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Antimicrobial, Anti-inflammatory, and Anti-oxidative Effects of Water- and Ethanol-extracted Brazilian Propolis

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Abstract Because it possesses anti-inflammatory, antifungal, antiviral, and tissue regenerative properties, propolis has been used for thousands of years in folk medicine for multiple purposes. Although the antimicrobial activity of propolis has already been demonstrated, very few studies have been conducted on bacteria of clinical relevance in dentistry. The aim of this study is to evaluate the antimicrobial, anti-inflammatory, and anti-oxidative activities of 0.1% and 1.0% propolis, both of water-extracted (proAQ) and ethanol-extracted (proAL) propolis, for industrial applications. In studies of antimicrobial activity, the growth of *Staphylococcus aureus* ATCC 35556, *Salmonella enteritidis* ATCC 12021, *Escherichia coli* O157:H7, and *Candida parapsilosis* KCCM 35428, all general food or clinical pathogens, were tested. The culture medium used was trypticase soy broth including 0.6% yeast extract; after 6 hr of incubation, the turbidities were measured at 620 nm with a spectrophotometer. The results indicate that the antimicrobial effects of both 1.0% proAQ and 1.0% proAL were greater against the growth of *S. aureus* ATCC 35556 and *C. parapsilosis* KCCM 35428 rather than those of *S. enteritidis* ATCC 12021 and *E. coli* O157:H7. Additionally, it appears that the anti-inflammatory effects of proAL are greater than those of proAQ. The anti-inflammatory effects were evaluated by measurement of the inhibition of hyaluronidase activity *in vitro*. At a 1% concentration, the anti-inflammatory effects of proAL were greater than those of proAQ. Finally, the anti-oxidative effects of 1% and 10% solutions of each extract sample were measured according to the TBA method at 40°C for 1, 2, 3, and 5 days and were compared with 1.0% BHT. The results indicate that the anti-oxidative effects at 0.1% for both proAQ and proAL were not significantly different than the anti-oxidative effects at 1.0% BHT ($p<0.05$). Thus, it appeared that the alcohol-extracted propolis had greater antimicrobial, anti-inflammatory, and anti-oxidative effects than the water-extracted propolis. This is based on the presumption that major biofunctional components were fat-soluble, rather than water-soluble.

Keywords: propolis, antimicrobial effect, anti-inflammatory effect, anti-oxidative effect, flavonoid

Introduction

Propolis, a resinous substance collected by bees from various plant sources and mixed with secreted beeswax, is a multifunctional material used by bees in the construction, maintenance, and protection of their honeycombs (1, 2). Propolis is a non-toxic, natural product with multiple pharmacological effects and a complex chemical composition. Several compounds have been identified in propolis, and three distinct chemical groups are reportedly present: flavonoids, cinnamic acid derivatives, and terpenoids (3-6). Flavonoids have been considered the main biologically active compounds in propolis (2-7).

Propolis exhibits a broad range of biological activities, including anti-inflammatory, antibacterial, antiviral, anti-AIDS, anesthetic, and cytostatic properties (1-5, 8). Recently, propolis preparations have been used in the production of functional foods and cosmetics. The medical application of propolis preparations has led to increased interest in its chemical compositions and its origin, and many polyphenolic compounds have thus far been identified in propolis from different regions. Bees modify the action of β-glucosidase, a compound which hydrolyzes flavonoid heterosides into flavonoid aglycone and is secreted during propolis collection (5). In nature, the main polyphenols of propolis are flavonoid aglycones, which are accompanied by phenolic acids and their esters, phenolic aldehydes, alcohols, ketones, and so on.

Recently, the antimicrobial effects of propolis on growth of *S. aureus* and *Streptococcus mutans*, which are involved in dental disease (9-12). Although research on the antimicrobial effects of propolis has been done with regard to dental medicine, little is known about its antimicrobial activity against other food pathogens or skin diseases. Research in this area should provide important information which is relevant to the food industry and to dermatologic medicine (10-13).

The anti-oxidative effects of propolis have been reported (14). The main anti-oxidative components contained in propolis are polyphenols. It has been well understood that polyphenols scavenge peroxide radicals at the beginning of lipid auto-oxidation, which stabilizes the resonance structure and delays the oxidative reaction. Since propolis contains a variety of polyphenols, the anti-oxidative effects of each component and its concentration may differ.

