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Inhibitory Effect of Methyl Gallate and Gallic Acid on Oral Bacteria

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This study examined the ability of methyl gallate (MG) and gallic acid (GA), the main compounds of gallotannins in Galla Rhois, to inhibit the proliferation of oral bacterial and the *in vitro* formation of *Streptococcus mutans* biofilms. The antimicrobial activities of these compounds were evaluated *in vitro* using the broth microdilution method and a beaker-wire test. Both MG and GA had inhibitory effects on the growth of cariogenic (MIC<8 mg/ml) and periodontopathic bacteria (MIC=1 mg/ml). Moreover, these compounds significantly inhibited the *in vitro* formation of *S. mutans* biofilms (MG, 1 mg/ml; GA, 4 mg/ml; *P*<0.05). MG was more effective in inhibiting bacterial growth and the formation of *S. mutans* biofilm than GA. In conclusion, MG and GA can inhibit the growth of oral pathogens and *S. mutans* biofilm formation, and may be used to prevent the formation of oral biofilms.

***Keywords*:** antibacterial activity, Galla Rhois, gallic acid, methyl gallate, oral bacteria

Dental plaque formation is a complex and dynamic process that involves the initial acquisition of an organic film with the subsequent colonization by bacterial species (Marsh and Bradshaw, 1995). There is a clear relationship between the number of dental caries and *Streptococcus mutans* inhabiting the plaque (Keene and Shklair, 1974). The destruction of periodontal tissue due to prolonged inflammation is initiated by bacterial colonization and invasion around the teeth (Nordland *et al.*, 1987).

Herbal materials can be effective agents in controlling the oral pathogens (Estafan *et al.*, 1998). Therefore, the effective suppression of dental plaque formation and periodontal pathogens using herbal composites may be a viable approach for preventing biofilm-induced oral diseases, such as dental caries and periodontal disease. It was suggested that effective substances that provide clinical benefits for plaque control should have an antimicrobial effect on pathogenic bacteria and an inhibitory effect on the adhesive properties of bacteria but with no side effects (Brecx, 1997). Galla Rhois is an outgrowth of plant (*Rhus chinensis* L.) tissue caused by a mite parasite (*Schlechtendalia chinensis* Bell) that is rich in gallotannins, and has been used in traditional Oriental medicine as antibacterial and anti-inflammatory drugs (Namba *et al*., 1993). Several authors have reported the antimicrobial effects of Galla Rhois extracts and Galla Rhois-derived tannins. (Ahn *et al.*, 1994; Ahn *et al.*, 1998; Bae *et al.*, 1998). However, there are few reports of the effects of gallotannins on dental biofilms and oral pathogens. Our previous studies showed that Galla Rhois extracts had significant inhibitory effects on dental biofilms both *in vitro* and *in vivo* (Hong *et al.*, 2004; Cho *et al.*, 2005). Wu-Yuan *et al.* (1988) reported that extracts of *Melaphis chinensis* containing gallotannins, particularly methyl gallate (MG), and gallic acid (GA), had a significant inhibitory effect on the growth, water-soluble glucan synthesis and aggregation of *S. mutans*. Homer *et al*. (1990) compared five chewing stick extracts and tannins including MG and GA on the ability to prevent the proteolytic activities of some periodontopathic bacteria, such as *Bacteroides gingivalis*, *Bacteriodes intermedius*, and *Treponema denticola*. Therefore, MG and GA can be considered the main compounds of gallotannins in Galla Rhois that have inhibitory effects on dental biofilm formation and periodontopathic bacteria. However, previous studies did not report a direct growth-inhibitory effect of MG and GA on oral pathogenic bacteria. Moreover, it is unclear if they have differential growth-inhibitory effect on oral pathogenic bacteria.

This study evaluated and compared the antimicrobial activity of MG and GA against cariogenic and periodontopathic bacteria as well as against the formation of *S. mutans* biofilms.

Materials and Methods

Materials

Methyl gallate (MG), gallic acid (GA), chlorhexidine, and metronidazole were purchased from Sigma (USA).

