Essential Oil of *Curcuma longa* Inhibits *Streptococcus mutans* Biofilm Formation

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Abstract: *Curcuma longa* (*C. longa*) has been used as a spice in foods and as an antimicrobial in Oriental medicine. In this study, we evaluated the inhibitory effects of an essential oil isolated from *C. longa* on the cariogenic properties of *Streptococcus mutans* (*S. mutans*), which is an important bacterium in dental plaque and dental caries formation. First, the inhibitory effects of *C. longa* essential oil on the growth and acid production of *S. mutans* were tested. Next, the effect of *C. longa* essential oil on adhesion to saliva-coated hydroxyapatite beads (S-HAs) was investigated. *C. longa* essential oil inhibited the growth and acid production of *S. mutans* at concentrations from 0.5 to 4 mg/mL. The essential oil also exhibited significant inhibition of *S. mutans* adherence to S-HAs at concentrations higher than 0.5 mg/mL. *S. mutans* biofilm formation was determined by scanning electron microscopy (SEM) and safranin staining. The essential oil of *C. longa* inhibited the formation of *S. mutans* biofilms at concentrations higher than 0.5 mg/mL. The components of *C. longa* essential oil were then analyzed by GC and GC-MS, and the major components were α-turmerone (35.59%), germacrone (19.02%), α-zingiberene (8.74%), αr-turmerone (6.31%), *trans*-β-elemenone (5.65%), curlone (5.45%), and β-sesquiphellandrene (4.73%). These results suggest that *C. longa* may inhibit the cariogenic properties of *S. mutans*.

Keywords: *Curcuma longa*, dental biofilm, dental caries, *Streptococcus mutans*

Introduction

Dental caries is a major oral health problem and is the most prevalent chronic disease in the dental field. It is an infectious disease in which the hard tissues of the teeth, such as enamel, dentin, and cementum, are gradually and irreversibly destroyed (Hamada and Torii 1980; Wang and others 2007). *Streptococcus mutans* (*S. mutans*) is a bacterium that is known to play a significant role in the formation of dental plaques and caries in humans (Wiater and others 1999; Abdus Salam and others 2004). *S. mutans* adheres to the primary colonizers on the tooth surface that cause dental plaque (Matsumoto and others 1999; Abdus Salam and others 2004).

S. mutans produces a glucosyltransferase (GTFase) that synthesizes water-insoluble glucan, a bacterial extracellular polysaccharide, from the sucrose contained in foods (Hamada and Torii 1980; Wiater and others 1999). This glucan is well known to accelerate the maturation of dental plaque. *S. mutans* breaks down the carbohydrates in foods and releases organic acids, mainly lactic acid, as byproducts. These organic acids demineralize teeth and cause the development of dental caries (Kohler and others 1995).

Fluoride compounds are effective in eradicating the bacteria that cause dental plaque for the purpose of preventing dental caries (Guha-Chowdhury and others 1995). However, they are known to be cytotoxic at concentrations over 80 ppm (Jeng and others 1998). Several other agents have also been studied for the prevention of dental plaque formation (Pan and others 1999). However, these agents are not sufficiently effective to prevent dental plaque. Therefore, the development of new agents that have the capacity to protect against dental caries is important.

Curcuma longa L., which belongs to the Zingiberaceae family, is popularly known as turmeric (Korea Administration Food and Drug 2008). It has long been used in foods as a spice, and in Oriental medicine for the treatment of dental diseases such as toothache, gastritis, vomiting, jaundice, and contusion (Kim and others 1998, 2005). It is well known that *C. longa* also has several other effects, such as antibacterial, blood pressure lowering, whitening, antioxidative, and anticarcinogenic activities (An and others 2006; Sun and others 2009). In addition, it has been reported that *C. longa* can promote blood circulation (Adaramoye and others 2009). However, there is inadequate scientific evidence for the effect of *C. longa* on the ability of *S. mutans* to cause dental disease. In the present study, we show that an essential oil of *C. longa* inhibits the growth, acid production, adherence, and biofilm formation of *S. mutans*.