Additionally, the anti-inflammatory effects of flavonoids have been reported (15). It has been well understood that a small molecule, hyaluronic acid, activates inflammation of tissue (16). In general, many anti-inflammatory medicines

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are hyaluronidase inhibitors (17).

The functional properties of propolis may differ according to the extraction methods used during its preparation, as well the resulting differences in composition at each condition. It has also been reported that the functional effects of propolis are different depending on the bees and region of the world (18, 19).

Thus, the purpose of this study is to evaluate ethanol- and water-extracted propolis at different concentrations as an antimicrobial agent against food pathogens and skin diseases, as an anti-inflammatory agent inhibiting hyaluronidase, *in vitro*, and as an anti-oxidative or antiaging agent.

Materials and Methods

Sample preparation Two types of Brazilian propolis which had been extracted with 80% ethanol (proAL) or distilled water (proAQ) were purchased from the Hebron J company. The bacterial strains used for antimicrobial testing were: *S. aureus* ATCC 35556 and *S. enteritidis* ATCC 12021, which were taken from Konkuk University; *E. coli* O157:H7, which was taken from Daegu Haany University; and *C. parapsilosis* KCCM 35428, which was screened from hospital patients suffering from skin diseases. All strains were cultured in trypticase soy broth (Difco Laboratories, Sparks, USA) with 0.6% yeast extract media (TSB-YE) at 37°C for 24 hr and were used for further studies.

Determination of total solid, fat soluble components, and total carbohydrates in samples The solid contents of both proAL and proAQ were measured by drying methods at 105°C. Before drying each sample, 50 mL of each sample was put in a drying dish and was pretreated by evaporation of water or ethanol until very viscous. The fat-soluble content was separated using ethyl ether. Fifty mL of each sample and 50 mL of ethyl ether were mixed in a separatory funnel. After standing overnight, the ether layer was separated, washed three times with distilled water, and evaporated in a rotary vacuum evaporator (SB1000; Eyela, Tokyo, Japan). The concentrates were dried in a 105°C dry oven for 2 hr, and their fat-soluble components were weighed. To determine the total carbohydrate content of both samples, the Lane-Eynon method (20) was used after acid-hydrolysis in 10 mL of 1 N HCl with refluxing in a boiling water bath for 2 hr.

Antimicrobial activity The concentrations of the propolis samples (proAQ and proAL) were prepared at 0.1% and 1.0% of the total broth volume, respectively, and aseptically filtered with a 0.45 μm membrane filter (Durapore HVLP; Millipore Co., Billerica, USA). One mL of water-extracted or one mL of alcohol-extracted propolis was added to 10 mL of TSB-YE media, respectively. Four bacterial strains, *S. aureus* ATCC 35556, *S. enteritidis* ATCC 12021, *E. coli* O157:H7, and *C. parapsilosis* KCCM 35428, were individually inoculated in culture media at 10^4 cfu/mL, and then incubated at 37°C for 5 hr. The antimicrobial effect of each sample was detected by measuring the turbidity with a spectrophotometer (OPRON 3000; Hanson Co., Seoul, Korea) at 620 nm. Each experiment was performed in triplicate, and the data regarding the difference between control (0%) and 0.1% proAL (or proAQ)-treated samples and the data regarding the difference between proAL- and pro AQ-treated samples of each strain at the same concentration were analyzed using the Student's t-test ($p<0.05$).

