Inhibitory effects on Streptococcus mutans of antibacterial agents mixed with experimental fluoride varnish

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We investigated the inhibitory effect of antibacterial agents mixed with experimental fluoride varnish (EFV) on Streptococcus mutans. The antibacterial agents used were (1 and 10) mM of xanthorrhizol, bakuchiol, bavachalcone, isobavachromene, and bavachromene. Agar diffusion tests were performed on S. mutans (1.1×10⁹ CFU/mL), using antibacterial agents without and with EFV. Bavachalcone showed the highest inhibition zone without and with EFV at both (1 and 10) mM (p<0.05). All EFV with antibacterial agents showed greater inhibition and semi-inhibition zones than EFV alone (p<0.05). The cell viability of each antibacterial agent was not significantly different from the vehicle controls (p>0.05), except xanthorrhizol and bakuchiol at 1 mM. All antibacterial agents were effective, while antibacterial agents with EFV co-formulations were more effective than EFV alone. Bavachalcone was the most effective agent against S. mutans, indicating its potential usefulness with fluoride varnish in preventing dental caries.

Keywords: Fluoride varnish, Antibacterial agent, Streptococcus mutans, Dental caries

INTRODUCTION

Among the bacteria found in dental plaque, Streptococcus is known to be the major causative bacterial strain of dental caries. Among them, Streptococcus mutans (S. mutans) plays an important role in developing dental caries, and S. sobrinus, S. salivarius, S. sanguinis, and S. mitis have also been reported to have close association with dental caries in humans12,23.

Since fluoride in drinking water was found to reduce dental caries in the US during the 1940s, various fluoride compounds have been used to prevent dental caries25. Early topical fluoride products contained stannous fluoride (SnF₂) and sodium fluoride (NaF) solutions. The applicability of SnF₂ is limited, due to its disadvantages of being unstable, causing discoloration of the tooth surface, and bitter taste, inducing the development of acidulated phosphate fluoride (APF) gel14,19. However, water-soluble APF gel can be easily removed by water or saliva, and the gel can flow from the tray down the throat46. To address these disadvantages, fluoride varnish was developed15. Since fluoride varnish was introduced in Europe in 1964, it has received recognition as a safe and effective fluoride agent through numerous clinical trials8. Studies have reported that fluoride varnish is easy to use, less toxic than APF, and safe even when swallowed, since it is less absorbable in the body than other fluoride agents8,10. Fluoride varnish has been reported to reduce dental caries by 46 and 33% in permanent and deciduous teeth, respectively8. Through a study in which teeth were treated with fluoride varnish, 2% NaF solution, or 1.23% APF, and examined for 30 months, the fluoride varnish proved to be the most effective in reducing dental caries11.

Disinfectants or antibiotics, such as chlorhexidine, penicillin, erythromycin, and tetracycline, are known for their antibacterial effects12,13. Chlorhexidine added to fluoride varnish caused discoloration of teeth and dental prostheses, dysgeusia, and burning sensation in the oral mucosa14,15. Tetracycline, when used for children younger than 9 years old, also permanently discolors the teeth16. Other reported adverse effects of the antibiotics include oral mucosal desquamation, the build-up of calcium deposits, digestive disorders, hypersensitivity, and the emergence of drug-resistant bacterial strains17-19. Due to these adverse effects, studies are being conducted to develop antibacterial agents from natural sources with superior safety profiles26-28. However, despite the numerous studies conducted on natural antibacterial agents to date, studies on the incorporation of natural antibacterial agents into fluoride varnish are lacking, except for a study involving the addition of polys to fluoride varnish24.

