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NOTE

Antimicrobial Effect of Korean Propolis Against the Mutans Streptococci Isolated from Korean

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The aim of this study was to determine the optimal concentration of Korean propolis against clinical isolates of mutans streptococci (MS) from Koreans. The antimicrobial activity was evaluated using the minimum inhibitory concentration (MIC) and time-kill curves against mutans streptococci. The MIC_{90} values of propolis for MS were 35 μg/ml. Propolis had a bacteriostatic effect on *Streptococcus mutans* ATCC 25175^T and bactericidal effects on *Streptococcus sobrinus* ATCC 33478^T at > 2×MIC (70 μg/ml). These results suggest that the propolis can be used in the development of oral hygiene products for the prevention of dental caries.

***Keywords*:** antimicrobial effect, propolis, mutans streptococci, Koreans

The dental caries is a major oral disease with the major causative bacterial species being *Streptococcus mutans* and *Streptococcus sobrinus*, which inhabit the dental plaque in the human oral cavity (Whiley and Beighton, 1998). A gargling solution containing anti-microbial agents was developed to prevent dental caries. Antibiotics are the representative antimicrobial agents but they are not good agents for use as a component in gargling solutions due to the potential induction of resistance to antibiotics. Therefore, many studies have attempted to identify antimicrobial agents from natural extracts.

Propolis is the resinous material collected by honeybees from various plants source (Burdock, 1998). The precise composition of propolis varies with the geographic origin (Sonmez *et al*., 2005). In general, 95% components of propolis are resin, wax, essential aromatic oils, and pollen and the rest components are amino acids, minerals, ethanol, Vitamins A, B complex, E and the highly active mixture of compounds known as bioflavonoids (Marucci, 1995). Propolis is used widely as an antimicrobial agent in traditional medicine worldwide (Koo *et al*., 2000; Santos *et al*., 2002; Sonmez *et al*., 2005; Liberio *et al*., 2009). Generally, just type strains or a few wild type strains of mutans streptococci have been used to test the antimicrobial activity of natural extracts (Kujumgiev *et al*., 1999; Sonmez *et al*., 2005). However, previous studies have reported that the antimicrobial activity differs according to the type of mutans streptococci strains and the clinical strains isolated from the Korean oral cavity (Lee *et al*., 2003; Lim *et al*., 2003). This suggests that the proper concentration of natural extracts needs to be determined before developing effective oral hygiene products for use by Koreans by examining the Korean isolates of mutans streptococci. Therefore, this study examined the optimal concentration of the Korean propolis against the clinical isolates from the Korean oral cavity for development of the oral hygiene products, such as gargling solution and toothpastes.

The *S. mutans* ATCC 25175^T and *S. sobrinus* ATCC 33478^T were purchased from the American Type Culture Collection (ATCC, USA). The clinical strains of *S. mutans* (KCOM 1088, KCOM 1091, KCOM 1092, KCOM 1095, KCOM 1097, KCOM 1111, KCOM 1112, KCOM 1113, KCOM 1116, KCOM 1117, KCOM 1118, KCOM 1123, KCOM 1124, KCOM 1126, KCOM 1127, KCOM 1128, KCOM 2762, KCOM 1136, KCOM 1137, KCOM 1139, KCOM 1142, KCOM 1143, KCOM 1145, KCOM 1146, KCOM 1197, KCOM 1200, KCOM 1201, KCOM 1202, KCOM 1203, KCOM 1207, KCOM 1208, KCOM 1209, KCOM 1212, KCOM 1214, KCOM 1217, KCOM 1219, KCOM 1226) and *S. sobrinus* (KCOM 1061, KCOM 1150, KCOM 1151, KCOM 1152, KCOM 1153, KCOM 1157, KCOM 1158, KCOM 1159, KCOM 1185, KCOM 1191, KCOM 1193, KCOM 1196, KCOM 1221, KCOM 1228, KCOM 1218) were isolated from Koreans (Yoo *et al*., 2007) and obtained from the Korean Collection for Oral Microbiology (KCOM, Korea). All strains were cultured on a Todd Hewitt (TH, Difco, Lab., USA) broth or agar plates in a 37°C incubator in air containing 10% CO_2.