Materials and Methods

Materials

Brain heart infusion (BHI) broth, mitis-salivarius agar (MSA), and phenol red broth were purchased from Difco Laboratories (Detroit, Mich., U.S.A.). Glucose and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, Mo., U.S.A.). *S. mutans* ATCC 25175 was purchased from the American Type Culture Collection (ATCC, Rockville, Md., U.S.A.).

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Plant material and essential oil

C. longa was obtained from Changsanogapisasum Farm (Kunsan, South Korea) in 2009. Its identity was confirmed by Dr. Bong-Seop Kil in the Dept. of Natural Science at Wonkwang Univ. A voucher specimen (nr 09-11-02) is kept in the Herbarium of the Dept. of Oral Biochemistry, School of Dentistry, Wonkwang Univ. The root of the *C. longa* plant (4.9 kg) was sliced and dried. Then the dried *C. longa* material was placed in a 2-L round-bottom flask. Steam distillation was carried out in a Clevenger-type apparatus for 3 h. The essential oil was stored in a deep freezer (−70 °C) to minimize escape of the volatile compounds before use. The essential oil was then dissolved in DMSO to give the desired stock solution of the essential oil. The final concentration of DMSO was adjusted to 0.1% (v/v) in bacterial culture system. Control groups were treated with bacterial culture media containing 0.1% of DMSO.

Inhibition of bacterial growth

The growth of *S. mutans* was examined at 37 °C in tubes containing 0.95 mL of BHI broth with 1% glucose and various concentrations of *C. longa* essential oil. These tubes were inoculated with 0.05 mL of an overnight culture grown in BHI broth (cell density at inoculation: 5×10^5 colony-forming units [CFU]/mL), and incubated at 37 °C for 24 h. The optical density (OD) of cells was measured at 550 nm using a spectrophotometer. Each concentration of the extract was tested in triplicate.

Acid production

To examine the effect of *C. longa* essential oil on acid production by *S. mutans*, a previous method was used with a slight modification (Nakahara and others 1993). The filter-sterilized *C. longa* essential oil was added to 0.95 mL of phenol red broth containing 1% glucose, which was then inoculated with 0.05 mL of *S. mutans* seed culture (cell density at inoculation: 5×10^5 CFU/mL). The cultures were incubated at 37 °C for 24 h, and the pH was directly determined in the bacterial growth media using a pH meter (Corning Inc., Corning, N.Y., U.S.A.). The initial pH of BHI with various concentrations of *C. longa* essential oil was also determined before inoculation of *S. mutans*. Each concentration of the extract was tested in triplicate.

Bacterial adherence

The bacterial adherence assay was based on a previously described method (Liljemark and others 1981). Briefly, *S. mutans* was grown in BHI at 37 °C for 24 h. The cells were then diluted in BHI to approximately 10^8 CFU/mL. Thirty micrograms of hydroxyapatite beads (dia 80 μm; Bio-Rad, Hercules, Calif., U.S.A.) were coated with clarified human saliva (Hay and others 1971) for 1 h at room temperature. The saliva-coated hydroxyapatite beads (S-HAs) were washed 3 times with 0.01 M potassium phosphate buffer (KPB; pH 7.0) and immersed in the bacterial suspension (1×10^7 CFU/mL) with and without *C. longa* essential oil. After incubation of the S-HAs with the bacteria, accompanied by gentle agitation, for 90 min at 37 °C, the S-HAs were washed and transferred to a tube containing KPB. The *S. mutans* cells that had adsorbed onto the S-HAs were dispersed using a sonicator (50 W, 30 s; Fisher Scientific, Springfield, N.J., U.S.A.), diluted, and spread on MSA plates containing bacitracin (3.2 mg/mL). After incubation at 37 °C for 48 h, the number of bacterial colonies was counted on each MSA plate. Each concentration of the extract was tested in triplicate, and the number of CFUs was calculated.