Anti-inflammatory activity Hyaluronidase activity was determined by measuring the amount of N-acetyl-glucosamine formed from sodium hyaluronidate with a spectrophotometer. Fifty μL of bovine hyaluronidase (5,000 units/mL, Sigma-Aldrich Co., St. Louis, USA) dissolved in 0.1 M acetate buffer (pH 3.5) was mixed with 100 μL of a designated concentration of sample dissolved in 5.0% dimethyl sulfoxide. The mixture was then incubated in a water bath at 37°C for 20 min. The control group was treated with 100 μL of 5.0% dimethyl sulfoxide alone. The reaction mixture was added to 100 μL of 12.5 mM calcium chloride, and was then incubated in a water bath at 37°C for 20 min. This Ca^+-activated hyaluronidase was treated with 250 μL of sodium hyalunonidate(1.2 mg/mL) dissolved in 0.1 M phosphate buffer (pH 3.5), and was then incubated in a water bath at 37°C for 40 min. One hundred μL of 0.4 N sodium hydroxide and 100 μL of 0.4 M potassium borate were added to the reaction mixture. The reaction mixture was then incubated in a boiling water bath for 5 min. After cooling to room temperature, 3 mL of *p*-dimethyl-aminobenzaldehyde solution (4 g of *p*-dimethyl-aminobenzaldehyde dissolved in 350 mL of 100% acetic acid and 50 mL of 10 N hydrochloric acid) was added to the reaction mixture, which was then incubated in a water bath at 37°C for 20 min. The optical density of the reaction mixture was measured at 585 nm using a spectrophotometer (OPRON-3000; Hanson Co., Seoul, Korea). The inhibitory effect was expressed as follows:

Inhibitory effect (%) = $[(\Delta ODc - \Delta ODs)/\Delta ODs] \times 100$
(ΔODc; optical density of control, ΔODs; optical density of sample)

Anti-oxidative activity The TBA (thiobarbituric acid) method was used; proAQ and proAL samples were diluted with d-water and ethanol (1:10), respectively. Additionally, 1.0 % butylated hydroxyltoluene (BHT; Sigma-Aldrich Co.) in methanol was used as a standard reagent. One mL of linoleic acid (25 mg/mL in ethanol, Waco Co., Japan) and 0.1 mL of each sample were mixed, after which 2 mL of 0.2 M phosphate buffer (pH 7.0) was added to each. The control group was treated with 0.1 mL of distilled water. The mixture was incubated at 40°C for 1, 2, 3, and 5 days, and the reaction was stopped by the addition of 50 μL of 7.2% BHT. Next, 0.5 mL of reaction mixture, 0.25 mL of 20% trichloroacetic acid (TCA), and 0.1 mL of 0.67% 2-thiobarbituric acid in 0.025 N HCl were mixed and incubated in a boiling water bath for 15 min with occasional stirring. After cooling to 25°C, 0.5 mL of 70% TCA was added and the sample was centrifuged at 15,000 × g for 5 min. The optical density of the supernatant was detected using a spectrophotometer at 532 nm.

Statistical analysis Analysis of variance was performed

for the three samples using the SAS program. Duncan's test was used to verify the significance of the difference according to each treatment.

Results and Discussion

Solid and fat-soluble contents To compare the compositions of major functional components of both proAQ and proAL, the total solid, fat-soluble components, and carbohydrates of proAQ and proAL were detected as preliminary data for the evaluation of the bio-functionality of both samples. As shown in Table 1, the solid content of proAQ was a little greater than that of proAL, but the fat-soluble composition of proAL was greater than that of proAQ. It appeared that proAQ contained mostly carbohydrates as its main component. In the case of the fat-soluble portion of proAL, the wax composition was determined to be 1.35±0.05% (w/v) (data not shown). Thus, it appeared that the composition of effective components in proAQ and proAL (as antimicrobial, anti-inflammatory, and anti-oxidative agents) was a maximum of 1.35% (w/v) and 1.40% (w/v), respectively.

Antimicrobial activity The resistances of microorganisms to both proAQ and proAL were studied (Fig. 1 and Fig. 2, respectively). All strains used in this research were pathogens associated with foods or skin diseases, with which the propolis can be used. At the beginning of culture, the concentration of each strain was 10^4 cfu/mL. At 0.1% concentration of proAQ (or pro AL), the growth rates of each strain were significantly decreased ($p<0.05$). The results show that both proAQ and proAL have antimicrobial effects on the growth of all of the tested strains at concentrations of 0.1% or above. At 0.1% and 1.0% concentrations of proAQ and proAL in TSB-YE broth, the growth of *S. aureus* ATCC 35556 and *C. parapsilosis* KCCM 35428 were more inhibited than that of *S. enteritidis* ATCC 12021 and *E. coli* O157:H7, gram-negative bacteria.