Bacterial strains and growth conditions

The following bacterial strains were used in this study: *S. mutans* Ingbritt, *S. sobrinus* B-13, *Actinomyces viscosus* ATCC 15988, *Lactobacillus casei* ATCC 334, *L. acidophilus* ATCC 4356, and *L. salivarius* subsp. *salicinius* ATCC 11742 were used as the cariogenic bacteria. *Porphyromonas gingivalis*

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ATCC 33277, *Fusobacterium nucleatum* ATCC 10953, *Prevotella loescheii* ATCC 15930, and *Tannerella forsythia* ATCC 43037 were used as the periodontopathic bacteria. *S. mutans*, *S. sobrinus*, and *A. viscosus* were grown in Brain Heart Infusion broth (BHI broth; Difco, USA). *L. casei*, *L. acidophilus*, and *L. salivarius* subsp. *salicinius* were grown in De Man, Rogosa, Sharpe broth (MRS broth; Difco). The bacteria were incubated at 37°C for 16 h under aerobic conditions. *P. gingivalis*, *P. loescheii*, and *F. nucleatum* were grown in Brucella broth supplemented with yeast extract (1 mg/ml), hemin (10 μg/ml), and menadione (5 μg/ml) (Kang *et al*., 2008). *T. forsythia* was grown in Brucella broth supplemented with a yeast extract (0.5 mg/ml), hemin (5 μg/ml), menadione (0.5 μg/ml), N-acetylmuramic acid (10 μg/ml), and fetal bovine serum (10%). The bacteria were incubated anaerobically (85% N_2, 10% H_2, and 5% CO_2) at 37°C for 48 h.