Ethanol-extracted propolis was obtained as a dry extract from GABO FARMS (Korea), and dissolved in ethanol at a concentration of 448 mg/ml. The solutions were kept at 4°C until use.

The minimum inhibitory concentration (MIC) was deter-

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Table 1. MIC value of propolis against the clinical strains of mutans streptococci isolated from Koreans

Species and strains	MIC (μg/ml)	Species and strains	MIC (μg/ml)
S. mutans ATCC[a] 25175[T]	35	*S. mutans* KCOM 1200	17.5
S. mutans KCOM[b] 1054	35	*S. mutans* KCOM 1201	17.5
S. mutans KCOM 1085	17.5	*S. mutans* KCOM 1202	17.5
S. mutans KCOM 1087	35	*S. mutans* KCOM 1203	35
S. mutans KCOM 1088	17.5	*S. mutans* KCOM 1207	17.5
S. mutans KCOM 1091	17.5	*S. mutans* KCOM 1208	17.5
S. mutans KCOM 1092	17.5	*S. mutans* KCOM 1209	35
S. mutans KCOM 1095	35	*S. mutans* KCOM 1212	17.5
S. mutans KCOM 1097	35	*S. mutans* KCOM 1214	17.5
S. mutans KCOM 1111	35	*S. mutans* KCOM 1217	17.5
S. mutans KCOM 1112	35	*S. mutans* KCOM 1219	35
S. mutans KCOM 1113	35	*S. mutans* KCOM 1226	35
S. mutans KCOM 1116	35	*S. sobrinus* ATCC 33478[T]	35
S. mutans KCOM 1117	17.5	*S. sobrinus* KCOM 1061	35
S. mutans KCOM 1118	35	*S. sob*rinus KCOM 1150	35
S. mutans KCOM 1123	35	*S. sobrinus* KCOM 1151	35
S. mutans KCOM 1124	35	*S. sobrinus* KCOM 1152	35
S. mutans KCOM 1126	35	*S. sobrinus* KCOM 1153	35
S. mutans KCOM 1127	35	*S. sobrinus* KCOM 1157	35
S. mutans KCOM 1128	35	*S. sobrinus* KCOM 1158	17.5
S. mutans KCOM 2762	17.5	*S. sobrinus* KCOM 1159	35
S. mutans KCOM 1136	35	*S. sobrinus* KCOM 1185	35
S. mutans KCOM 1137	17.5	*S. sobrinus* KCOM 1191	35
S. mutans KCOM 1139	35	*S. sobrinus* KCOM 1193	70
S. mutans KCOM 1142	17.5	*S. sobrinus* KCOM 1196	35
S. mutans KCOM 1143	17.5	*S. sobrinus* KCOM 1221	35
S. mutans KCOM 1145	35	*S. sobrinus* KCOM 1228	35
S. mutans KCOM 1146	17.5	*S. sobrinus* KCOM 1218	35
S. mutans KCOM 1197	35		

[a] ATCC, American Type Culture Collection
[b] KCOM, Korean Collection for Oral Microbiology; T, Type strain

mined using a microdilution assay according to the NCCLS standard (NCCLS, 2000). The bacterial strains were cultured in TH broth at 37℃ in an incubator for 24 h and added into a 96-well plate to a final concentration of 1×10^6 CFU/ml. The ethanol-extract propolis solutions were then added to each well to a final concentration of 560, 280, 140, 70, 35, 17.5, or 8.8 μg/ml. The final ethanol concentration in each well was 1%. Ampicillin (100 μg/ml) was used as the positive control, and the culture medium only and culture medium plus 1% ethanol groups were used as the double negative control. After 24 h incubation under suitable conditions, the lowest concentration of ethanol-extracted propolis to inhibit visible growth was taken as MIC value.