At 1.0% concentration, the antimicrobial effect of proAL against *S. enteritidis* ATCC 12021 and *E. coli* O157:H7 (gram-negative) was greater than that of proAQ. However, the antimicrobial effect of proAQ against *S. aureus* ATCC 35556 was greater than that of proAL. The antimicrobial effects of proAQ and proAL against *C. parapsilosis* KCCM 35428 were not found to be significantly different ($p<0.05$).

When concentrations below 0.1% were used for either of the extracts, no antimicrobial effects were detected (data not shown). According to these results, the major components of the extracts (which have the antimicrobial effects), which are still unknown, are various and different. Thus, it is expected that propolis can be used as an antimicrobial additive or pharmaceutical supplement because of its broad spectrum of effectiveness against various pathogens.

Anti-inflammatory activity Hyaluronic acid, a mucopolysaccharide composed of alternating glucuronic acid and N-acetyl glucosamine residues, is the main component of the extracellular matrix and of biological fluids in tissue (21, 22). High molecular weight hyaluronic acid inhibits the phagocytic ability of macrophages, an important inflammation reaction (16). Hyaluronic acid also acts as an important regulator in repairing fetal wounds without scarring (23, 24). However, the degradation of hyaluronic acid to smaller molecules results in an increase in inflammation, angiogenesis, fibrosis, and collagen deposition in the

Table 1. The compositions of total solids, fat-soluble compounds, and carbohydrates of proAQ and proAL samples

	ProAQ	ProAL
Solid content (%)	3.64±0.04[1)]	3.56±0.03
Fat-soluble content (%)	0.24±0.02	3.35±0.05
Carbohydrates (%)	2.24±0.05	0.11±0.02
Others (%)	1.16±0.02	Trace

[1)]The values are mean±S.D.

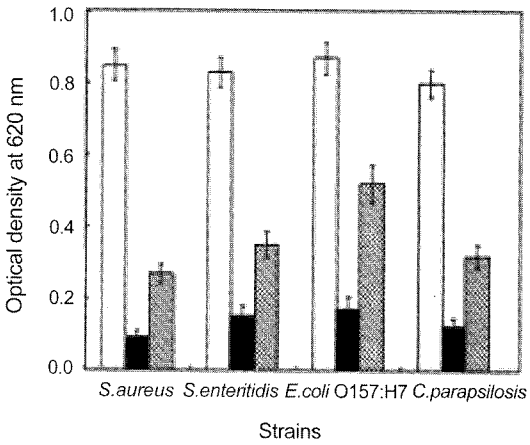

Fig. 1. The antimicrobial effect of water-extracted propolis (proAQ); the control was treated with 1 mL of sterilized distilled water instead of cultured broth. The propolis samples were diluted with sterilized distilled water (1:9 and 1:99), and 1 mL of each diluted sample was added to 9 mL of TSB-YE, respectively. Culture broth turbidity was measured at 620 nm after incubation for 5hr (□ , 0%; ▨ , 0. 1%; ■ , 1%).

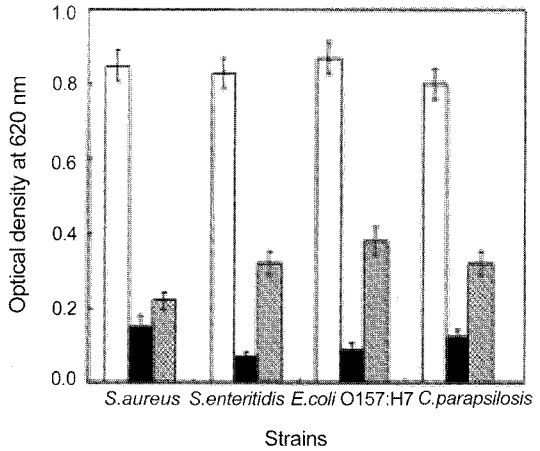

Fig. 2. The antimicrobial effect of alcohol-extracted propolis (proAL); the control was treated with 1 mL of sterilized distilled water instead of cultured broth. The propolis sample was diluted with 80% ethanol (1:9 and 1:99), and 1 mL of each diluted sample was added to 9 mL of TSB-YE, respectively (□ , 0 %; ▨ , 0. 1%; ■ , 1%).