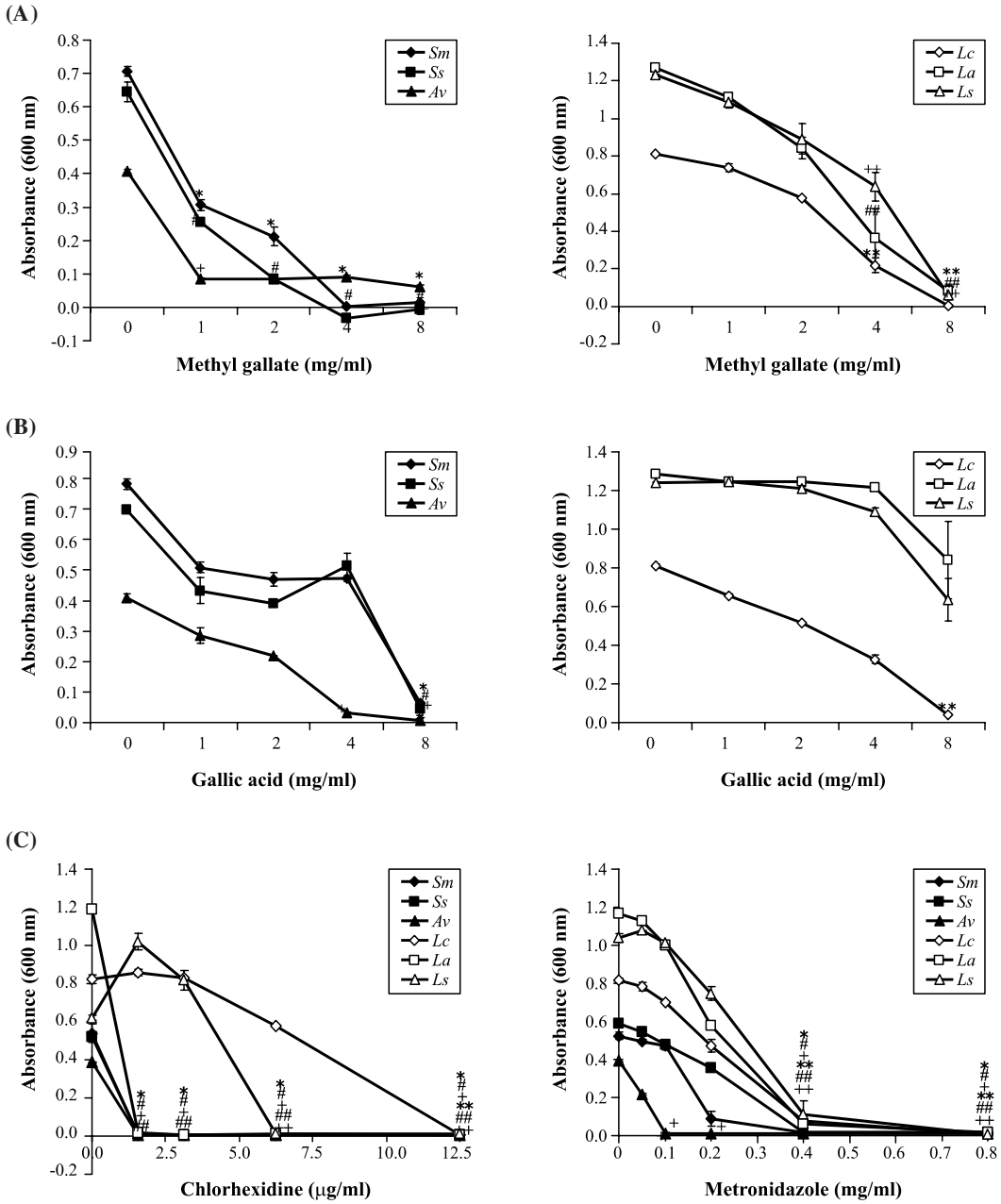

Fig. 1. Determination of the antibacterial activity of MG (A), GA (B), chlorhexidine or metronidazole (C) for cariogenic bacteria. Serial doses of MG and GA were added to the bacterial cultures in 96-well plates and incubated for 24 h. The growth of bacteria was determined by measuring the optical density of the cultures at 600 nm. The data is reported as the Mean±SD of triplicate wells. $^{*}P<0.05$, compound treated culture vs *Sm* control; $^{\#}P<0.05$, compound treated culture vs *Ss* control; $^{+}P<0.05$, compound treated culture vs *Av* control; $^{**}P<0.05$, compound treated culture vs *Lc* control; $^{\#\#}P<0.05$, compound treated culture vs *La* control; $^{++}P<0.05$, compound treated culture vs *Ls* control. *Sm*=*S. mutans*; *Ss*=*S. sobrinus*; *Av*=*A. viscosus*; *Lc*=*L. casei*; *La*=*L. acidophilus*; *Ls*=*L. salivarius.*

The bacteria in the logarithmic growth phase were used in all experiments.

Evaluation of antibacterial activity

The antibacterial activity assays of MG and GA were carried out using a broth microdilution method following the Clinical and Laboratory Standards Institute guidelines (Citron and Hecht, 2003; Jorgensen and Turnidge, 2003). The logarithmic phase cultures of the bacteria were added to the culture medium containing a series of MG and GA dilutions in the wells of microtiter plates, and their growth was assessed after a period of incubation. The inoculum size was controlled by measuring the optical density at 600 nm and extrapolating the CFU/ml using preset standard curves. Successive two-fold dilutions of MG and GA were prepared in a 100 μl volume, and 100 μl each of bacteria was added to the prepared plates. The final inoculum concentrations of the periodontopathic bacteria and cariogenic bacteria were 1×10^6 CFU/ml and 5×10^5 CFU/ml, respectively. The wells of the plates included one growth and one sterile control. Chlorhexidine and metronidazole were used as controls. After incubation under aerobic or anaerobic conditions at 37°C for 24 h, the level of microbial growth was measured using a microplate reader at 600 nm. The minimal inhibitory concentration (MIC) was defined as the lowest dilution of MG and GA that restricted growth to an absorbance of ≤0.1.

Evaluation of inhibitory activity on plaque accumulation

Beaker-wire tests were performed as previously described to determine the effects of MG and GA on *in vitro* plaque accumulation (Kang *et al.*, 2006). Briefly, MG or GA was added at various concentrations (0.0625 mg/ml, 0.25 mg/ml, 1 mg/ml, and 4 mg/ml) to BHI medium (pH 6.5) containing 5% sucrose, 0.5% yeast extract (Difco), and 0.1 M of MES (2-[N-Morpholino] ethanesulfonic acid monohydrate). *S. mutans* (1×10^6 CFU/ml) was inoculated, and three stainless steel wires (Dentaurum, Germany) were hung on the lid and immersed into each beaker and incubated under slow agitation at 37°C for 24 h. The wires were weighed, and the wet weights of plaque accumulation on the wires were determined by subtracting the weight of the wire.