The bactericidal activity was also evaluated using the time-kill curves on the type strains of *S. mutans* (ATCC 25175[T]) and *S. sobrinus* (ATCC 33478[T]). The time-kill curves were assessed at the following propolis concentrations: 0.5× MIC, 1× MIC, 2× MIC, and 4× MIC. The control curve was obtained in the culture medium for each strain. The bacteria were inoculated in TH broth and incubated overnight in a 37℃ incubator. Liquid media containing propolis at the above-mentioned concentrations were inoculated with 10^6 CFU/ml of an overnight culture and incubated in a 37℃ incubator. At 0, 3, 6, 12, and 24 h after inoculating with the bacteria, each bacterial culture solution was diluted 100- or 10,000-folds and plated onto a TH agar plate. The agar plate was incu-

Table 2. Antimicrobial effect of propolis against mutans streptococci isolated from Koreans

Species	Concentration of propolis (μg/ml)		
	MIC_{50}	MIC_{90}	MIC_{100}
S. mutans (n=40)	35	35	35
S. sobrinus (n=15)	35	35	70
Total (n=55)	35	35	70

MIC_{50}, MIC_{90}, and MIC_{100} minimal inhibitory concentration needed to inhibit the growth of 50, 90, and 100% of mutans streptococci, respectively

bated in a 37°C incubator for 24 h and the bacterial colonies were counted.

KB cells, oral epithelial carcinoma cell line, were obtained from ATCC. The KB cells were grown in MEM medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2.

A MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] assay was performed to test the cell toxicity of ethanol-extracted propolis on the KB cells. Eighty percentage confluent KB cell monolayers in 24-well plates were incubated with various propolis concentrations (140, 70, 35, and 17.5 μg/ml) and 1% ethanol as a control in the growth medium at 37°C in humidified air containing 5% CO_2 for 24 h. The medium in each well was changed to a new culture medium containing a 10% MTT solution (Sigma, USA) and cultured 3 h under the same culture conditions. The isopropanol (Sigma) was placed in each well at a volume of 300 μl. The culture plate was well shaken. The sample was aliquoted on a 96-well plate at a volume of 200 μl. The absorbance was measured at a wavelength of 595 nm. At this time, the experimental and control groups were assigned three wells each. This procedure was individually repeated three times.

The antimicrobial effects of propolis were examined by measuring the minimum inhibitory concentration using the microdilution method. The MIC of propolis against the type strains of mutans streptococci, *S. mutans* ATCC 25175^T and *S. sobrinus* ATCC 33478^T, was 35 μg/ml each (Table 1). The MIC of propolis against the clinical strains of mutans streptococci ranged from 17.5 to 70 μg/ml (Table 1). However, the MIC_{50} and MIC_{90} was 35 μg/ml each (Table 2). Many studies have reported that propolis from different localities worldwide have antimicrobial activity against mutans streptococci (Ikeno *et al.*, 1991; Steinberg *et al.*, 1996; Melliou and Chinou, 2004; Uzel *et al.*, 2005). In those studies, type strains or a few strains of mutans streptococci were used. The MIC_{90} value of propolis against 55 strains of mutans streptococci was the same regardless of the type of mutans streptococci strain. Therefore, these results suggest the proper concentration of the propolis for developing an oral hygiene product such as a gargling solution to prevent dental caries for Koreans.