Table 2. The inhibitory effect of proAQ and proAL on hyaluronidase

Sample	Concentration (%)	Inhibitory effect (%)[1)]
Control	0	0.02±0.01 [2)d]
ProAL	0.1	31.85±0.35[b]
	1.0	98.85±0.16[a]
ProAQ	0.1	14.85±0.23[c]
	1.0	96.84±0.54[a]

[1)]Inhibitory effect (%) = [(ΔODc -ΔODs)/ΔODs]×100 (ΔODc; Optical density of control, ΔODs; Optical density of sample).
[2)]The values are mean±S.D. Values followed by the same letter are not significantly different ($p<0.05$).

healing of wounds (23, 24). Hyaluronidase is an endo-hexosaminidase which initiates the degradation of high molecular weight hyaluronic acid. In these studies, the anti-inflammatory effects of proAQ and proAL were evaluated by detecting their inhibitory effect on hyaluronidase *in vitro*. The results of this study show that both proAQ and proAL have inhibitory effects on hyaluronidase (Table 2). However, it appears that the inhibitory effect of proAL was greater than that of proAQ. At 0.1% concentrations of both proAL and proAQ, the inhibitory effect of proAL was two times greater than that of proAQ. However, no inhibitory effects against hyaluronidase were detected at concentrations below 0.1% for both samples. Judging from these results, it may be presumed that the compounds which have anti-inflammatory effects in propolis are various, and its composition differs according to the solvent and its concentration during extraction.

Anti-oxidative activity Anti-oxidative activity was measured by the oxidation of linoleic acid. As an oxidation product of linoleic acid, malonaldehyde produces a red color by reacting with thiobarbituric acid (TBA), which results from the condensation of two molecules of TBA with one molecule of malonaldehyde. The results of the examination for antioxidant activity are illustrated in Fig. 3

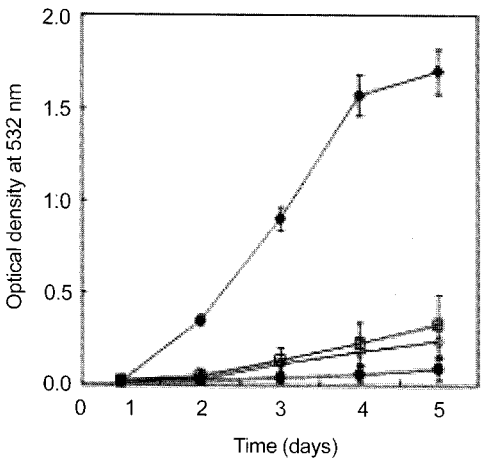

Fig. 3. The anti-oxidative effect of proAQ and proAL; proAQ and proAL were diluted with d-water and 80% ethanol (10:1), respectively. The anti-oxidative effects were measured according to the TBA method (◆, Control; □, ProAQ;◇, ProAL; ●, 1.0% BHT).

and indicate that all of the proAL and proAQ samples have anti-oxidative activities ($p<0.05$). Considering the solid contents of proAQ and proAL (Table 1), the anti-oxidative effects of both proAL and proAQ were at least ten times greater than that of 1.0% BHT. This is because the solid contents of proAQ and proAL used in these experiments were about 0.14% (w/v) when compared with the 1.0% BHT sample. At the same concentrations, it appeared that the anti-oxidative effect of proAQ was a little lower than that of proAL, but this difference was not significant ($p<0.05$).

As a result, the bio-functional effects in proAL were shown to be greater than those in proAQ. Therefore, it is presumed that the major components which produce antimicrobial, anti-inflammatory, or anti-oxidative effects are fat-soluble, rather than water-soluble. The components and their compositions may be different depending on the hydrophobicity of extraction solvents. Additionally, it is presumed that the major components which have anti-oxidative effects in propolis are polyphenols and flavonoids and, in the case of proAQ, they may exist as glycosides. The isolation and quantitative determination of each flavonoid or major compound in proAQ and proAL as an antioxidant or an antimicrobial agent will be pursued in future studies.

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