Research Article

Chamaecyparis obtusa Suppresses Virulence Genes in Streptococcus mutans

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Chamaecyparis obtusa (C. obtusa) is known to have antimicrobial effects and has been used as a medicinal plant and in forest bathing. This study aimed to evaluate the anticariogenic activity of essential oil of C. obtusa on Streptococcus mutans, which is one of the most important bacterial causes of dental caries and dental biofilm formation. Essential oil from C. obtusa was extracted, and its effect on bacterial growth, acid production, and biofilm formation was evaluated. C. obtusa essential oil exhibited concentration-dependent inhibition of bacterial growth over 0.025 mg/mL, with 99% inhibition at a concentration of 0.2 mg/mL. The bacterial biofilm formation and acid production were also significantly inhibited at the concentration greater than 0.025 mg/mL. The result of LIVE/DEAD® BacLight™ Bacterial Viability Kit showed a concentration-dependent bactericidal effect on S. mutans and almost all bacteria were dead over 0.8 mg/mL. Real-time PCR analysis showed that gene expression of some virulence factors such as brpA, gfbB, gtfC, and gtfD was also inhibited. In GC and GC-MS analysis, the major components were found to be α-terpinene (40.60%), bornyl acetate (12.45%), α-pinene (11.38%), β-pinene (7.22%), β-phellandrene (3.45%), and α-terpinolene (3.40%). These results show that C. obtusa essential oil has anticariogenic effect on S. mutans.

1. Introduction

Dental caries is the most common infectious oral disease that has afflicted humans including children and adolescents [1]. It is a multifactorial disease, which is caused by detrimental changes in bacterial ecology due to formation of a biofilm that adheres to the tooth surface [2]. During the past few decades, many reports worldwide showed an overall decreasing trend of dental caries. However, recent studies have reported an alarming increase in caries prevalence, especially among the underprivileged groups [3].

S. mutans can colonize the oral cavity and form bacterial biofilm. It has the ability to survive in an acidic environment and interact with other microorganisms colonizing this ecosystem [2].

Caries results from an imbalance between demineralization and remineralization of tooth structure. Acidogenic bacteria ferment dietary carbohydrates, thereby producing organic acids, which initiate dissolution of tooth enamel and breakdown of dental tissue [4]. The extent of the pH fall is influenced by numerous factors, including the composition of the microflora, as well as the type and frequency of sugar intake [5].

S. mutans produce glucosyltransferase (GTF) enzyme which is recognized as virulence factors in the etiology of dental caries. GTF enzymes synthesize extracellular glucans.
and contribute significantly to the dental plaque matrix's polysaccharide formation [6]. The sucrose-dependent mechanism of plaque formation is based on GTF produced by S. mutans in combination with glucan-binding proteins (GBPs). The synthesized glucans provide the possibility of both bacterial adhesion to the tooth enamel and adhesion of the microorganisms to each other [2].

Deminerization can be reversed by calcium and phosphate, together with fluoride, diffusing into the tooth and depositing a new veneer on the crystal remnants in the noncavitated lesion, and is known as remineralization [4]. Fluoride has been used as the "first choice" for the prevention of dental caries [7], and other anticariogenic natural products or compounds like xylitol have also been introduced [8].

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C. obtusa is a tropical tree species found in Japan and the southern region of South Korea, and essential oil is extracted from leaves and twigs of the C. obtusa tree. The essential oil has several types of terpenes and has been commercially used in soaps, toothpaste, and cosmetics as a functional additive [9]. The essential oil of C. obtusa is a concentrated hydrophobic liquid containing volatile compound with natural antibiotic properties that protect against harmful insects, animals, and microorganisms. Inhalation of this essential oil is known as C. obtusa aromatherapy or C. obtusa forest bathing [10] and has been shown to exert antibacterial and antifungal effects [11].

This study was performed to analyze anticariogenic effect of C. obtusa on S. mutans and to determine its chemical composition using a gas chromatography (GC)/gas chromatography-mass spectrometry (GC-MS) analysis.

