International Journal of Oral Biology, Vol. 36, No. 1, March 31 2011, p. 7~11

International Journal of Oral Biology

Antimicrobial Effects of Ursolic Acid against Mutans Streptococci Isolated from Koreans

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(received December 13, 2010 ; revised January 28, 2011 ; accepted February 11, 2011)

Ursolic acid is a triterpenoid compound present in many plants. This study examined the antimicrobial activity of ursolic acid against mutans streptococci (MS) isolated from the Korean population. The antimicrobial activity was evaluated by the minimum inhibitory concentration (MIC) and time kill curves of MS. The cytotoxicity of ursolic acid against KB cells was tested using an MTT assay. The MIC_{90} values of ursolic acid for *Streptococcus mutans* and *Streptococcus sobrinus* isolated from the Korean population were 2 μg/ml and 4 μg/ml, respectively. Ursolic acid had a bactericidal effect on *S. mutans* ATCC 25175^T and *S. sobrinus* ATCC 33478^T at > 2 × MIC (4 μg/ml) and 4 × MIC (8 μg/ml), respectively. Ursolic acid had no cytotoxic effect on KB cells at concentrations at which it exerted antimicrobial effects. The results suggest that ursolic acid can be used in the development of oral hygiene products for the prevention of dental caries.

Key words: antimicrobial effect, *Streptococcus mutans*, *Streptococcus sobrinus*, ursolic acid.

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Introduction

The low pH of the organic acids produced by the carbohydrate metabolism of bacteria in dental plaque causes dental caries, resulting in demineralization of the dental hard tissue. Among the oral bacteria, mutans streptococci (MS) have been identified as the major causative bacteria of dental caries (Loeshe, 1986). MS are composed of seven species according to 16S rRNA gene sequence analysis (Kawamura *et al*., 1995). Of them, *Streptococcus mutans* and *Streptococcus sobrinus* are isolated mainly from humans and are strongly associated with human dental caries (Loeshe, 1986).

Fluorides and chlorhexidine are effective compounds for the prevention of dental caries and are the major component of oral hygiene products, such as toothpaste, gargling solutions and dental floss (Emilson, 1994; Schaeken *et al*., 1991). However, the intake of fluoride compound might cause abdominal pain and its excessive intake produces side effects, such as mottled teeth (Spencer & Do, 2008). According to these side effects, Koreans have generally rejected water fluoridation. The excessive use of chlorhexidine, which is used as an oral gargling solution, can also cause the discoloration of teeth (Tredwin *et al*., 2005).

Recently, multiple tests have been performed to determine the effective chemicals from many plants that have been used as antimicrobial agents in the Korean population for a long time to overcome the side effects of chemical compounds, such as fluoride and chlorhexidine. It was reported that ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), which is a derivative of

triterpenoid saponins (Liu, 1995), has a higher degree of the anti-bacterial activity against pathogenic bacterial strains, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Fontanay *et al*., 2008). Ursolic acid can suppress dental plaque formation by inhibiting the glucosyltransferase activity of *S. mutans* OMZ 176 (Kozai *et al*., 1987).

In general, the antimicrobial tests for the screening of anticaries agents are performed by measuring the minimum bactericidal concentration (MBC) and/or minimum inhibitory concentration (MIC) against the type of strains or few wild type strains of MS. According to previous studies, the MIC of a water-extract of green tea on the clinical strains of MS isolated from Korean population was different from that of the type strains of MS (Lim *et al*., 2003; Lee *et al*., 2003). These results suggest that the appropriate concentration of anticaries agents in oral hygiene products, such as gargling solution or toothpastes, should be determined from clinical isolates obtained from the Korean population. The purpose of this study was to determine the antimicrobial activity and optimal ursolic acid concentration using 40 clinical strains of *S. mutans* and 15 clinical strains of *S. sobrinus* isolated from the Korean population for the development of oral hygiene products containing ursolic acid for Koreans.

