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ORIGINAL ARTICLE

Anti-biofilm and bactericidal effects of magnolia bark-derived magnolol and honokiol on *Streptococcus mutans*

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ABSTRACT

Dental caries affects people of all ages and is a worldwide health concern. *Streptococcus mutans* is a major cariogenic bacterium because of its ability to form biofilm and induce an acidic environment. In this study, the antibacterial activities of magnolol and honokiol, the main constituents of the bark of magnolia plants, toward planktonic cell and biofilm of *S. mutans* were examined and compared with those of chlorhexidine. The minimal inhibitory concentrations of magnolol, honokiol and chlorhexidine for *S. mutans* were 10, 10 and 0.25 μg/mL, respectively. In addition, each agent showed bactericidal activity against *S. mutans* planktonic cells and inhibited biofilm formation in a dose- and time-dependent manner. Magnolol (50 μg/mL) had greater bactericidal activity against *S. mutans* biofilm than honokiol (50 μg/mL) and chlorhexidine (500 μg/mL) at 5 min after exposure, while all showed scant activity against biofilm at 30 s. Furthermore; chlorhexidine (0.5–500 μg/mL) exhibited high cellular toxicity for the gingival epithelial cell line Ca9-22 at 1 hr, whereas magnolol (50 μg/mL) and honokiol (50 μg/mL) did not. Thus; it was found that magnolol has antimicrobial activities against planktonic and biofilm cells of *S. mutans*. Magnolol may be a candidate for prevention and management of dental caries.

Key words honokiol, magnolia bark, magnolol, *Streptococcus mutans*.

Dental caries is an infectious disease caused by bacterial colonization of tooth surfaces. In spite of promotion of oral health care worldwide, dental caries is considered the most prevalent human disease, affecting 80–90% of the world's population (1). Severe caries can progress to pulpitis, apical periodontitis, and even loss of teeth. Furthermore, systemic diseases, such as cardiovascular diseases, can be induced as a result of caries progression (2, 3). Therefore, detection and treatment of caries is generally considered very important. However, in recent years, interest has shifted from treatment to prevention and use of fluorides and reduced sugar intake have been shown to dramatically decrease the prevalence and severity of dental caries. These approaches are undoubtedly major means of reducing dental caries.

Streptococcus mutans, a gram-positive bacterium that resides in the oral cavity, is the primary cause of formation of dental caries (4, 5). *S. mutans* has a greater ability to form biofilm, known as dental plaque, than other species and also metabolizes various carbohydrates into lactic acid (6). However, it is not possible to neutralize its activities within biofilm because the limited access for saliva and subsequent low pH environment contribute to demineralization of tooth enamel, leading

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List of Abbreviations: BHI-S, brain heart infusion containing 0.5% sucrose; CHX, chlorhexidine.

to cariogenic destruction of teeth (7). Therefore, inhibition of *S. mutans* by controlling plaque is essential for successful control and prevention of dental caries (8).

Plaque control is the most important factor for maintaining an appropriate and healthy oral environment. Although tooth brushing is generally regarded as the best means of mechanical plaque control, chemical control with antimicrobial agents such as CHX is often used for regions that are difficult to reach with a toothbrush. However, because of its inherent ability to resist antibiotics and antimicrobial rinses, most such agents are largely ineffective against biofilm. In addition, the minimal inhibitory and minimal bactericidal concentrations of antibiotics for effects on biofilm-growing bacteria are reportedly up to 100–1000-fold higher than for planktonic bacteria (9). Therefore, researchers have investigated agents that may be capable of sterilizing biofilm cells.

There are various formulations, including mouth rinses, gels and toothpastes, for delivering CHX to actively treat periodontal disease and prevent progression of caries. This antiseptic agent has superior activity for both sterilizing and inhibiting oral microorganisms and that activity is long-lasting because of its ability to adsorb onto the pellicles of the enamel surfaces of teeth (10). However, CHX is reportedly cytotoxic for human gingival fibroblasts and gingival epithelial cells *in vitro* (11). Moreover, long-term use of CHX may stain the teeth, change taste sensation, and even result in anaphylactic reactions (12–14). Therefore, medicinal plants with fewer and less severe adverse reactions have recently been utilized to achieve oral hygiene in place of CHX (15–18).