2. Materials and Methods

2.1. Plant Material and Essential Oil. C. obtusa was collected in October 2013 from the Jeollanam-do province, South Korea. Fresh leaves and twigs of C. obtusa (1kg) were ground mechanically and hydrodistilled for 3 hours using a Clevenger-type apparatus. The yield of the C. obtusa essential oil was 1.08% of yellow pale oil, based on the fresh weight of the plant. The C. obtusa essential oil was stored in a deep freezer (−70°C) to minimize the loss of volatile compounds.

2.2. Inhibition of Bacterial Growth. S. mutans (ATCC 25175) was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in brain heart infusion (BHI; Difco, Detroit, MI, USA) broth under aerobic condition at 37°C. The growth of S. mutans was examined at 37°C in 0.95 mL of brain heart infusion broth containing various concentrations of the C. obtusa. These tubes were inoculated with 0.05 mL of an overnight culture grown in the BHI broth (final: 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.

2.3. Acid Production. Acid production by S. mutans was examined to evaluate the effect of C. obtusa essential oil using a modification of previously described method [12]. The C. obtusa essential oil was filter-sterilized using membrane filter with 0.2 μm pore size and added to 0.95 mL of the phenol red broth containing 1% glucose, which was then inoculated with 0.05 mL of the seed culture of S. mutans. After 24 h of cultivation, the pH of the cultures was determined using a pH meter (Corning, Inc., Corning, NY, USA). Three replicates were measured for each concentration of the test extract.

2.4. Biofilm Formation Assay. The biofilm assay was based on a previously described method [13, 14]. Biofilm formation was measured by staining with safranin and observed by scanning electron microscopy (SEM). C. obtusa essential oil was added to BHI broth containing 0.1% sucrose in 35 mm polystyrene dishes. The cultures were then inoculated with a seed culture of S. mutans (final: 5 × 10^5 CFU/mL) and incubated for 24 h at 37°C. After incubation, the supernatants were removed, and the dishes were rinsed with distilled water. Biofilm formation was stained with 0.1% safranin and photographed. The bound safranin was released into the stimulated cells with 30% acetic acid and the absorbance of the solution was measured at 530 nm. In addition, biofilm on 35 mm polystyrene dishes was observed by SEM [15]. The biofilm formed on the dishes was rinsed with distilled water, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 24 h, and dehydrated with ethanol gradient series (60%, 70%, 80%, 90%, 95%, and 100%). Then, the samples were freeze-dried, sputter-coated with gold (108A sputter coater; Cressington Scientific Instruments, Inc., Watford, England, United Kingdom), and observed by SEM (JSM-6360, JEOL, Tokyo, Japan).

2.5. Confocal Laser Scanning Microscopy. To determine the bactericidal effect of C. obtusa essential oil on S. mutans, staining with LIVE/DEAD® BacLight™ Bacterial Viability Kit was performed and examined by confocal laser scanning microscopy. The cultured S. mutans in BHI was diluted using BHI media to 1 × 10^7 CFU/mL and treated with the essential oil at 37°C under aerobic conditions. After 30 min of incubation, the bacteria were washed with PBS and stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA), prepared according to the manufacturer’s protocol. After 15 min of staining, bacteria were observed using confocal laser scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany).

2.6. Real-Time Polymerase Chain Reaction Analysis. A real-time PCR was performed to examine the effect of C. obtusa essential oil on virulence factor gene expression of S. mutans. The subminimal inhibitory concentration (0.025–0.1 mg/mL) of the essential oil was used. After 24 h of culture, total RNA was isolated from S. mutans using a TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized. The amplification was performed using a StepOnePlus Real-Time PCR system with SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). 16S rRNA was used as an internal control.
2.7 GC and GC-MS Analysis. GC analysis was performed on a Hewlett-Packard model 6890 series gas chromatograph with a flame ionization detector (FID) and a split ratio of 30:1 using DB-5HT fused silica capillary columns (30 m × 0.25 mm, i.d., 0.25 μm film thickness). The temperature of the column was programmed from 40°C to 230°C, at 2°C/min and then kept constant at 230°C for 20 min. The injector and detector temperatures were 230°C and 250°C, respectively. The gas carrier used was nitrogen, at a flow rate of 0.80 mL/min. Peak areas were measured by electronic integration and the relative amounts of the individual components were determined based on the peak areas. The GC-MS analysis was carried out on an Agilent Technologies 7890A GC and 5975C mass selective detector (MSD) operating in EI mode at 70 eV, fitted with a DB-5MS fused silica capillary column (30 m × 0.25 mm, i.d., 0.25 μm film thickness). The column temperature was programmed from 40°C to 230°C at 2°C/min and then kept constant at 230°C for 20 min. The injector and ion source temperatures were 250°C, respectively. The gas carrier was helium at a flow rate of 1.0 mL/min.