Biofilm assay

The biofilm assay was based on a previously described method (Petersen and others 2004). *C. longa* essential oil was added to BHI broth containing 0.1% sucrose in 35 mm polystyrene dishes, 24-well plates (Nunc, Copenhagen, Denmark), or 24-well plates containing resin teeth (Endura, Shofu Inc., Kyoto, Japan). The cultures were then inoculated with a seed culture of *S. mutans* (cell density at inoculation: 5×10^5 CFU/mL). After cultivating for 24 h at 37 °C, the supernatant was removed, and the dishes or wells were rinsed with distilled H_2O. Biofilm formation in the wells was measured by staining with 0.1% safranin. The bound safranin was released from the stained cells with 30% acetic acid, and the absorbance of the solution was measured at 530 nm. The biofilm that formed on the surface of the resin teeth was also stained with 0.1% safranin, and photographed.

Scanning electron microscopy

The biofilm that formed on the 35-mm polystyrene dishes was also analyzed by SEM using a modification of previously described methods (Nakamiya and others 2005). The biofilm on the dishes was rinsed with distilled H_2O and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C for 24 h. After gradual dehydration with increasing concentrations of ethyl alcohol (60%, 70%, 80%, 90%, 95%, and 100%), the sample was freeze-dried. The specimens were then sputter-coated with gold (108A sputter coater; Cressington Scientific Instruments Inc., Watford, U.K.). To examine the biofilim, a JSM-6360 scanning electron microscope (JEOL, Tokyo, Japan) was used.

GC and GC-MS analysis

GC analysis was performed using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, Calif., U.S.A.). The sample was injected into an HP-5MS column (60 m × 0.25 mm, 0.25 μm thickness). The temperature of the column was programmed from 50 °C to 240 °C at 2 °C/min, and then kept constant at 240 °C for 40 min. The injector and detector temperatures were 250 °C. The carrier gas was N_2 at a flow rate of 1.2 mL/min.

GC-MS analysis was performed using an Agilent 6890 gas chromatograph with a 5970 Quadrupole MSD. The sample was injected into a DB-5 column (50 m × 0.25 mm, 0.25 μm thickness). The temperature of the column was programmed from 50 to 240 °C at 2 °C/min, and then kept constant at 240 °C for 40 min. The injector and detector temperatures were 250 °C. The carrier gas was He at a flow rate of 1.2 mL/min.

Statistical analysis

All experiments were performed in triplicate. Data were analyzed using the statistical package for social sciences (SPSS, Chicago, Ill., U.S.A.). The data are expressed as the mean ± standard deviation. The differences between the means of the experimental and control groups were evaluated by Student's *t*-test. A P value of < 0.05 was considered statistically significant.

Results and Discussion

Inhibition of bacterial growth

C. longa is reportedly used in traditional Oriental medicine to treat gastritis, vomiting, jaundice, contusion, and dental disease such as toothache (Kim and others 1998, 2005). It is well known that *S. mutans* is a major bacterium responsible for the formation of dental plaque and dental caries (Wiater and others 1999). In

this study, we investigated the potential effects of *C. longa* essential oil on the cariogenic properties of *S. mutans*, such as growth, acid production, adherence, and the ability to produce bacterial biofilms. First, the antimicrobial activity of *C. longa* essential oil against *S. mutans* was tested. *S. mutans* was treated with 0.25, 0.5, 1, 2, and 4 mg/mL of *C. longa* essential oil. As shown in Figure 1A, the essential oil (0.25 to 4 mg/mL) inhibited the growth of *S. mutans* in a dose-dependent manner, and significant inhibition was observed at concentrations higher than 0.5 mg/mL in comparison to the control group ($P < 0.05$). In addition, the antibacterial activity of the essential oils was not significantly changed even if the essential oil was stored in sealed glass vial at room temperature for 1 d (Data not shown). These results showed that *C. longa* essential oil inhibited the growth of *S. mutans*. Therefore, the fact that *C. longa* essential oil suppressed the growth of *S. mutans* supports the scientific rationale that native inhabitants used *C. longa* for the treatment of dental diseases.