Materials and Methods

Bacterial strains and growth conditions

The *S. mutans* ATCC 25175^T and *S. sobrinus* ATCC 33478^T were purchased from the American Type Culture Collection (ATCC, Manassas, VA, 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 obtained from the Korean Collection for Oral Microbiolgy (KCOM, Gwangju, Korea). All strains were cultured in Todd Hewitt broth or on agar plates (Difco, Lab., Detroit, MI. USA) in a 37°C incubator in air containing 10% CO_2.

Determination of the minimum inhibitory concentration (MIC)

The MIC was performed by a microdilution assay according to the NCCLS standard (NCCLS, 2000). The bacterial strains were cultured in TH broth at 37°C in an incubator for 24 hours and then added to a 96-well plate to a final concentration of 1×10^6 CFU/ml. The ursolic acid (Sigma, St. Louis, MO, USA) solutions were added to each well to a final concentration of 1, 2, 4, 8, and 16 μg/ml. At this time, ursolic acid was dissolved in dimethyl sulfoxide (DMSO; Sigma). The final concentration of DMSO in each well was 1%. The 1% DMSO in the medium and medium only group was used as the double negative groups. The ampicillin (concentration of 100 μg/ml) was used as a positive control. After 24 hr incubation under the appropriate conditions, the lowest concentration of ursolic acid that inhibited visible growth was considered to be the MIC.

Time- kill assay

The bactericidal activity was also evaluated from 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 ursolic acid concentrations: 0.5 × MIC, 1 × MIC, 2 × MIC, and 4 × MIC. The control curve was obtained in a culture medium for each strain. The bacteria were inoculated in a TH broth and incubated overnight in a 37°C incubator. The liquid media containing ursolic acid at the above mentioned concentrations were inoculated with 1×10^6 CFU/ml of an overnight culture and incubated in a 37°C incubator. At 0, 3, 6, 12, and 24 hrs after inoculating bacteria, each bacterial culture solution was diluted 100- or 10,000-folds and plated onto a TH agar plate. The agar plate was incubated in a 37°C incubator for 24 h and the bacterial colonies were counted.

KB cells culture

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

MTT assay

A MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay was performed to test the cytotoxicity of ursolic acid on KB cells. The 80% confluent NHGF cell monolayers in the 24-well plates were incubated with 32, 16, 8, 4, and 2 μg/ml of ursolic acid and 1% DMSO as a control in 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 with a 10% MTT solution (Sigma, USA) and cultured for 3 h in under same culture conditions. Isopropanol (Sigma, USA) was placed in each well at a volume of 300 μl to dissolve the formazan crystals. 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 repeated individually three times. In the experimental group and the control group, all the experimental results were expressed as mean ± SD (SD: standard deviation).

Results

MIC measurements were performed to determine if ursolic acid has an anti-bacterial effect on the type strains of MS (*S. mutans* ATCC 25175^T and *S. sobrinus* ATCC 33478^T). The MIC value of ursolic acid was 2 μg/ml for both *S. mutans* ATCC 25175^T and *S. sobrinus* ATCC 33478^T (Table 1). To determine the appropriate concentration of ursolic acid as an anti-carious agent, the MIC_{50} and MIC_{90} were measured with 40 and 15 clinical strains of *S. mutans* and *S. sobrinus* isolated from the oral cavity of Koreans (Table 2).

Time-kill kinetic studies were performed to determine if the activity of ursonic acid was bactericidal or bacteriostatic. Ursonic acid exhibited bactericidal activity against *S. mutans*

Table 1. Minimum inhibitory concentration of ursolic acid against the clinical isolates of mutans streptococci from Koreans