Magnolia bark, a herbal material obtained from *Magnolia officinalis* and other species of the Magnoliaceae family, has been used for centuries in traditional Chinese medicines and Japanese remedies for anxiety, sleeping disorders and allergic diseases. Magnolia bark extract produced from dried stems, roots or branch bark of *M. officinalis* is also a constituent of some dietary supplements and cosmetic products (19). Magnolol and honokiol, the most abundant antimicrobial constituents of magnolia bark, reportedly have antimicrobial effects on oral bacteria, including *S. mutans* (20). However, there are few reports about the bactericidal activities of magnolol and honokiol against biofilm.

The purpose of this study was to investigate the anti-biofilm and bactericidal effects of magnolol and honokiol on *S. mutans* biofilm.

MATERIALS AND METHODS

Bacterial culture and reagents

S. mutans MT8148 was grown statically at 37°C in BHI broth (Becton Dickinson, Sparks, MD, USA) under aerobic conditions for 24 hr. The overnight cultures were inoculated into fresh BHI until bacterial growth had reached the exponential growth phase (OD 0.4 at 620 nm), then used for planktonic and biofilm assays. Magnolia bark methanol extract, magnolol and honokiol were prepared as previously described (21) and dissolved in DMSO for use in the experiments. For comparison, CHX digluconate solution (Sigma-Aldrich, St. Louis, MO, USA) was also used.

Antimicrobial activities of magnolia bark methanol extract, magnolol and honokiol

The inhibitory activities of magnolia bark methanol extract, magnolol and honokiol against bacterial growth and bacterial biofilm formation were examined using 96-well plates (Corning, Corning, NY, USA). Ten microliter aliquots of *S. mutans* grown to the exponential phase were inoculated into 200 μL of BHI broth for planktonic assays and BHI-S for biofilm assays. Magnolia bark methanol extract, magnolol, honokiol and CHX were separately added to these bacterial cultures and incubated at 37°C for 24 hr. The inhibitory activity against bacterial growth was measured at a wavelength of 620 nm using a microplate reader (Thermo Fisher Scientific, Kanagawa, Japan). Inhibition of biofilm formation in the static biofilm inhibition assays was analyzed by utilizing crystal violet staining, the OD being measured at 571 nm.

Bactericidal activity of magnolol and honokiol against planktonic cells and biofilm

The bactericidal activities of the agents against planktonic cells were analyzed using standard plating methods. Magnolol (10, 20 and 50 μg/mL), honokiol (10, 20 and 50 μg/mL) and CHX (0.5, 20, 50 and 500 μg/mL) were added to *S. mutans* culture media for 30 s, 5 min and 1 hr, then diluted and inoculated onto Mitis–Salivalius agar plates (Becton, Dickinson) and then incubated under aerobic conditions at 37°C for 2–3 days.

To assess the bactericidal activities of magnolol and honokiol against biofilm, *S. mutans* was incubated at 37°C for 24 hr in BHI-S broth, during which biofilms formed on the bottoms of the glass dishes (Greiner Bio-one GHBH, Frickenhausen, Germany). The biofilms were washed with PBS to remove unbound cells, then treated for 30 s with magnolol (50 μg/mL), honokiol (50 μg/mL) or CHX (50, 500 μg/mL) or for 5 min with these agents or CHX (1200 μg/mL). Next, the biofilms were stained for 15 min at room temperature in the dark using a LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Eugene, Oregon, USA), according to the

manufacturer's instructions, and then observed using a Zeiss LSM700 scanning laser confocal microscope with ZEN image software (Carl Zeiss MicroImaging GHBH, Jena, Germany). Additionally, biofilms treated with these agents for 5 min were detached from the dishes by scraping and dispersed in PBS by vortexing for 120 s, after which viability was assessed by plating the bacteria on Mitis–Salivarius agar plates.

Cellular toxicity of magnolol and honokiol for a human gingival cell line

The human gingival cell line Ca9-22 was purchased from RIKEN Bioresource Center (Ibaraki, Japan). The cells were grown to 90% confluence in minimum essential medium (Life Technologies, Grand Island, NY, USA) containing 10% FBS (Japan Bio Serum, Hiroshima, Japan) and 1% penicillin–streptomycin (Wako Pure Chemical Industries, Osaka, Japan) at 37°C in 5% CO_2, then seeded at a density of 2×10^5 cells/mL into 96-well plates. Once the cells had reached 80–90% confluence, they were carefully washed with PBS and treated with magnolol (10, 20, 50 μg/mL), honokiol (10, 20, 50 μg/mL), or CHX (0.5, 20, 50, 500 μg/mL) for 5 min or 1 hr. After treatment, the cells were carefully washed with PBS to remove any residual activities of the agent and cellular viability was assessed using a methyl thiazolyl tetrazolium assay. The OD of the colored solution was quantified at a wavelength of 571 nm using a microplate reader.