To find the effective antibacterial agent on S. mutans, we used agar diffusion tests to screen more than 100 single compounds from the Korea Chemical Bank. We found that compounds extracted from Psoralea corylifolia and substances with similar chemical formulae inhibit the growth of S. mutans. The extracts of Psoralea corylifolia have been reported to possess antibacterial, antitumor, antioxidant, anti-inflammatory, antifungal, and immunomodulatory activity29. P. corylifolia contains many bioactive compounds, including coumarins (psoralen, isopsoralen,
psoralidin, and angelicin), flavonoids (neo-bavaisoflavone, bavachalcone, isobavachalcone, bavachin, bavacinin, corilin, corilifolin corilifol, and 6-prenyllarigenin), and meroterpenes (bakuchiol and 3-hydroxybakuchiol). Among the components of *P. corylifolia*, only bakuchiol was reported to inhibit oral microorganisms. However, the antibacterial effects of other components, such as bavachalcone, isobavachromene, and bavachromene, have not been investigated before now. Xanthorrhizol isolated from java turmeric (*Curcuma xanthorrhiza* Roxb.), which was reported to have fast bactericidal activity against *S. mutans*, was used for comparison.

The objective of the present study was to develop an effective antibacterial experimental fluoride varnish (EFV) against dental caries-inducing *S. mutans*, by adding four components of *P. corylifolia* (bakuchiol [BAK], bavachalcone [BCC], isobavachromene [IBC], and bavachromene [BCM]), in comparison to xanthorrhizol (XAN).

**MATERIALS AND METHODS**

**Antibacterial agents**

XAN, BAK, BCC, IBC, and BCM were each diluted to (1 and 10) mM using dimethyl sulfoxide (DMSO, Table 1). The positive and negative controls were 10 µg/mL ampicillin and phosphate-buffered saline (PBS), respectively, while DMSO was used as the vehicle control.

**Fabrication of EFV**

With 45 wt% rosin (KR-610, Arakawa Chemical Industries, Osaka, Japan) as a base, 50 wt% solvent (ethanol, absolute ≥ 99.7%, Merck, Darmstadt, Germany) and 5 wt% NaF were added to formulate the EFV. The components were placed in a double boiler at 80–100°C on a hot plate (RCH-3, Tokyo Rikakikai, Tokyo, Japan) and mixed at 240 rpm using an overhead stirrer (RW20DZM.n, IKA Korea, Seoul, Korea) for 30 min.

**Formulation of EFV with antibacterial agents**

The antibacterial agents were diluted with DMSO to (2 and 20) mM solutions, which were mixed in a ratio of 1:1 with the EFV to prepare (1 and 10) mM antibacterial agents in EFV. The vehicle control was a 1:1 mixture of fluoride varnish and DMSO.

**Agar diffusion test**

The inhibitory effects of the five different antibacterial agents on *S. mutans* (ATCC 25175) were assessed using the agar diffusion test. For the primary culture, *S. mutans* was cultured in brain heart infusion (BHI) culture medium in a CO₂ incubator at 37°C for 24 h. Then, 200 µL of the cultured *S. mutans* suspension was inoculated into the BHI medium and cultured for 6 h as the secondary culture, which was diluted with BHI broth to obtain bacterial concentrations of 1.1×10¹⁰ CFU/mL. After placing a 6-mm-diameter paper disc on top of the agar medium, 5 µL each of the (1 and 10) mM antibacterial agents was dropped on the paper discs. After culturing for 24 h in a 37°C CO₂ incubator, the diameters of the inhibition zones were measured at right angles, and the average was determined as the inhibition zone (n=6).

The antibacterial agents and the positive control showed two clearly distinguishable zones, a bacterial zone, and a completely transparent inhibition zone with no bacterial growth. In contrast, when the fluoride varnish was included, a translucent semi-inhibition zone appeared, which was less transparent than the inhibition zone, but still distinguishable from the bacterial zone (Fig. 1). Microscopic observation of the semi-inhibition

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**Table 1** Information on antibacterial agents investigated in this study

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>Code</th>
<th>Chemical structure</th>
<th>Molecular weight</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthorrhizol</td>
<td>XAN</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>218.3</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Bakuchiol</td>
<td>BAK</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>256.4</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Bavachalcone</td>
<td>BCC</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>324.4</td>
<td>Chemfaces</td>
</tr>
<tr>
<td>Isobavachromene</td>
<td>IBC</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>322.4</td>
<td>Chemfaces</td>
</tr>
<tr>
<td>Bavachromene</td>
<td>BCM</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>322.4</td>
<td>Chemfaces</td>
</tr>
</tbody>
</table>
zones reveals the bacterial density to be lower than that of the bacterial zone [Fig. 1 (c)]. The diameters of the semi-inhibition zone formed around the paper discs were measured at right angles, and the average value was determined as the semi-inhibition zone.