The identification of individual components was based on comparisons with Wiley 7n/NIST 05 mass spectra libraries and retention indices with reference to literature data. Linear retention indices were calculated against those of n-paraffin (C6–C26) series [16].

2.8 Statistical Analysis. All experiments were performed in triplicate. Data were analyzed using SPSS software 12.0 (Chicago, IL, USA). The data are expressed as the mean ± standard deviation values. The statistical analysis was done using Student's t-test. Values of p < 0.01 were considered statistically significant.

3. Results

3.1 Inhibition of Bacterial Growth. After extraction of Chamaecyparis obtusa essential oil by hydrodistillation, the antibacterial activity of the oil was tested against S. mutans. C. obtusa essential oil significantly inhibited the growth of S. mutans in a concentration-dependent manner. The bacteria were treated with 0.025, 0.05, 0.1, and 0.2 mg/mL of C. obtusa essential oil. When treated with 0.025 mg/mL of the essential oil, the bacterial growth was significantly inhibited in comparison to the control group (p < 0.05). The positive control (0.1% NaF) also showed antibacterial activity (Figure 1).

3.2 Inhibition of Acid Production. To investigate whether C. obtusa essential oil can inhibit S. mutans organic acid production, the bacteria were cultured in the presence of various concentrations (0.025–0.2 mg/mL) of the essential oil and the change in pH was measured. The pH of the control declined to 5.30 after bacterial culture, while the initial pH of the media before bacterial culture was 7.40. However, the addition of 0.025, 0.05, 0.1, and 0.2 mg/mL of C. obtusa essential oil resulted in pH levels of 5.38, 5.73, 7.12, and 7.40, respectively. These results indicate that C. obtusa essential oil may inhibit the organic acid production by S. mutans.

3.3 Inhibition of Biofilm Formation. To determine whether C. obtusa essential oil inhibits biofilm formation by S. mutans, the bacteria were cultured in the presence of various concentration of C. obtusa essential oil in polystyrene dishes. Biofilm formation was studied using safranin staining, and absorbance was measured at 530 nm. The biofilm formation by S. mutans was significantly inhibited by treatment with C. obtusa essential oil in a dose-dependent manner over 0.025 mg/mL of C. obtusa essential oil. When treated with 0.1% NaF (positive control), complete inhibition was shown (Figure 2). SEM results were consistent with those of safranin staining (Figure 3).

3.4 Bactericidal Effect. To evaluate bactericidal effect of C. obtusa essential oil, S. mutans were cultured in presence of high concentrations (0.2–1.6 mg/mL) of the essential oil, stained with LIVE/DEAD BacLight™ Bacterial Viability Kit and observed using confocal laser scanning microscopy. Treatment with C. obtusa essential oil decreases living bacteria (green fluorescence labeled cell stained by SYTO® 9) and increases dead bacteria (red fluorescence labeled cell stained by PI). The bactericidal effect of C. obtusa essential oil was also observed in a dose-dependent manner (Figure 4).

![Figure 1: Bacterial growth inhibition of Streptococcus mutans (S. mutans) by Chamaecyparis obtusa (C. obtusa) essential oil. The optical density (A550) was read using a spectrophotometer. *P < 0.01 compared to the control group.](image)

![Figure 2: SEM result was consistent with those of safranin staining.](image)

![Figure 3: SEM result was consistent with those of safranin staining.](image)

![Figure 4: Bactericidal effect of Chamaecyparis obtusa (C. obtusa) essential oil.](image)
Essential oils are volatile components mainly obtained by distillation of plant and consist of a mixture of various terpenoids. Terpenes are the therapeutic chemical substances present in medicinal plants [19]. Essential oils extracted from various plants are known to have antimicrobial activity [20]. Some natural derivatives like extracts from Myristica fragrans, Lippia sidoides, Hyptis pectinata, Carcuma longa, and Baccharis dracunculifolia have been proved to be effective against S. mutans [21–25]. This study was performed to evaluate anticariogenic activity of C. obtusa essential oil on S. mutans.