Statistical analysis

Data were analyzed statistically by one-way analysis of variance with Tukey's or Dunnett's multiple comparisons test using Graph Pad Prism Software ver. 6.05 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Magnolia bark methanol extract, magnolol and honokiol inhibit S. mutans growth and biofilm formation in a dose-dependent manner

Magnolia bark methanol extract significantly inhibited the growth of *S. mutans* MT8148 with a minimal inhibitory concentration of 40 μg/mL (Fig. 1a). In addition, concentrations >30 μg/mL showed inhibitory activity against biofilm formation (Fig. 1b). Therefore, we next examined the activities of magnolol and honokiol, the main antibacterial constituents of magnolia bark, against *S. mutans*. Both inhibited *S. mutans* growth and biofilm formation in a dose-dependent manner (Fig. 2). CHX showed those activities at a lower

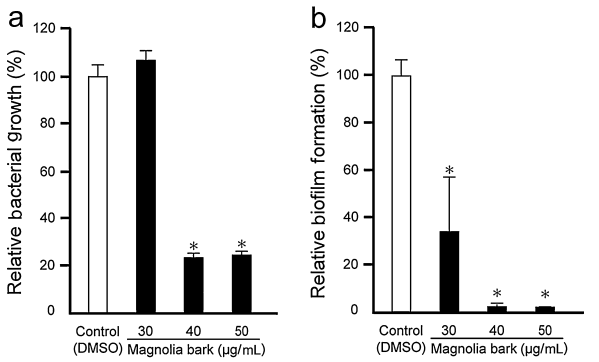

Fig. 1. Inhibitory effects of magnolia bark methanol extract on growth and biofilm formation of *S. mutans*. (a) *S. mutans* was inoculated into BHI broth and cultured with various concentration of magnolia bark methanol extract for 24 hr at 37°C. The OD of each well was measured at 620 nm. (b) *S. mutans* was inoculated into BHI-S broth and cultured with various concentrations of magnolia bark methanol extract for 24 hr at 37°C. Biofilms formed on well surfaces were stained with crystal violet and then eluted using acetic acid. The OD of each well was then measured at 571 nm. Results are shown as the mean ± S.D. of quadruplicate determinants. $^*P < 0.05$, as compared with control.

concentration than did magnolol and honokiol. The minimal inhibitory concentrations of magnolol, honokiol and CHX were 10, 10 and 0.25 μg/mL, respectively.

Magnolol and honokiol exert bactericidal effects

We speculated that the inhibitory effects of both magnolol and honokiol on bacterial growth and biofilm formation are attributable to their bactericidal activity; thus, we performed colony counts. As shown in Figure 3, both components exerted bactericidal activity against *S. mutans* planktonic cells in a dose- and time-dependent manner. Treatment with magnolol or honokiol at concentrations of 50 μg/mL resulted in a greater than 90% decrease in viable *S. mutans* at 5 min and 1 hr after exposure. However, no significant bactericidal activity was seen after 30 s. In contrast, 500 μg/mL (0.05%) of CHX, which is the maximum concentration used in Japan, showed significant bactericidal activity at 30 s.

Magnolol penetrates biofilm and sterilizes S. mutans organisms

Biofilm is known to be resistant to antibiotics because of their poor penetration, thus we examined whether the tested antibacterial agents are effective against *S. mutans* biofilm. Magnolol, honokiol and CHX did not produce visible reduction in biofilm mass at 30 s or 5 min after treatment (Fig. 4a–m). Furthermore, these agents demonstrated scant bactericidal activity at 30 s

