a crude index that does not fully reflect the chemical complexity of propolis (17). Although ten reference compounds, mainly phenolic constituents, were quantified by HPLC, terpenoids and other non-phenolic constituents were not systematically characterized and specifications based on multiple marker compounds, batch-to-batch consistency, LC-MS-based fingerprinting and the bioavailability of individual constituents were not evaluated. In addition, the individual and combined contributions of its constituents, including whether they act synergistically or additively, remain to be elucidated (17). Third, the pharmacokinetic properties of BBbK100, including the absorption, metabolism and plasma and gingival tissue concentrations of its major constituents, as well as its long-term effects, were not evaluated. Although artepillin C has been shown to be absorbed after oral administration in rats (35), the extent to which BBbK100 constituents reach periodontal tissues after oral administration remains unclear and local delivery formulations may be required to achieve effective concentrations at the site of disease (35). Fourth, the antibacterial activity of BBbK100 was evaluated only against planktonic bacteria; its effects on established biofilms, bacterial adherence to host cells, extracellular polymeric substance production and the expression of virulence factors such as gingipains and fimbriae were not examined. Moreover, because the *in vivo* model was induced by LPS rather than by live bacterial infection, the *in vivo* relevance of its antibacterial activity could not be assessed. Fifth, no standard antimicrobial agent such as chlorhexidine was included as a positive control and the concentrations of BBbK100 achievable in saliva or gingival crevicular fluid were not determined. Sixth, inflammation *in vitro* was induced using a single concentration (1 μg/ml) of *P. gingivalis* LPS. Although *P. gingivalis* is regarded as a keystone pathogen in periodontitis (36) and its LPS has been used to induce inflammatory responses in human periodontal ligament fibroblasts (18), this model does not reproduce the complexity of polymicrobial periodontitis. In addition, only three pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β), which were selected due to their central roles in osteoclastogenesis and alveolar bone resorption, were evaluated and other mediators involved in periodontitis were not assessed. Furthermore, the signaling pathways underlying the observed effects, such as NF-κB and Nrf2/HO-1, were not directly examined; thus, the aforementioned mechanisms proposed remain hypothetical. These aspects should be investigated in future research, including studies using established multi-species biofilm models, infection-based periodontitis models and local delivery formulations.

In conclusion, the present study demonstrated that EEP BBbK100, a standardized mixture of Brazilian green, Brazilian brown and Korean propolis, exerted antioxidant and anti-inflammatory effects in cellular and animal models of periodontitis. BBbK100 protected HPLF cells against oxidative stress, attenuated LPS-induced pro-inflammatory cytokine expression and inhibited the planktonic growth of key periodontal pathogens. Furthermore, in an LPS-induced periodontitis rat model, BBbK100 increased antioxidant enzyme expression in gingival tissue and partially restored plasma SOD activity and IL-6 levels. By combining propolis from multiple origins with a defined total flavonoid content, BBbK100 may help reduce the compositional variability of single-origin extracts. Collectively, these findings suggest that BBbK100 is a potential natural therapeutic candidate for the prevention and treatment of periodontitis, although further studies, including dose-ranging, pharmacokinetic and clinical studies, are warranted to validate its efficacy and safety. In particular, given that propolis is a well-recognized contact allergen, with caffeic acid esters identified as key sensitizers and systemic contact dermatitis has also been reported after oral ingestion (37,38), clinical studies of BBbK100, which contains caffeic acid derivatives such as CAPE, should exclude individuals with known allergy to propolis or other bee products and should include careful monitoring for hypersensitivity reactions.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SKK and MKK contributed equally to the present study. SKK and MKK performed the experiments. SKK drafted the initial version of the manuscript and MKK made major contributions to the revision of the manuscript. SWL and SHL generated and analyzed the data and prepared the graphical outputs. JYB conceived and designed the study, interpreted the data, acquired the funding, supervised the study, and critically revised and finalized the manuscript. SKK and MKK confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present experimental protocol was approved by the Dankook University Institutional Animal Care and Use Committee (Cheonan, South Korea; approval no. DKU-25-002).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, artificial intelligence tools were used to improve the readability and language of the manuscript, to check the bibliographic details of the references, and to generate images, and subsequently, the authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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