To find out which component of EFV contributes to the formation of semi-inhibition zone, agar diffusion test was conducted on EFV with and without NaF. And the semi-inhibition zones were compared with that of the antibacterial agent mixed with EFV.

**Cell viability test using MTT**

L-929 cells were cultured using Roswell Park Memorial Institute (RPMI) medium (RPMI-1640, Life Technologies, NY, USA) mixed with 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin 100X, Life Technologies). For cell attachment, 90 µL of cell suspension (1×10⁴ cells/well) was dispensed into each well of a 96-well plate, which was placed in a 5% CO₂ incubator (MCO175, Sanyo Electric Biomedical, Osaka, Japan) at 37°C for 24 h. Then, 10 µL of each antibacterial agent at concentrations of (0, 50, 100, 200, and 1,000) µM was added, and cultured for an additional 24 h. For the vehicle control, 0 µM (0.2% DMSO) was used, and only 10 µL of the culture medium was applied as the negative control (n=3). After 24 h, the culture medium was carefully removed, and 50 µL of 5 mg/mL MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, St. Louis, MO, USA) solution was added, and incubated for 2 h in the CO₂ incubator. Then, the MTT solution was removed, and 100 µL of isopropanol added. After swaying the plate, an enzyme-linked immunoassay (ELISA) reader (Spectra MAX250, Molecular Devices, San Jose, CA, USA) was used to measure the absorbance at 570 nm. The cell viability was calculated as the percentage of negative control. If the cell viability was reduced to under 70%, it could be regarded as having cytotoxic potential.²⁹

**Statistical analysis**
The statistical analysis was performed using the statistical package for the social sciences (IBM SPSS Statistics) version 24. The cell viability was analyzed using a Kruskal-Wallis test, while the agar diffusion data were analyzed by one-way analysis of variance (ANOVA), with post hoc test using Duncan’s multiple range test (α=0.05). A t-test was performed to analyze the significance between 1 and 10 mM of the antibacterial agents (α=0.05).

**RESULTS**

*Inhibition zone in agar diffusion test*

Figure 2 (a) shows the inhibition zones of the antibacterial agents in the agar diffusion test. The negative control and the vehicle control, DMSO, did not form any inhibition zone around the paper disc of 6-mm-diameter, indicating that it did not affect the antibacterial activity against *S. mutans*. All antibacterial agents showed antibacterial effect. At (1 and 10) mM, BCC showed the highest antibacterial effect, followed by BAK (p<0.05).

Figure 2 (b) shows the inhibition zones of the antibacterial agents mixed with EFV. The vehicle control, DMSO in EFV, showed a slight antibacterial effect against *S. mutans*. At (1 and 10) mM, all the antibacterial agents in EFV showed significantly higher antibacterial activity than the vehicle control (p<0.05). At both (1 and 10) mM, BCC showed the highest antibacterial activity (p<0.05).

As a result of t-test, the inhibition zones of 10 mM antibacterial agents were significantly greater than 1 mM in both Figs. 2 (a) and (b).

*Sem-inhibition zone in agar diffusion test*

The vehicle control (DMSO in EFV) and all experimental groups that contain EFV showed semi-inhibition zones (Fig. 3). Since no semi-inhibition zone was observed in the positive control group that did not contain EFV, the results of the inhibition zone are shown for the positive control group. The semi-inhibition zones of all experimental groups were significantly higher than the inhibition zone of the positive control and the semi-inhibition zone of the vehicle control (p<0.05). In the comparison of the experimental groups of 1 mM, the BCC and XAN groups showed significantly higher semi-inhibition zones than other groups (p<0.05), with no
significant differences from BAK ($p>0.05$).

The EFV without and with NaF showed semi-inhibition zones [Figs. 4 (a) and (b)], while with the antibacterial agents alone, no semi-inhibition zone was observed [Fig. 4 (c)]. In EFV without NaF, the semi-inhibition zone was 10.7 mm [Fig. 4 (a)]; and in EFV with NaF, 15.3 mm [Fig. 4 (b)]; whereas for the antibacterial agent mixed with EFV, the zone became much larger (25.0 mm) [Fig. 4 (d)].