Adherence inhibition assay

Adherence inhibition assays were done using the protocol of Loo *et al*. (2000). Briefly, BHI broth containing 2% sucrose and various concentrations of MG or GA (from 0.0625 to 4 mg/ml) was inoculated with overnight cultures of *S. mutans*. Controls consisted of cells grown in BHI broth with sucrose only. Media and non-adherent bacterial cells were decanted from the wells, and the remaining loosely bound cells were removed by rinsing with distilled water twice. The plates were then blotted on paper towels and air dried, and adherent bacteria were stained with 50 μl of 0.1% crystal violet for 15 min at room temperature. After rinsing twice with 200 μl of distilled water each time, bound dye was extracted from the stained cells by using 200 μl of 99% ethanol. The adherent bacteria were measured using a microplate reader at 595 nm.

Cytotoxicity of MG and GA

Cytotoxicity of MG and GA were assessed by a modified MTT assay (Mosmann, 1983). KB cells, a human mouth epithelial cell line, were grown in 5% CO_2 at 37°C in minimal essential medium with Earle's salts (Hyclone, USA) containing 2 mmol/L L-glutamine (MEM), 10% fetal bovine serum and 50 μg/ml gentamicin. KB cells were treated with MG or GA (1~8 mg/ml) for 24 h. Cultures of the control group were left untreated. MTT solution (5 mg/ml) was added to each well culture plate which was then covered with aluminum foil for 4 h at 37°C under 5% CO_2. Consecutively, the medium was removed and DMSO was added to extract the MTT formazan. An automatic microplate reader read the absorbance of each well at 540 nm.

Statistics

Each experiment was carried out in triplicate and the mean value was analyzed further. Statistical analysis was conducted using SPSS version 12.0 (Statistical packages for Social Science version 12.0; SPSS Inc., USA). A Mann-Whitney test was used to compare the groups to identify any statistically significant differences in the experiments.

Results

Determination of antibacterial activity of MG and GA for cariogenic bacteria

The antibacterial activity of MG and GA against *S. mutans*, *S. sobrinus*, *A. viscosus*, *L. casei*, *L. acidophilus*, and *L. salivarius* were determined using a broth microdilution method. As shown in Fig. 1A, the growth of *A. viscosus* was inhibited completely by a low dose of MG (MIC=1 mg/ml). *S. mutans* and *S. sobrinus* showed intermediate sensitivity to MG (MIC=2~4 mg/ml), whereas the growth of *Lactobacillus* spp. was inhibited completely at a relatively high concentration (MIC=8 mg/ml). Fig. 1B shows the results of the MIC of GA for all the bacterial strains. The MIC of GA for *A. viscosus*, *S. mutans*, and *S. sobrinus* were 4 mg/ml, 8 mg/ml, and 8 mg/ml, respectively. While the MIC of GA for *L. casei* was 8 mg/ml, both *L. acidophilus* and *L. salivarius* showed a low sensitivity to GA (MIC>8 mg/ml). Chlorhexidine and metronidazole also showed strong antimicrobial activities against cariogenic bacteria (Fig. 1C). *A. viscosus*, *S. mutans*, and *S. sobrinus* showed a very high sensitivity to chlorhexidine (MIC=1.56 μg/ml). *Lactobacillus* spp. also showed a high sensitivity to chlorhexidine (*L. acidophilus*, 1.56 μg/ml; *L. salivarius*, 6.25 μg/ml; *L. casei*, 12.5 μg/ml). The MIC of metronidazole for *A. viscosus*, *S. mutans* and *S. sobrinus* were 0.1 mg/ml, 0.2 mg/ml, and 0.4 mg/ml, respectively. The MIC of metronidazole for *L. casei*, *L. acidophilus*, and *L. salivarius* were 0.4 mg/ml, 0.4 mg/ml, and 0.8 mg/ml, respectively.