Inhibition of acid production

S. mutans is considered one of the most cariogenic of all the oral bacteria found in human dental plaque. It is able to metabolize dietary sugars and produce organic acids such as lactic acid and formic acid. This acidic environment in the mouth can lower the pH of dental plaque, demineralize the tooth enamel, and cause dental caries (Kohler and others 1995). The inhibitory effect of *C. longa* essential oil on the acid production of *S. mutans* was examined. The bacterial cultures were exposed to the *C. longa* essential oil and the pH was monitored. As summarized in Table 1, there was an obvious decrease in pH in the control group; however, the pH decrease was substantially inhibited in the presence of the extract (0.5 to 4 mg/mL). These results suggest that *C. longa* essential oil may inhibit the production of organic acid by *S. mutans*.

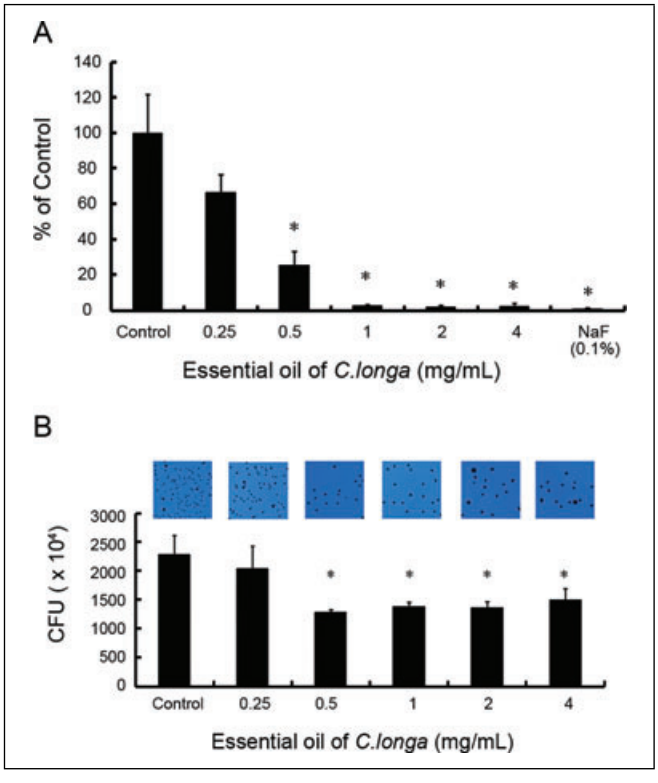

Figure 1–Effect of *C. longa* essential oil on the growth and adherence to saliva coated hydroxyapatite bead (S-HAs) of *S. mutans*. (A) *S. mutans* was inoculated into BHI broth with various concentrations of *C. longa* essential oil and was incubated for 24 h at 37 °C. Antibacterial activity against *S. mutans* is shown in the presence of the essential oil at concentrations ranging from 0.25 to 4 mg/mL. (B) The colony-forming units (CFU) of *S. mutans* that adhered to 30 mg of saliva-coated hydroxyapatite beads incubated at various concentrations of *C. longa* essential oil. When cultured in the presence of 0.5 to 4 mg/mL of the essential oil, adherence ability of *S. mutans* was significantly repressed. *, significantly different from the control group ($P < 0.05$) as determined by independent sample *t*-tests of the mean values.

Inhibition of bacterial adherence and *S. mutans* biofilm

The initial step in the formation of dental plaque is adhesion of *S. mutans* to tooth surfaces (Matsumoto and others 1999). Inhibition of *S. mutans* adherence to the tooth surface is important for the prevention of plaque formation. Cell surface hydrophobicity plays a role in the adherence of oral bacteria to the tooth surface (Rosenberg and others 1983). In a previous study, mutant strains of *S. mutans*, *S. sanguis*, and *S. salivarius* that had lost their cell surface hydrophobicity were incapable of adhering to S-HAs (Westergren and Olsson 1983).