To evaluate anticariogenic properties of C. obtusa essential oil, S. mutans was used because these bacteria is considered as a major cause for the formation of dental caries [2]. Our results showed that growth of S. mutans was suppressed by treatment with C. obtusa essential oil. Furthermore, the results of LIVE/DEAD® BacLight™ Bacterial Viability Kit also showed that C. obtusa essential oil has a bactericidal effect against S. mutans. These results suggested that C. obtusa essential oil has a potential for anticariogenic effect, which is interesting since the inhibition of the growth of S. mutans is one of the strategies for prevention of dental caries.

Growth of S. mutans was suppressed by treatment with C. obtusa essential oil in a concentration-dependent manner above the concentration of 0.025 mg/mL. In dental plaque formation, pH is one of the major factors, since low pH leads to demineralization of hydroxyapatite and favors the cariogenicity [26]. S. mutans can metabolize dietary sugars and produce organic acid. Low-pH environment in the biofilm matrix results in dissolution of enamel, thus initiating the pathogenesis of dental caries. Therefore, the alteration of pH is used as an indicator to determine the effect of anticariogenic agents [27]. In this study, C. obtusa essential oil inhibited the decrease of pH induced by S. mutans and the result suggests that C. obtusa essential oil may inhibit dental caries through inhibition of acid production by S. mutans.

Biofilms are communities of microorganisms that adhere to biological or abiotic substrata and produce an extracellular matrix typically comprising of polysaccharides and proteins. Dental plaque is a kind of biofilm found on a tooth surface, embedded in a matrix of host and bacterial polymers [28]. In biofilm, known as plaque in the oral cavity, the interaction of specific bacteria with constituents of the diet results in caries [27]. Biofilm formation by S. mutans was also inhibited by treatment with C. obtusa essential oil cultured on polystyrene dishes. These results suggested that C. obtusa essential oil directly inhibits the biofilm formation by S. mutans.

Furthermore, several virulence gene factors of S. mutans are associated with various aspects of cariogenicity such as acid tolerance, bacterial adhesion, and biofilm formation [2]. The brpA has been shown to contribute to biofilm formation and plays a major role in cell envelope biogenesis/homeostasis and regulation of stress response as well as in acid tolerance [29, 30]. The gpbB, which encodes surface-associated glucan-binding protein (GBP), mediates binding of bacteria to glucans and enables development of biofilm. The gtfC and gtfD encode glucosyltransferases (GTase) which are essential virulence factor in plaque development and are responsible for glucans formation from sucrose [2].

**Figure 2:** Inhibitory effect of C. obtusa essential oil on biofilm formation by S. mutans, as observed by safranin staining. (a) Control; (b) 0.025 mg/mL; (c) 0.05 mg/mL; (d) 0.1 mg/mL; (e) 0.2 mg/mL; and (f) positive control (0.1% NaF). *Significance was determined at *p* < 0.01 compared to the control group.

However, low concentration (lesser than 0.2 mg/mL) of the essential oil did not show bactericidal effect (data not shown).

### 4. Discussion

Fluoride plays an important role in the prevention and control of dental caries. However, an unfortunate side effect of fluoride is fluorosis. Ingestion of fluoride before 2 to 3 years of age is considered critical for possible fluorosis in the permanent dentition [17]. Therefore, natural products are currently receiving special attention as a good alternative to synthetic chemical substances for the prevention of dental caries [18].
Table 2: Gas chromatography and gas chromatography-mass spectrometry (GC/GC-MS) analysis of the essential oil isolated from C. obtusa.