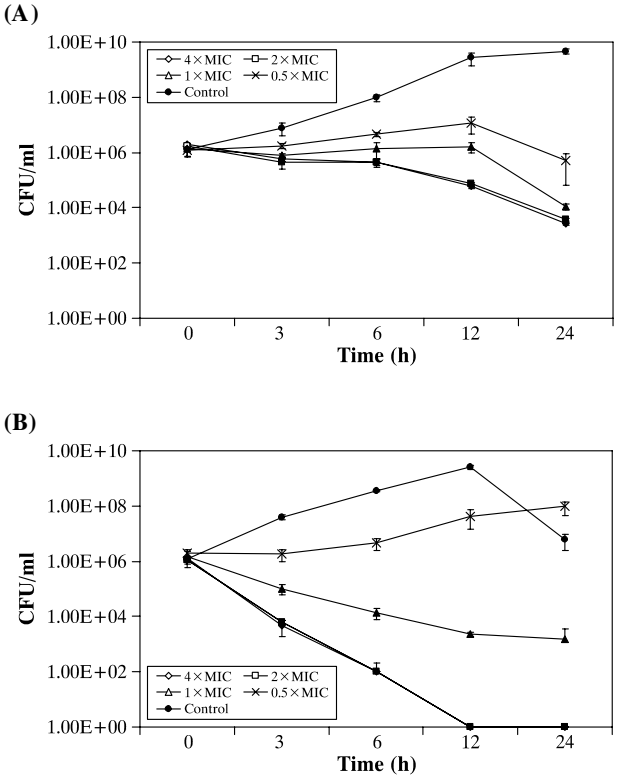

Fig. 1. Time-kill curves of propolis against (A) *S. mutans* ATCC 25175^T and (B) *S. sobrinus* ATCC 33478^T at different propolis concentrations.

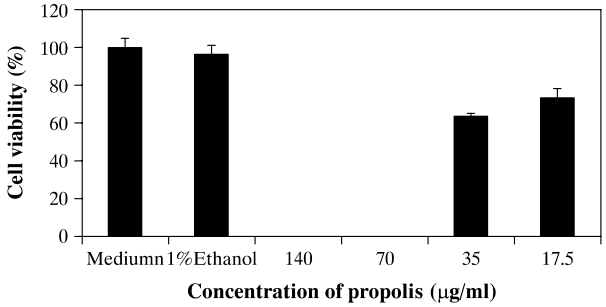

Fig. 2. Effects of propolis on the cell viability of KB cells

A time-kill assay was performed to determine if the antimicrobial effect of propolis is due to the bactericidal or bacteriostatic effect. The data suggests that propolis has a bacteriostatic effect on *S. mutans* ATCC 25175^T to a concentration up to 4X MIC (Fig. 1A) and bactericidal effect on *S. sobrinus* ATCC 33478^T at concentrations > 2× MIC (Fig. 1B).

The propolis used in this study was composed of approximately 30 different compounds according to high-performance liquid chromatography (data not shown). In a further study, the antimicrobial effects of the fractions of the propolis need to be determined to identify the component with the most effective antimicrobial efficiency against mutans streptococci.

To determine the cytotoxicity of propolis, a MTT assay was performed using KB cells, which are a carcinoma cell line originating from oral keratinocytes cells. The data showed that the cell viability of KB cells at 35 and 17.5 μg/ml propolis was approximately 66% and 76%, respectively (Fig. 2). The cell viability of KB cells was 4.7% at propolis concentrations >70 μg/ml (Fig. 2). According to the MTT test of propolis on normal human gingival fibroblast (NHGF) cells, the cell viability in 17.5, 35, 70, and 140 μg/ml propolis was 97%, 124%, 117%, and 4.7%, respectively (data not shown). The main reason for the difference in the cell toxicity of propolis between the KB and NHGF cells is unclear at present but it may be the difference in the resistance to the propolis according to the cell type. The cell viability tests for the chemicals or natural extracts on human cells were performed *in vitro*. The human oral tissue cells *in vivo* may be more resistant to these chemicals than those *in vitro*, because oral tissue cells *in vivo* can be supplied nutrients continuously by blood and supported

better repair mechanisms rather than those *in vitro*. Overall, these results suggest that a propolis concentration 35 μg/ml can be used *in vivo* for the prevention of dental caries.

In summary, propolis at concentrations >35 μg/ml has antimicrobial activity against 90% of the mutans streptococci strains (55 strains) isolated from Koreans. Moreover, the propolis can be useful in the development of oral hygiene products for the prevention of dental caries.

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