In the present study, we tested the inhibitory effect of *C. longa* essential oil on the adhesion of *S. mutans* to S-HAs. The adherence of S. *mutans* cultured in the presence of 0.25 mg/mL of essential oil was not significantly different from that of the control group. However, when the bacteria were cultured in the presence of 0.5 to 4 mg/mL of the essential oil, adherence was significantly repressed (Figure 1B), but the inhibition of water-insoluble glucan did not show a dose-dependent manner well. Maximal inhibition was reached at 0.5 mg/mL and inhibition rate from 0.5 to 4 mg/mL did not show a big difference. This result suggests that the efficacy of *C. longa* essential oil on the inhibition of water-insoluble glucan may be not high. However, this is the first report that *C. longa* suppressed the synthesis of water-insoluble glucan.

Also, to determine whether the *C. longa* essential oil inhibits the formation of *S. mutans* biofilm, the bacteria were treated with *C. longa* essential oil, and then biofilm formation was observed by safranin staining and SEM. As shown in Figure 2, *C. longa* essential oil (0.5 to 4 mg/mL) inhibited the formation of *S. mutans* biofilm. Furthermore, the inhibitory effect of the essential oil at 2 to 4 mg/mL was higher than that of the positive control (0.1% NaF). SEM photographs (Figure 3A) emphasize the results obtained by safranin stain (Figure 2). *S. mutans* attached to and aggregated on the surface of 35-mm polystyrene dishes, and visibly formed biofilm in the control group; however, biofilm formation was decreased in the presence of *C. longa* essential oil at concentrations higher than 0.5 mg/mL. Biofilm formation was also decreased in the presence of the positive control. Also, the essential oil reduced the adherence of *S. mutans* to S-HAs at concentrations higher than 0.5 mg/mL compared to the control group. Therefore,

Table 1–The pH of *S. mutans* cultures incubated with various concentrations of *C. longa* essential oil.

Conc. (mg/mL)	pH (before incubation)	pH (after incubation)
0 (Control)	7.27 ± 0.05	5.17 ± 0.15
0.25	7.23 ± 0.05	5.13 ± 0.20
0.5	7.20 ± 0.00	5.73 ± 0.36*
1.0	7.13 ± 0.05	6.67 ± 0.23*
2.0	7.13 ± 0.05	7.17 ± 0.40*
4.0	7.10 ± 0.00	7.18 ± 0.20*
NaF (0.1%)	7.00 ± 0.00	7.40 ± 0.00*

Values represent the mean ± SD obtained from triplicate experiments. *, significantly different from the control group ($P < 0.05$) as determined by independent sample *t*-tests of the mean values.

further studies are needed to investigate whether *C. longa* can reduce the surface hydrophobicity of *S. mutans*.

Inhibition of *S. mutans* biofilm formation on the surface of resin teeth

Adhesion of *S. mutans* to pellicle-coated tooth surfaces is the first step in the formation of dental plaque, a type of biofilm on teeth. Biofilm formation increases the bacterial resistance to both the host defense system and antimicrobials. Dental caries and periodontitis are caused by dental biofilm, and GTFase is well known as an important factor for the maturation of dental biofilm (Yoshida and Kuramitsu 2002).

We observed biofilm formation on the surface of resin teeth (Figure 3B). *C. longa* essential oil (0.5 to 4 mg/mL) inhibited the formation of *S. mutans* biofilm on the surface of resin teeth. The positive control also showed an inhibitory effect on *S. mutans* biofilm formation. Furthermore, *S. mutans* biofilm formation was inhibited by *C. longa* essential oil at concentrations from 2 to 4 mg/mL. In addition, the SEM data of *S. mutans* biofilm formation were similar to the safranin stain data. Additionally, the *C. longa* essential oil (2 to 4 mg/mL) also inhibited *S. mutans* biofilm formation on resin tooth surfaces. The effects of the essential oil observed on biofilm formation as well as acid production of *S. mutans* may be due to a mortality of *S. mutans*. The results from these studies suggest that *C. longa* may be considered useful for the prevention of dental plaque.