Determination of the antibacterial activity of MG and GA for periodontopathic bacteria

The antibacterial activity of MG and GA against *P. gingivalis*, *F. nucleatum*, *P. loescheii*, and *T. forsythia* was also determined. As shown in Fig. 2A, MG at low doses (MIC=1 mg/ml) completely inhibited the growth of all bacteria ($P<0.05$). GA at low doses (MIC=1 mg/ml) also significantly

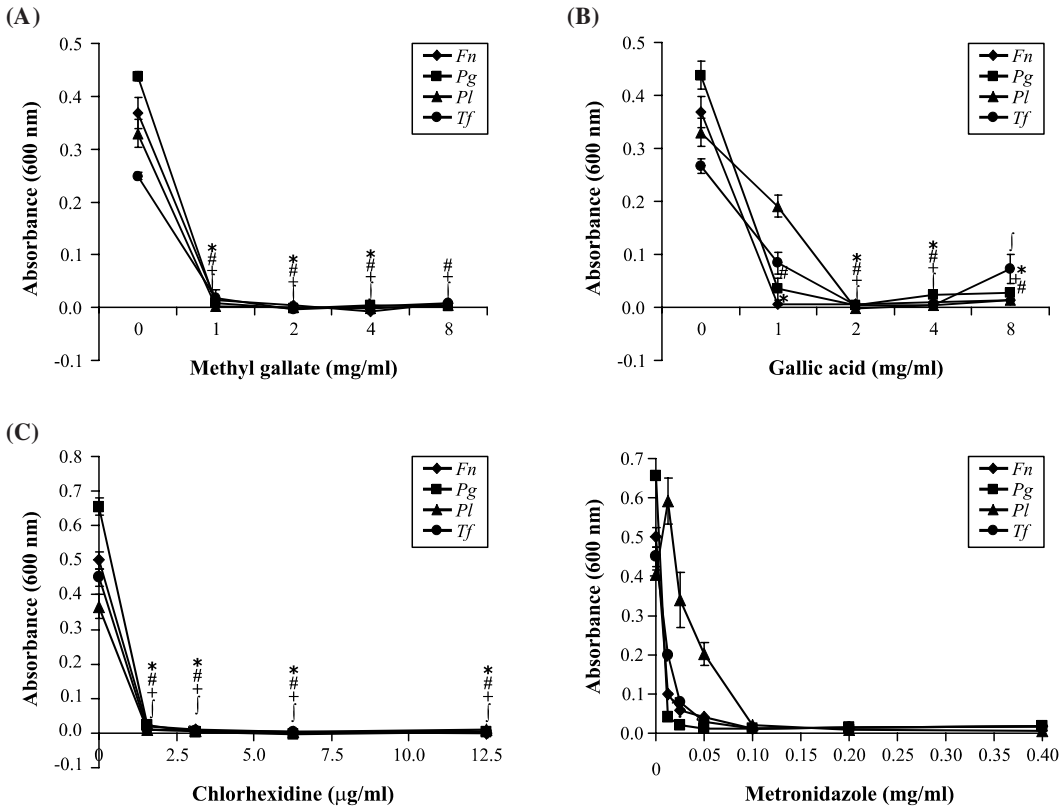

Fig. 2. Determination of the antibacterial activity of MG (A), GA (B), chlorhexidine or metronidazole (C) for periodontopathic bacteria. Serial doses of MG and GA were added to the bacterial cultures in 96-well plates and incubated for 24 h. The growth of bacteria was determined by measuring the optical density of the cultures at 600 nm. The data is reported as the Mean±SD of triplicate wells. $^{*}P<0.05$, compound treated culture vs *Fn* control; $^{\#}P<0.05$, compound treated culture vs *Pg* control; $^{+}P<0.05$, compound treated culture vs *Pl* control; $^{\int}P<0.05$, compound treated culture vs *Tf* control. *Fn=F. nucleatum*; *Pg=P. gingivalis*; *Pl=P. loescheii*; *Tf=T. forsythia.*

inhibited the growth of all bacteria, whereas the MIC of *P. loescheii* was 2 mg/ml ($P<0.05$) (Fig. 2B). Chlorhexidine and metronidazole also showed strong antimicrobial activities against periodontopathic bacteria. All tested periodontopathic bacteria showed a very high sensitivity to chlorhexidine (MIC=1.56 μg/ml). The MIC of metronidazole for *P. gingivalis*, *F. nucleatum*, and *T. forsythia* were 12.5 to 25 μg/ml, whereas for *P. loescheii* was 0.1 mg/ml (Fig. 2C).