Fig. 2 Inhibition zone in agar diffusion test.
(a) Inhibition zone of antibacterial agents with vehicle control (V cont) as DMSO, (b) Inhibition zone of antibacterial agents in EFV with V cont as DMSO in EFV. Positive control (+Cont) is ampicillin (10 µg/mL) and negative control (−Cont) is PBS. Different uppercase and lowercase letters indicate significant differences at (1 and 10) mM concentrations, respectively, by Duncan’s multiple range test at $\alpha=0.05$. XAN: xanthorrhizol, BAK: bakuchiol, BCC: bavachalcone, IBC: isobavachromene, BCM: bavachromene

Fig. 3 Semi-Inhibition zone of antibacterial agents in EFV using agar diffusion test with vehicle control (V cont) as DMSO in EFV. Positive and negative controls (+Cont and −Cont), ampicillin (10 µg/mL) and PBS. Different uppercase and lowercase letters indicate significant difference at (1 and 10) mM concentrations by Duncan’s multiple range test at $\alpha=0.05$. XAN: xanthorrhizol, BAK: bakuchiol, BCC: bavachalcone, IBC: isobavachromene, BCM: bavachromene

Fig. 4 Semi-inhibition zone.
(a) Images of fluoride varnish without sodium fluoride (NaF), (b) Fluoride varnish with NaF, (c) Antibacterial agent alone (BCC), (d) Antibacterial agent in fluoride varnish with NaF. I: Inhibition zone, SI: Semi inhibition zone, B: bacterial zone

Fig. 5 Cell viability using MTT test was calculated as the percentage of the negative control.
The vehicle control was expressed as 0 µM (0.2% DMSO in a medium). XAN: xanthorrhizol, BAK: bakuchiol, BCC: bavachalcone, IBC: isobavachromene, BCM: bavachromene. Different uppercase letters indicate significant differences by a Kruskal-Wallis test at $\alpha=0.05$. 

significant differences from BAK ($p>0.05$).
Cell viability using CCK-8 assay
There were no significant differences among the vehicle controls (0 µM) of each antibacterial agent group (p>0.05, Fig. 5). The viability of the 1,000 µM BCC, IBC, and BCM-treated cells was not significantly different from those treated with the vehicle controls (p>0.05). In the case of XAN and BAK, 1,000 µM showed significantly lower cell viabilities (under 70%) than the vehicle controls.

DISCUSSION
To develop an antibacterial fluoride varnish, we investigated the antibacterial activities of 4 components of P. corylifolia in comparison of xanthorrhizol with and without EFV. The inhibition zones of the antibacterial agents indicated that the most effective antibacterial agent against S. mutans was BCC, followed by BAK, and IBC, BCM, and XAN at both (1 and 10) mM. Those of IBC and BCM were not significantly different from XAN. Thus, we newly confirmed three effective antibacterial agents against S. mutans: BCC, IBC, and BCM. Among these, BCC was proved to have higher antibacterial activity than BAK and XAN, which were reported previously\(^{27,28}\). When the antibacterial agents were mixed with EFV, the inhibition zone of BCC was also the highest, and there were no significant differences among the other experimental groups.

The reasons for the antibacterial effects to differ for each substance can be the differences of chemical structure and molecular weight. Table 1 shows the difference of the number of hydroxyl groups and aromatic hydrocarbon, and the length of hydrocarbon chain. The greater the number of hydroxyl groups and the longer the length of hydrocarbon seem to increase the effectiveness of the inhibition of S. mutans. The extraction condition and the type of solvent during the extraction process can affect the antibacterial activity of the substances. Searching for other compounds with similar structures may lead to the discovery of additional effective antibacterial compounds.