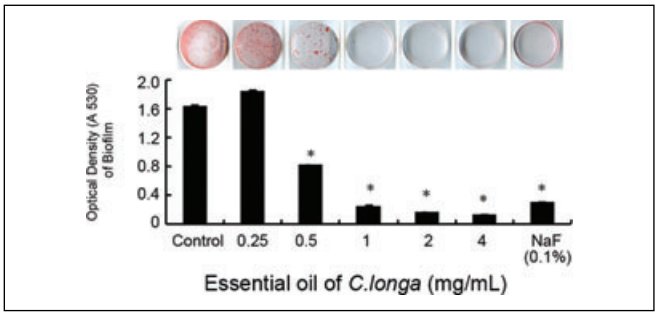

Figure 2–Effect of *C. longa* essential oil on *S. mutans* biofilm formation. *S. mutans* was inoculated into BHI broth with various concentrations of *C. longa* essential oil and incubated for 48 h at 37 °C. The biofilms that formed on the dish surface were measured by staining with 0.1% safranin. The bound safranin was released from the stained cells with 30% acetic acid, and the absorbance of the solution was measured at 530 nm. *C. longa* essential oil at 0.5 to 4 mg/mL inhibited the formation of *S. mutans* biofilm. *, significantly different from the control group ($P < 0.05$) as determined by independent sample *t*-tests of the mean values.

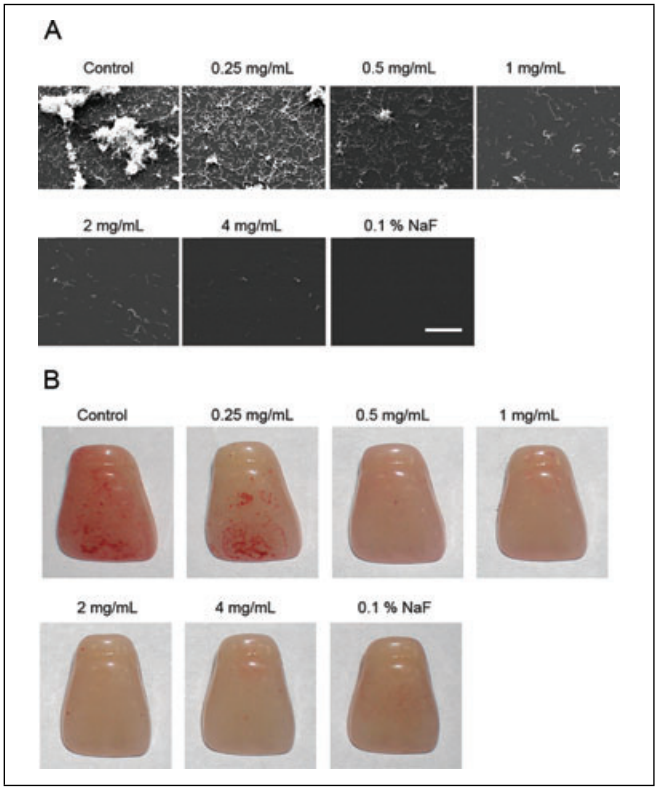

Figure 3–(A) SEM of *S. mutans* biofilms grown in *C. longa* essential oil. SEM of *S. mutans* biofilms grown in *Curcuma longa* essential oil. Biofilm formation was decreased in the presence of *C. longa* essential oil at concentrations higher than 2 mg/mL. (B) The effect of *C. longa* essential oil on biofilm formation on resin tooth surfaces. *S. mutans* biofilms on resin tooth surfaces were incubated in *C. longa* essential oil. *C. longa* essential oil (0.5 to 4 mg/mL) inhibited the formation of *S. mutans* biofilm on the surface of resin teeth. 0.1% NaF was used as positive control. Scale bar represented 50 μm.