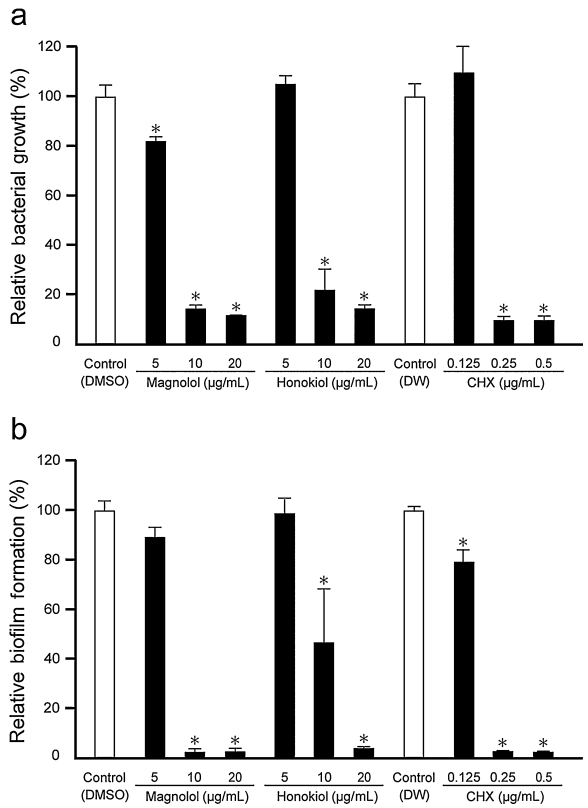

Fig. 2. Inhibitory effects of magnolol, honokiol, and CHX on growth and biofilm formation of *S. mutans*. (a) *S. mutans* was inoculated into BHI broth and cultured with various concentrations of magnolol, honokiol or CHX for 24 hr at 37°C. The OD of each well was measured at 620 nm. (b) *S. mutans* was inoculated into BHI-S broth and cultured with various concentrations of magnolol, honokiol or CHX for 24 hr at 37°C. Biofilms formed on well surfaces were stained with crystal violet and eluted using acetic acid. The OD of each well was then measured at 571 nm. Results are shown as the mean ± S.D. of quadruplicate determinants. $^*P < 0.05$, as compared with control. DW, distilled water.

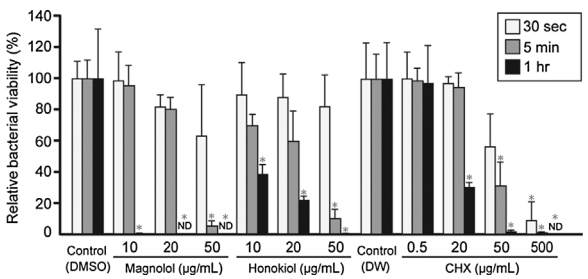

Fig. 3. Bactericidal effects of magnolol and honokiol. *S. mutans* was inoculated into BHI broth and cultured with various concentrations of magnolol, honokiol or CHX for 30 s, 5 min or 1 hr at 37°C. The percent of viable bacteria was determined by colony count. Results are shown as the mean ± S.D. of quadruplicate determinants. $^*P < 0.05$, as compared with control for each time point. ND, not detected.

(Fig. 4a–f). However, magnolol demonstrated greater bactericidal activity against the bottom of biofilm than did 500 and even 1200 μg/mL of CHX at 5 min (Fig. 4h, l, m). The bactericidal activity of honokiol against *S. mutans* biofilm at 5 min was weaker than that of magnolol (Fig. 4i). Consistent with these findings, as shown in Figure 4n, magnolol exhibited significant higher bactericidal activity than CHX and decreased viable *S. mutans* in biofilm by more than 99% at 5 min.

Magnolol and honokiol are less cytotoxic than CHX for a human gingival cell line

Next, we examined the cytotoxicity of magnolol, honokiol and CHX for the human gingival cell line Ca9-22. CHX exhibited significant cytotoxicity against those cells in a dose- and time-dependent manner (Fig. 5). We found that CHX at a concentration of 500 μg/mL decreased viable gingival cells by more than 95% after only 5 min, suggesting that CHX kills not only bacteria but also host epithelial cells. On the other hand, we observed no significant decrease in viability of magnolol- and honokiol-treated gingival cells for up to 1 hr after exposure.

DISCUSSION

Magnolia bark has received great attention for its pharmacological features, such as its anti-cancer (22), anti-inflammatory (23), anti-oxidant (24), anti-Alzheimer (25) and anti-atherosclerosis (26) effects, and is used for treatment of various diseases. Magnolol and honokiol, the main antimicrobial constituents of magnolia bark, reportedly display antimicrobial effects against a variety of oral bacteria such as *Staphylococcus aureus*, *Porphyromonas gingivalis* and *Enterococcus faecalis* (27, 28). Moreover, it has been reported that magnolol inhibits glucosyltransferase produced by *Streptococcus milleri* (29). However, few studies have investigated whether these extracts have anti-biofilm effects. In the present study, we found that magnolol (>10 μg/mL) and honokiol (>10 μg/mL) not only have bactericidal effects on planktonic *S. mutans*, but also anti-biofilm effects, the latter being attributable to their bactericidal effects.