| Retention time (min) | Retention index | Compound               | Peak area (%) |
|---------------------|----------------|------------------------|---------------|
| 8.849               | 801            | n-Hexanal              | 0.18          |
| 11.982              | 852            | trans-2-Hexenal        | 0.28          |
| 13.110              | 870            | n-Hexanol              | 0.06          |
| 15.125              | 902            | Bornylene              | 0.12          |
| 16.419              | 919            | Tricyclene             | 0.77          |
| 16.763              | 925            | α-Thujene              | 0.12          |
| 17.742              | 936            | α-Pine (40.60%)        | 11.38         |
| 18.650              | 952            | Camphene               | 2.87          |
| 20.228              | 975            | Sabinene               | 0.46          |
| 21.345              | 980            | δ-3-Carene (3.45%)     | 0.37          |
| 22.047              | 983            | β-Pine (7.22%)         | 40.60         |
| 24.483              | 1023           | α-Terpine (40.60%)     | 40.60         |
| 24.937              | 1029           | Limonene (0.57)        |               |
| 25.474              | 1036           | β-Phellandrene (3.45%) |               |
| 27.337              | 1061           | γ-Terpine (0.21)       |               |
| 28.393              | 1072           | cis-Sabinene hydrate   | 0.35          |
| 29.742              | 1089           | α-Terpinolene (3.40%)  |               |
| 29.983              | 1092           | Dehydro-p-cymene (0.53)|               |
| 30.874              | 1102           | trans-Sabinene hydrate | 0.09          |
| 31.769              | 1115           | 1-Octen-3-yl acetate   | 0.06          |
| 33.199              | 1133           | trans-p-2-Menth-1-ol   | 0.05          |
| 36.312              | 1173           | Cryptone               | 0.11          |
| 36.461              | 1175           | p-Cymen-8-ol (0.10)    |               |
| 36.965              | 1181           | Terpinen-4-ol (0.23)   |               |
| 37.889              | 1193           | α-Terpin (0.11)        |               |
| 38.960              | 1207           | Verbenone (0.09)       |               |
| 39.699              | 1217           | Fenchyl acetate (0.07) |               |
| 42.535              | 1256           | Linalyl acetate (0.08) |               |
| 45.552              | 1296           | Bornyl acetate (12.45) |               |
| 49.502              | 1352           | α-Terpinyl acetate (0.62)|            |
| 51.422              | 1380           | α-Copaene (0.28)       |               |
| 53.780              | 1414           | α-Cedrene (0.52)       |               |
| 54.015              | 1417           | β-Caryophyllene (0.16) |               |
| 54.331              | 1422           | β-Cedrene (0.25)       |               |
| 55.009              | 1431           | cis-Thujopsene (0.07)  |               |
| 55.419              | 1434           | β-Gurjunaene (0.06)    |               |
| 56.378              | 1453           | α-Humulene (0.37)      |               |
| 56.976              | 1462           | cis-Murola-4(4)=dien (0.24)|        |
| 57.656              | 1472           | β-Cadinene (0.06)      |               |
| 57.927              | 1476           | Germacrene D (0.13)    |               |
| 58.150              | 1490           | β-Himachalene (0.10)   |               |
| 59.003              | 1492           | α-Murolene (0.16)      |               |
| 59.519              | 1497           | α-Chamigrene (0.05)    |               |
| 60.024              | 1508           | γ-Cadinene (0.38)      |               |

Table 2: Continued.

| Retention time (min) | Retention index | Compound               | Peak area (%) |
|---------------------|----------------|------------------------|---------------|
| 61.079              | 1525           | δ-Cadinene (0.15)      |               |
| 61.522              | 1530           | Cadina-1,4-diene (0.09)|               |
| 61.937              | 1539           | Selina-3,7(11)-dien (0.07)|             |
| 63.011              | 1551           | Elemol (0.07)          |               |
| 63.406              | 1562           | trans-Nerolidol (0.25) |               |
| 64.651              | 1578           | Spathulenol (0.05)     |               |
| 64.916              | 1586           | Caryophyllene oxide (0.10)|             |
| 66.435              | 1603           | Cedrol (0.97)          |               |
| 67.079              | 1622           | 1-epi-Cubenol (0.08)   |               |
| 67.835              | 1635           | γ-Eudesmol (0.58)      |               |
| 68.123              | 1239           | r-Cadinol (0.57)       |               |
| 68.964              | 1648           | α-Cadinol (0.09)       |               |
| 69.973              | 1670           | Bulnesol (0.07)        |               |
| 70.829              | 1685           | α-Bisabolol (0.13)     |               |
| 82.676              | 1898           | Rimuene (0.27)         |               |
| 84.836              | 1926           | Hibaene (2.33)         |               |
| 85.085              | 1945           | Pimaradiene (0.10)     |               |
| 86.747              | 1977           | 13-Isopimaradiene (1.42)|             |
| 88.068              | 1998           | Dolabradiene (0.09)    |               |
| 90.446              | 2051           | Dehydroabietane (0.07) |               |

Total 97.38

The retention index on DB-5HT column.