Table 2–GC and GC-MS analysis of the essential oil isolated from *C. longa*.

tR (min)	Component	Retention index	Peak area (%)
5.742	Toluene	768	*t*
7.967	2-Furfuryl alcohol	846	*t*
16.297	1,8-Cineole	1032	0.03
19.724	Terpinolene	1088	0.03
20.517	2-Nonanol	1099	0.13
24.637	*trans*-Dihydro-α-terpineol	1168	*t*
25.290	4-Terpineol	1178	*t*
25.824	*p*-Cymen-8-ol	1186	0.07
26.174	α-Terpineol	1191	0.09
26.205	2-Decanone	1194	*t*
26.877	*cis*-3-Hexadecene	1207	0.04
27.762	*cis*-Anethole	1251	*t*
32.793	2-Undecanone	1293	0.05
33.975	2-Methoxy-4-vinylphenol	1312	0.10
35.465	δ-Elemene	1337	0.05
35.626	Piperitenone	1338	0.06
38.870	β –Elemene	1391	0.46
39.696	α-Cedrene	1406	0.05
40.449	β –Caryophyllene	1419	0.39
41.312	γ-Elemene	1434	0.22
42.415	α-Humulene	1452	0.04
42.749	*trans*-β-Farnesene	1458	0.19
44.296	*ar*-Curcumene	1482	0.91
45.542	α-Zingiberene	1502	8.74
45.981	β-Bisabolene	1511	1.11
47.055	β-Sesquiphellandrene	1531	4.73
47.237	*trans*-γ-Bisabolene	1535	0.18
48.526	Germacrene B	1558	0.58
49.864	*ar*-Turmerol	1592	0.21
50.535	Dihydro-*ar*-turmerone	1594	0.87
51.315	*trans*- β -Elemenone	1608	5.65
53.164	*ar*-Turmerone	1643	6.31
56.175	α-Turmerone	1697	35.59
56.849	Germacrone	1711	19.02
58.768	Curlone	1750	5.45
59.271	Bisabolone	1760	1.84
60.481	*trans*-α-Atlantone	1783	1.05
69.455	Palmitic acid	1972	0.33
73.506	Hexadecane-1,2-diol	2062	0.04
75.448	Nonadecanone	2106	*t*
76.995	Linoleic acid	2143	0.35
77.208	Linolenic acid	2148	0.12
Total			99.50

t: Trace (peak area less than 0.02%).

GC and GC-MS analysis of *C. longa* essential oil

In the previous study, roots of *C. longa* has been reported to contain 2.2% of essential oil, and the essential oil from India contained mainly 1,8-cineole (11.2% to 14.6%), α-turmerone (11.1%), β-caryophyllene (9.8%), αr-turmerone (7.3% to 41.4%), β-sesquiphellandrene (7.1%), tumerol (20.0%), and α-altantone (2.4%) (Raina and others 2002). In our investigation, the results from the GC and GC-MS analysis of the *C. longa* essential oil are shown in Table 2. The results of the analysis revealed the major components such as α-turmerone (35.59%), germacrone (19.02%), α-zingiberene (8.74%), α-turmerone (6.31%), *trans*-β-elemenone (5.65%), curlone (5.45%), and β-sesquiphellandrene (4.73%). This result shows that Korean *C. longa* had high contents of α-turmerone (35.59%), germacrone (19.02%), and α-zingiberene (8.74%) in the roots compared to the Indian *C. longa*, but Korean *C. longa* had low contents of 1,8-cineole (0.03%), β-sesquiphellandrene (4.73%), and tumerol compared to the Indian *C. longa*.

Conclusions

In summary, this study showed the ability of *C. longa* essential oil to inhibit the bacterial growth, acid production, adherence to HAs, and biofilm formation of *S. mutans*. In addition, using GC and GC-MS analysis, the major compounds of *C. longa* essential oil, such as α-turmerone (35.6%), germacrone (19.0%), α-zingiberene (8.7%), *ar*-turmerone (6.3%), *trans*-β-elemenone (5.7%), curlone (5.5%), and β-sesquiphellandrene (4.7%), were identified. These results provide the scientific rationale for the use of *C. longa* for the treatment of dental diseases by native inhabitants and suggest that *C. longa* essential oil may be useful for the prevention and treatment of oral microbial diseases.

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