Extracellular material produced by microorganisms, termed matrix, accounts for over 90% of formed biofilms and consists of extracellular polymeric substances, mainly polysaccharides, proteins, extracellular DNA and lipids, which prevent the penetration of antimicrobial agents (30). Many studies have therefore focused on the bactericidal activity of antimicrobial agents against

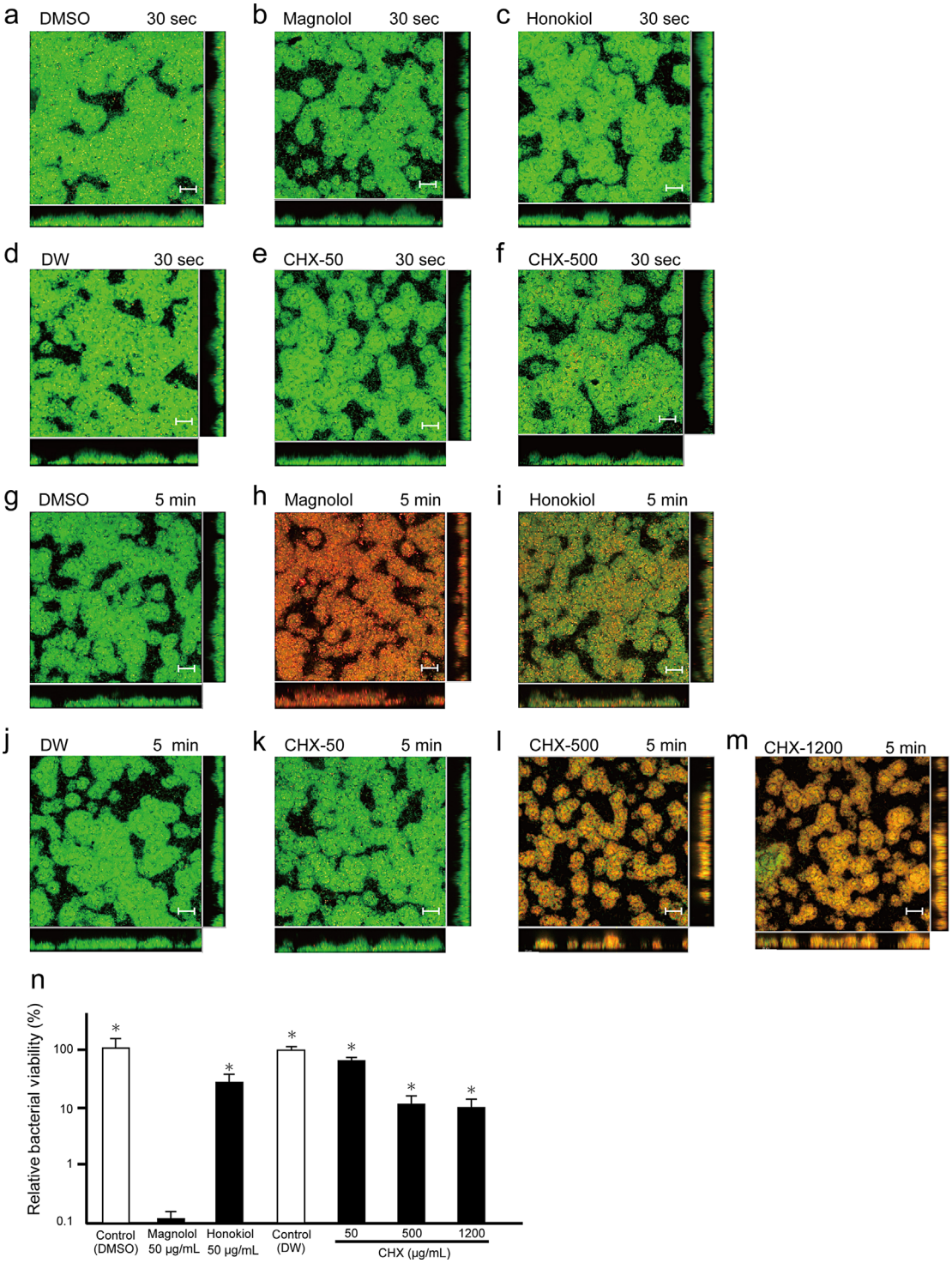

Fig. 4. Bactericidal activity of magnolol and honokiol toward *S. mutans* biofilm shown by fluorescent microscopic analysis. Bacteria were labeled using a Live/Dead staining kit: live bacteria appear fluorescent green (SYTO 9) and dead bacteria fluorescent red (propidium iodide). All samples were assessed using a confocal laser scanning microscope. Samples were treated for (a–f) 30 s or (g–l) 5 min with (a, g) 0.5% DMSO, (d, j) DW; (b, h), 50 μg/mL of magnolol; (c, i), 50 μg/mL of honokiol; (e, k), 50 μg/mL of CHX, (f, l), 500 μg/mL of CHX; and (m) 1200 μg/mL of CHX. Biofilm bottom and side views are presented. All independent experiments were performed three times and representative images are shown. Scale bar, 10 μm. The percent of viable bacteria in biofilm treated with the agents for 5 min was determined by plating bacteria in Mitis–Salivarius agar (n). Results are shown as the mean ± S.D. of quintuplicate determinants. $^{*}P < 0.05$, as compared with magnolol. DW, distilled water.

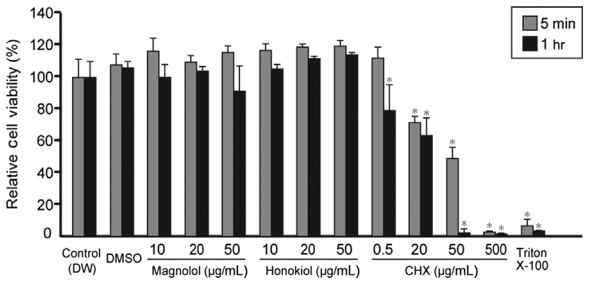

Fig. 5. Cytotoxic effects of magnolol, honokiol and CHX. Human gingival cell line Ca9-22 were grown to 90% confluence in minimum essential media at 37°C in 5% CO_2, then treated with various concentrations of magnolol, honokiol and CHX for 5 min or 1 hr. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to determine cell viability. Results are shown as the mean ± S.D. of quadruplicate determinants. $^*P < 0.05$, as compared with control.

biofilm. For example, cationic agents such as CHX kill bacteria more effectively than anionic or non-ionic agents because cationic agents are readily attracted to the negatively charged microbial cell surface (31). On the other hand, it has also been reported that mass diffusion in biofilm is affected by molecular weight and electrical charge (32–34). Cationic agents have low diffusibility because of their binding ability (35, 36), suggesting