In this study, to evaluate correlation between inhibitory effect by C. obtusa essential oil and virulence factors expression, we determined the mRNA expression level of several virulence factors using a real-time PCR analysis. We evaluated the gene expression level of brpA, gbpB, gtfC, and gtfD. C. obtusa essential oil significantly inhibited the transcription level of brpA, gbpB, gtfC, and gtfD. Based on our results of GC/GC-MS analysis, the major components included α-terpinene (40.60%), bornyl acetate (12.45%), α-pinene (11.38%), β-pinene (7.22%), β-phellandrene (3.45%), and α-terpinolene (3.40%) (Table 2). Although the biological activities of C. obtusa essential oil are not yet fully understood, some previous study reported that several types of terpenes of C. obtusa have been shown to exert antibacterial and antifungal effect [11]. Recent studies also reported other beneficial properties of C. obtusa, such as pharmacological activities for the treatment of atopic dermatitis [31], improvement on cognitive function of the central nervous system on rat experimental model [10], and promotion of hair growth [32]. In addition, C. obtusa has antinociceptive and anti-inflammatory properties, which increases its applicability in oral care [9, 33]. Based on our results, we conclude that C. obtusa may have a possible practical use against the cariogenic bacteria within the mouth and recommend further investigation of C. obtusa on periodontopathic and cariogenic bacteria.
Figure 3: Inhibitory effect of *C. obtusa* essential oil on biofilm formation by *S. mutans*, as observed by scanning electron microscopy. (a) Control; (b) 0.025 mg/mL; (c) 0.05 mg/mL; (d) 0.1 mg/mL; (e) 0.2 mg/mL; and (f) positive control (0.1% NaF). Scale bar = 10 μm.

Figure 4: Bactericidal effect of *C. obtusa* essential oil. Cultured *S. mutans* was treated with *C. obtusa* extract (0.2–1.6 mg/mL) and stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit. The stained bacteria were observed by confocal laser scanning microscopy. Treatment with *C. obtusa* decreased green-labeled living bacteria (SYTO® 9 stain) and increased red-labeled dead bacteria (PI stain) in a dose-dependent manner. (a) Control; (b) 0.2 mg/mL; (c) 0.4 mg/mL; (d) 0.8 mg/mL; (e) 1.6 mg/mL; and (f) positive control (0.1% NaF). Bar = 100 μm.

5. Conclusions

This study has proved that *C. obtusa* essential oil exhibited significant inhibition of bacterial growth, acid production, and biofilm formation by *S. mutans*. Also *C. obtusa* essential oil showed bactericidal effect. Furthermore, *C. obtusa* essential oil also inhibited the transcription level of several virulence factors such as *brpA*, *gbpB*, *gtfC*, and *gtfD* of *S. mutans*. In GC and GC-MS analysis, the major components were α-terpinene (40.60%), bornyl acetate (12.45%), α-pinene (11.38%), β-pinene (7.22%), β-phellandrene (3.45%), and α-terpinolene (3.40%). Therefore, the results of this study
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Figure 5: Inhibitory effect of C. obtusa essential oil on virulence factor gene expression. S. mutans was cultured and treated with subminimal inhibitory concentration (0.025–0.1 mg/mL) of C. obtusa extract, and real-time polymerase chain reaction (PCR) analysis was performed. The brpA, gbpB, gtfC, and gtfD expressions were significantly inhibited at 0.025 mg/mL of C. obtusa. Each value is expressed as mean ± standard deviation. *Significance was determined at *p < 0.01 when compared with the control group.

indicate that C. obtusa essential oil showed good anticariogenic effect on S. mutans and appear to be a promising new agent that may prevent dental caries. Further studies are needed to develop the new medicine for clinical use.

Competing Interests
The authors declare that there are no competing interests.

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