When the antibacterial agents were combined with EFV, they showed significantly larger inhibition and semi-inhibition zones than for EFV alone. In particular, the semi-inhibition zones of the antibacterial agents in EFV showed an approximately 200% increase, compared to the inhibition zone of the antibacterial agents alone. Semi-inhibition zones appeared in the EFV alone, and the antibacterial agents combined with EFV. Thus, this phenomenon seemed to be induced by the effects of the fluoride varnish. In Fig. 4, the semi-inhibition zone of EFV with NaF was larger than that of EFV without NaF; whereas for the antibacterial agent mixed with EFV, the zone became much larger. This indicates that among the components of the fluoride varnish, rosin also has an inhibitory effect, but the effect became stronger when NaF added. All the antibacterial agents incorporated into EFV created a synergy effect on the inhibition of S. mutans. The semi-inhibition zones of XAN, BAK, and BCC of 1 mM were significantly larger than those of 10 mM (Fig. 3). It may be caused by the fact that semi-inhibition zone was affected not only by the antibacterial agents but also by the rosin and NaF components of EFV (Fig. 4). When the antibacterial agents combined with varnish, the inhibition zone was not consistent with the concentration of the antibacterial agents\(^{24}\). Microscopic observation showed that the semi-inhibition zone revealed less bacterial density than the bacterial zone (Fig. 1). The production of the semi-inhibition zone indicates a certain level of inhibitory effects on the bacteria, although the bacterial inhibition is not as much as in the inhibition zone. However, it is meaningful to have the ability to suppress the growth of bacteria beyond the level of the inhibition zone.

The mechanisms of antibacterial agents are based on the inhibition of cell-wall synthesis, protein synthesis, metabolic process, and DNA replication\(^{29}\). To prevent dental caries induced by cariogenic bacteria, three strategies are applied: Inhibition of the synthesis of water-insoluble glucans by glucosyltransferase, inhibition of the initial adhesion of S. mutans, and inhibition of the growth of S. mutans by antibacterial agents\(^{27,31}\). Although BCC was found to inhibit some gram positive bacteria\(^{32}\), it was not tested against S. mutans. The mechanism of the antibacterial activity of XAN is not yet known. According to Katsura et al.\(^{27}\), BAK showed bactericidal effect against S. mutans and also inhibited the adhesion of S. mutans. Therefore, the other derivatives of P. corylifolia with similar chemical structure are expected to have similar mechanism to BAK. However, the mechanism of the antibacterial activity of BAK is still under investigation and further studies are required to clearly prove the mechanism of the other antibacterial agents.

For cell viability, most antibacterial agents showed higher viability. In particular, BCC, IBC, and BCM, even at 1 mM, showed equivalent cell viability to the vehicle controls. Therefore, BCC, IBC, and BCM can be considered to be applied safely up to 1,000 µM. However, XAN and BAK at 1 mM showed lower cell viability than 70%. An antibacterial agent can be regarded as having cytotoxic potential if its cell viability is lower than 70%. In this study, we tested cell viability at different concentrations, to find the appropriate concentration for high cell viability with antibacterial activity. Further studies are needed to determine the optimal concentrations of the antibacterial agents for use in the mouth.

To prevent dental caries, the antibacterial agents investigated in the present study may be applied not only in fluoride varnish and dental materials, but also as ingredients in candies, gum, and oral rinse products. Further studies are necessary to assess the effect of the antibacterial agents on other cariogenic bacteria and normal flora in the mouth. In addition, it is necessary to assess the long-term sustainability of the activity of the antibacterial agents incorporated into the fluoride varnish.

In summary, all antibacterial agents used in the study showed effective antibacterial activities against S. mutans. Among them, we newly confirmed three
effective antibacterial agents, BCC, IBC, and BCM, against S. mutans, other than BAK and XAN, which were reported previously. The incorporation of the antibacterial agents into EFV resulted in superior antibacterial activities, compared with that of fluoride varnish alone. Among the antibacterial agents, BCC was proven to inhibit S. mutans most effectively with and without fluoride varnish. Therefore, we propose that antibacterial fluoride varnish containing BCC has clinical effectiveness in dentistry to prevent dental caries.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant, funded by the Korea government (MSIT) (No. 2018R1A2B6002088). The chemical library used in this study was kindly provided by the Korea Chemical Bank (http://www.chembank.org/) of the Korea Research Institute of Chemical Technology. The biological resources used in this research were distributed by KCTC (http://kctc.kribb.re.kr/).

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