that CHX requires a prolonged reaction time to sterilize biofilm (37). In the present study, CHX did not show a sufficient bactericidal effect against the bottom of the biofilm or the surface layer for up to at least 5 min after exposure. Although magnolol had a negligible effect on disrupting matured biofilm, such as degradation of *S. mutans*-derived extracellular matrix, it showed greater bactericidal action against the bottom of the biofilm after 5 min than did CHX.

Our previous study demonstrated that *S. mutans* biofilm is less permeable to macromolecular than to low molecular weight substances and that non-ionic agents have greater penetration than cationic or anionic agents (Sakaue Y *et al*., 2013, unpublished data). Magnolol is a non-ionic agent of molecular weight 266.33 g/mol, which is lower than that of CHX (505.446 g/mol). This may explain, at least in part, why magnolol was more effective toward the bottom of *S. mutans* biofilm than CHX in the present experiment.

Low toxicity is desirable for antimicrobial agents utilized in the mouth. Magnolia bark has low genotoxicity and even has an anticlastogenic effect *in vivo* (38, 39), as well as having low toxicity for human epithelial cells and fibroblasts (40, 41). In our study, magnolol and honokiol were both much less toxic for gingival epithelial cell lines than CHX, at least up to 1 hr after exposure. These results suggest that it is safe to use these extracts in the mouth.

In conclusion, we found that magnolol shows good penetration of and a bactericidal effect on *S. mutans* biofilm, as well as low toxicity for gingival epithelial cells compared with CHX. However, because oral biofilms are not formed by a single species of bacterium and other caries-associated bacteria have been reported, our *in vitro* biofilm model may not reflect actual oral biofilm. Although further investigation is required to determine the bactericidal activities of magnolol toward biofilm formed by multiple oral bacteria, its remarkable bactericidal effects on *S. mutans* biofilm suggest that this novel plant extract agent may help in prevention and management of dental caries.

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DISCLOSURE

None of the authors have conflicts of interest associated with this study to report.

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