Species & strains	MIC (μg/ml)	Species & strains	MIC (μg/ml)
S. mutans ATCC[1] 25175^T	2	*S. mutans* KCOM 1200	2
S. mutans KCOM[2] 1054	2	*S. mutans* KCOM 1201	2
S. mutans KCOM 1085	2	*S. mutans* KCOM 1202	2
S. mutans KCOM 1087	2	*S. mutans* KCOM 1203	2
S. mutans KCOM 1087	2	*S. mutans* KCOM 1207	2
S. mutans KCOM 1091	2	*S. mutans* KCOM 1207	2
S. mutans KCOM 1092	2	*S. mutans* KCOM 1209	2
S. mutans KCOM 1095	2	*S. mutans* KCOM 1212	2
S. mutans KCOM 1097	2	*S. mutans* KCOM 1214	2
S. mutans KCOM 1111	2	*S. mutans* KCOM 1217	2
S. mutans KCOM 1112	2	*S. mutans* KCOM 1219	4
S. mutans KCOM 1113	2	*S. mutans* KCOM 1226	2
S. mutans KCOM 1116	2	*S. sobrinus* ATCC 33478^T	2
S. mutans KCOM 1117	2	*S. sobrinus* KCOM 1061	2
S. mutans KCOM 1118	2	*S. sobrinus* KCOM 1150	2
S. mutans KCOM 1123	2	*S. sobrinus* KCOM 1151	2
S. mutans KCOM 1124	2	*S. sobrinus* KCOM 1152	2
S. mutans KCOM 1126	2	*S. sobrinus* KCOM 1153	2
S. mutans KCOM 1127	2	*S. sobrinus* KCOM 1157	2
S. mutans KCOM 1128	2	*S. sobrinus* KCOM 1158	2
S. mutans KCOM 2762	2	*S. sobrinus* KCOM 1159	2
S. mutans KCOM 1136	2	*S. sobrinus* KCOM 1185	4
S. mutans KCOM 1137	2	*S. sobrinus* KCOM 1191	2
S. mutans KCOM 1139	2	*S. sobrinus* KCOM 1193	4
S. mutans KCOM 1142	2	*S. sobrinus* KCOM 1196	4
S. mutans KCOM 1143	2	*S. sobrinus* KCOM 1221	2
S. mutans KCOM 1145	2	*S. sobrinus* KCOM 1228	2
S. mutans KCOM 1146	2	*S. sobrinus* KCOM 1218	2
S. mutans KCOM 1197	2		

ATCC, America Type Culture Collection; KCOM, Korean Collection for Oral Microbiolgoy

Table 2. Inhibitory effect of ursolic acid against mutans streptococci isolated from Koreans

Species (n=59)	Concentration of ursolic acid (μg/ml)		
	MIC_{50}	MIC_{90}	MIC_{100}
S. mutans (n=40)	2	2	4
S. sobrinus (n=15)	2	4	4
Total (n=55)	2	2	4

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

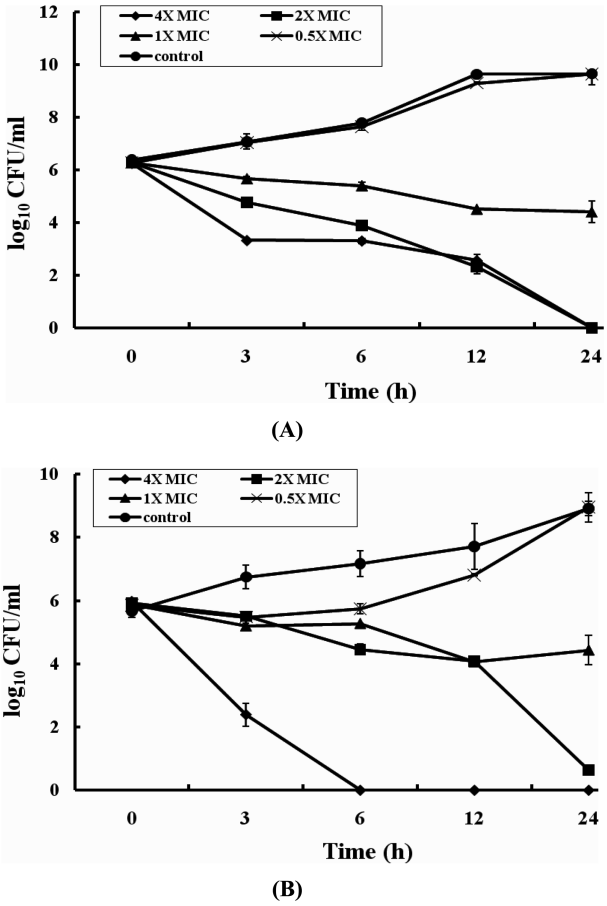

Fig. 1. Time kill curves of ursolic acid on (A) *S. mutans* ATCC 25175^T and (B) *S. sobrinus* ATCC 33478^T at different ursolic acid concentrations.