Inhibitory effects of MG and GA on *S. mutans* biofilm formation

The effect of MG and MA on the formation of *S. mutans* biofilms was evaluated using beaker-wire tests. The addition of MG and GA reduced the level of *S. mutans* biofilm formation, which was dependent on the concentration of these tannins (Fig. 3). Each wet weight of the artificial biofilm on the wires was reduced significantly by addition of MG (1 mg/ml and 4 mg/ml) and GA (4 mg/ml) ($P<0.05$). The weights of *S. mutans* biofilm (mg) were as follows: control (198.5±51.8); MG (1 mg/ml, 41.6±11.8; 4 mg/ml, 1.7±0.6) and GA (1 mg/ml, 170.2±29.1; 4 mg/ml, 61.2±21.7). At 4 mg/ml, both MG and GA significantly inhibited the formation of the artificial *S. mutans* biofilms by 79.0% and 69.2%, respectively ($P<0.05$).

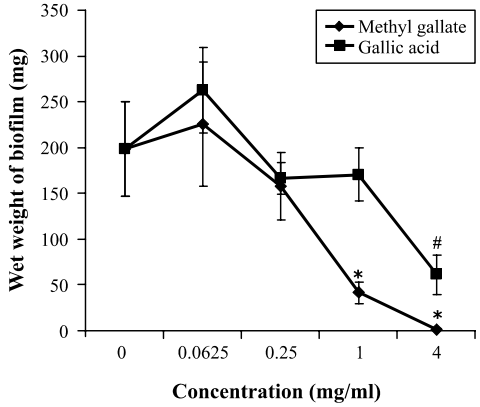

Fig. 3. Effect of MG and GA on *in vitro* plaque accumulation. MG or GA was added at various concentrations to the *S. mutans* cultures. The level of plaque accumulation on the wires immersed in the cultures was measured. The data is reported as the Mean ±SD of the results from three replicate experiments. $^{*}P<0.05$, MG treated culture *versus* control; $^{\#}P<0.05$, GA treated culture *versus* control.

Table 1. Inhibitory effects of MG and GA on *in vitro* adherence

Conc. (mg/ml)	Adherence inhibition on *S. mutans* (% of control)	
	MG	GA
0 (control)	0.0±0.0	0.0±0.0
0.0625	20.1±8.0	-16.3±9.5
0.25	-5.0±3.6	-6.0±0.1
1	87.2±0.3[a]	2.7±4.6
2	88.5±0.5[a]	80.3±2.0[a]
4	94.4±1.2[a]	97.0±0.4[a]

Data are expressed at the Mean±SD of a representative experiment performed in triplicate. [a] $P<0.05$ as compared with control.

Inhibitory effects of MG and GA on *in vitro* adherence

In vitro adherence of *S. mutans* was significantly inhibited by addition of MG (1∼4 mg/ml) and GA (2∼4 mg/ml) compared to control ($P<0.05$). More than 80% inhibition was achieved at MG (1 mg/ml) and GA (2 mg/ml). At a concentration from 1 to 4 mg/ml, both MG and GA inhibited adherence of *S. mutans* in a dose-dependent manner (Table 1).

Cytotoxicity

Cell viability was measured using MTT assay. The viabilities of KB cells incubated with MG at concentrations of 1, 2, 4 and 8 mg/ml for 24 h were 100.0±6.0%, 110.0±10.9%, 95.0 ±1.6%, and 90.0±7.2% of the control value, respectively. The viabilities of KB cells with GA (1∼8 mg/ml) for 24 h were 94.6±5.1%, 90.0±3.7%, 50.0±0.2%, and 45.0±0.9% of the control value, respectively. No obvious cytotoxic effects of MG (1∼8 mg/ml) were observed. However, the presence of GA (4∼8 mg/ml) significantly decreased cell viability ($P<0.05$) (Fig. 4).

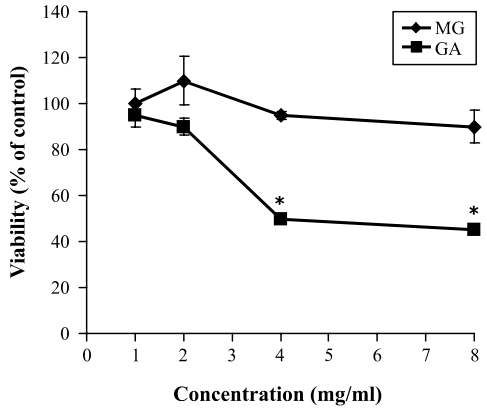

Fig. 4. Cytotoxicity of MG and GA on KB cells. KB cells were treated with MG and GA (1, 2, 4 or 8 mg/ml) for 24 h. Untreated cells were used as a control. Cell viability was assessed by an MTT assay. Data are expressed at the Mean±SD of a representative experiment performed in triplicate. *$P<0.05$ for GA treated culture vs control.

Discussion

It was reported that *S. mutans* insoluble glucan is a major component in the development of dental caries. Moreover, *S. mutans* can colonize the tooth enamel surface and initiate plaque formation by their ability to make extracellular polysaccharides from sucrose using glucosyltransferase (Gibbons and Houte, 1975; Hamada and Slade, 1980; Jacquelin *et al.*, 1995). Considerable efforts have been made to inhibit the formation of *S. mutans* biofilm using a variety of antibacterial agents and chemicals, including chlorhexidine, triclosan, sanguinarine, and fluoride (Shani *et al*., 2000; Shapiro *et al*., 2002).

Several authors have suggested that effective antimicrobial agents from herbal extracts along with their derived materials against these cariogenic and periodontopathic bacteria can form an important part preventing dental caries and periodontal disease, particularly those compounds that affect dental plaque formation (Watanabe *et al.*, 2000; Kim *et al.*, 2008).

In this study, both MG and GA, which are the main compounds in the gallotannins of Galla Rhois, showed antibacterial activity against all the cariogenic organisms tested, whereas some *Lactobacillus* spp. showed low sensitivity to GA. Among the cariogenic bacteria tested, *A. viscosus* was most susceptible to these compounds. *A. viscosus* may play an important role in the formation of supragingival plaque on the primary teeth of children (Tang *et al.*, 2003). In addition, MG exhibited more potent inhibitory activity against the growth of oral pathogens and the formation of *S. mutans* biofilms than GA.

In this study, MG and GA at relatively low doses also inhibited the growth of all the periodontopathic bacteria tested. This result is supported by Homer *et al.* (1990), who reported that low concentrations of MG and GA inhibited the growth of three periodontopathic bacteria including *P. gingivalis*. As the periodontopathic bacteria used in this study are considered to play major roles in periodontitis and dentoalveolar infections (Loesche *et al.*, 1985; Slots and Ting, 1999), it is important to control these bacteria in order to prevent periodontal disease. Although MG and GA showed less powerful antimicrobial effect than positive controls, they showed significant effectiveness on antimicrobial activity against both cariogenic and periodontopathic bacteria tested comparing with negative control. Therefore, MG and GA may be considered useful antimicrobial agents against oral pathogens. GA is well known for its antimicrobial properties (Saxena *et al*., 1994; Sato *et al*., 1997). With regard to the antibacterial activity of some alkyl esters of GA, it was reported a major dependence of relative antimicrobial activity on their relative lipophilic properties, suggesting a possible site of action within or internal to the cytoplasmic membrane (Boyd and Beveridge, 1981). Mechanisms of action might be proposed to explain MG or GA antimicrobial activities including inhibition of extracellular microbial enzymes required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation. Anti-adhesion might be one mechanism in action in antimicrobial activity of MG and GA in general. In this study, the *in vitro* adherence of *S. mutans* was inhibited by MG and GA.

Although Galla Rhois has been used as a traditional oriental medicine, the safety of MG and GA to the oral tissues has not been tested. In this study, MG was more effective antimicrobial agents than GA. In addition, no obvious cytotoxic effects, following treatment of KB cells with MG for 24 h, were detected by an MTT test, thus indicating that therapeutic use of MG for dental treatment without any serious adverse effects may be recommended. However, GA at final concentrations of 4 and 8 mg/ml showed cytotoxicity. Further study using an *in vivo* model will be needed to evaluate the efficacy and safety for oral use. In addition, determining the action mechanisms of these compounds will be pivotal to the future therapeutic use of MG.

In conclusion, MG and GA can inhibit the growth of cariogenic and peiodontopathic bacteria as well as the *in vitro* formation of *S. mutans* biofilms. This suggests that MG may be good candidates for controlling dental caries and periodontal disease.

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