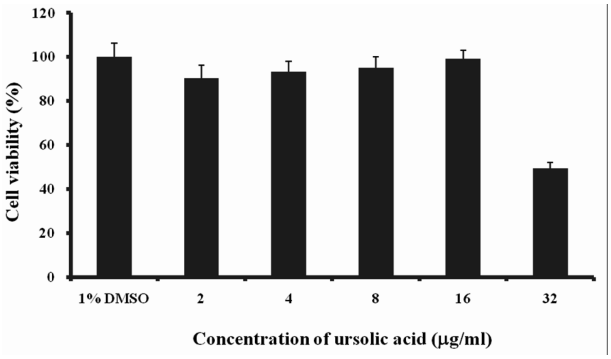

Fig. 2. Cytotoxicity of ursolic acid on KB cells.

ATCC 25175^T and *S. sobrinus* ATCC 33478^T when added to the culture medium at concentrations higher than 4 × MIC and 2 × MIC, respectively, for 24 h (Fig 1).

This study examined whether ursolic acid has a cytotoxic effect on KB cells at the concentration at which ursolic acid has a bactericidal effect on the MS using a MTT assay. The data showed that ursolic acid up to 16 μg/ml (8 × MIC) had no cytotoxic effect on KB cells (Fig 4).

Discussion

The MIC_{50} value of ursolic acid against the clinical strains of MS was the same as that of the type strains of them. The MIC_{90} value against the clinical isolates of *S. sobrinus* was 2 times higher than that for the type strains of *S. sobrinus* (ATCC 33478^T). Twenty percent of the clinical strains of *S. sobrinus* had a higher MIC value than the type strain (ATCC 33478^T). However, the MIC_{90} value for all clinical strains of MS was the same as that of the type strains of *S. mutans* and *S. sobrinus*. The result of the time kill assay suggest that > 8 μg/ml of ursolic acid can be used as an antimicrobial agent to prevent dental caries.

Ursolic acid had no cytotoxic effect on the normal human oral keratinocytes and human gingival fibroblast cells upto 16 μg/ml ursolic acid (data not shown). The cytotoxicity tests for the chemicals or natural extracts on the human cells were performed *in vitro*. Human oral tissue cells *in vivo* could be more resistant to the chemicals than those under *in vitro* conditions because the oral tissue cells *in vivo* can be supplied with nutrients continuously by blood and have a better repair mechanism than those under *in vitro* conditions. Considering these factors, an ursolic acid concentration > 16 μg/ml can be used *in vivo* for the prevention of dental caries. However, the optimal concentration of ursolic acid should be determined by further clinical studies. Nevertheless, these results suggest that ursolic acid can be useful in the development of anticaries agents for humans.

According to previous studies, the MIC values of the clinical strains for the crude natural extract had a broad range (Lim *et al*., 2003; Lee *et al*., 2003). However, in this study, the range of MIC values of ursolic acid on the clinical isolates of MS was relatively narrow, from 2 to 4 μg/ml. The reason for this is unclear but it appears that the susceptibility of MS vary according to crude natural extracts and clinical isolates. Therefore, the antibacterial effects of a single compound on the clinical isolates of MS may be not need to be tested.

In conclusion, ursolic acid at < 16 μg/ml had a bactericidal effect on MS without toxicity to oral tissue cells. Accordingly, ursolic acid can be used for the development of oral hygiene products, such as toothpaste, oral gargling solutions and dental floss.

Acknowledgments

This study was supported by a grant from the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A091074).

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