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Inhibition of *Streptococcus mutans* biofilm accumulation and development of dental caries *in vivo* by 7-epiclusianone and fluoride

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Abstract

7-Epiclusianone (7-epi), a novel naturally occurring compound isolated from *Rheedia brasiliensis*, effectively inhibits the synthesis of exopolymers and biofilm formation by *Streptococcus mutans*. In the present study, the ability of 7-epi, alone or in combination with fluoride (F), to disrupt biofilm development and pathogenicity of *S. mutans in vivo* was examined using a rodent model of dental caries. Treatment (twice-daily, 60s exposure) with 7-epi, alone or in combination with 125 ppm F, resulted in biofilms with less biomass and fewer insoluble glucans than did those treated with vehicle-control, and they also displayed significant cariostatic effects *in vivo* ($p < 0.05$). The combination 7-epi + 125 ppm F was as effective as 250 ppm F (positive-control) in reducing the development of both smooth- and sulcal-carries. No histopathological alterations were observed in the animals after the experimental period. The data show that 7-epiclusianone is a novel and effective antibiofilm/anticaries agent, which may enhance the cariostatic properties of fluoride.

Keywords

benzophenone; *Streptococcus mutans*; dental caries; biofilms; fluoride; extracellular polysaccharides

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Introduction

Dental caries is among the most prevalent chronic human infections disease affecting both children and adults worldwide (Petersen et al. 2005; Dye et al. 2008). Colonization of tooth surfaces by mutans streptococci is associated with the etiology and pathogenesis of dental caries in humans (Loesche 1986; Beighton 2005). The ability of these organisms, particularly *Streptococcus mutans*, to synthesize extracellular glucans from sucrose using glucosyltransferases (Gtfs) is a major virulence factor (Yamashita et al. 1993). The Gtfs secreted by *S. mutans* (particularly GtfB and GtfC) bind avidly to the pellicle formed on the tooth surface and to surfaces of other oral microorganisms, and are highly active in the adsorbed state (Schilling and Bowen 1988; Vacca-Smith and Bowen 1998). The insoluble glucans synthesized by surface-adsorbed GtfB and GtfC provide specific binding sites for bacterial colonization on the tooth surface and to each other, modulating the formation of tightly adherent biofilms (Schilling and Bowen 1992; Paes Leme et al. 2006; Xiao and Koo 2010, Koo et al. 2010).

If dental biofilm is allowed to remain on tooth surfaces and is exposed to dietary carbohydrates frequently (especially sucrose), *S. mutans* as a member of the biofilm community will continue to synthesize polysaccharides and metabolize the sugars to organic acids. The elevated amount of extracellular polysaccharides (EPS) increases the biofilm stability and structural integrity, and provides protection to the bacteria from inimical influences of antimicrobials and other environmental assaults (Paes Leme et al. 2006). In addition, the ability of *S. mutans* to utilize some extra- and intracellular polysaccharides as short term storage compounds offers an additional ecological benefit, simultaneously increasing the amount of acid production and the extent of acidification. The persistence of this acidic environment leads to the selection of a highly acid tolerant flora (Marquis et al. 2003; Beighton 2005); the low pH environment within the matrix of the plaque results in demineralization of adjacent enamel, thus initiating the dental caries process. Therefore, EPS and acidification of the biofilm matrix are critical for the formation and establishment of cariogenic dental plaque (Bowen 2002; Marsh 2003), and offer primary targets for chemotherapeutic intervention (Koo et al. 2002; Koo and Jeon 2009).

Recently, a highly bioactive tetraprenylated benzophenone (7-epiclusianone) from the fruits of *Rheedia brasiliensis*, a native edible plant from Amazon region in Brazil, was isolated (Almeida et al. 2008). This naturally occurring compound markedly reduced glucan synthesis by GtfB and GtfC, and also disrupted the glycolytic activity of *S. mutans* cells. Furthermore, topical applications of 7-epiclusianone (7-epi) affected the formation and acidogenicity of *S. mutans* biofilms (Murata et al. 2008), indicating that it could be a potentially useful adjunctive anti-biofilm and cariostatic agent.

Fluoride in various preparations is the mainstay for caries prevention (Clarkson and McLoughlin 2000). However, as currently used it does not offer complete protection against the disease (Featherstone 2006). Fluoride exerts its major effects by reducing enamel-dentine demineralization and enhancing remineralization of early caries lesions. Furthermore, it also affects to some extent the biological activities of cariogenic streptococci, eg the inhibition of glucan synthesis and acidogenicity (Marquis et al. 2003). Thus, if an additional agent enhances the overall disruptive effects with respect to biofilm virulence, a way would be open for improving the cariostatic properties of fluoride without increasing its exposure (NIH 2001; Koo 2008).

Therefore, the aims of this study were (1) to examine whether the biological activities of 7-epi can effectively reduce the pathogenicity of *S. mutans* *in vivo* using a well-established rodent model of dental caries (Bowen et al. 1988), and (2) to determine whether the

inclusion of this compound could result in anticaries preparations with a lower concentration of fluoride.

Material and methods

Test agents

The fruits of *R. brasiliensis* were collected from trees growing under controlled conditions at the herbarium of the Federal University of Viçosa – UFV (latitude 20°45'14" south and longitude 42°52'55" west), Minas Gerais state, Brazil, where the voucher specimen (#VIC2604) is deposited. Dried and powdered *R. brasiliensis* fruit pericarps (1000 g) were extracted by maceration with 3 l of *n*-hexane at room temperature, filtered, and then dried, using a rotary evaporator under reduced pressure at 45°C (Santos et al. 1999; Murata et al. 2010). This procedure was repeated five times, yielding of 80 g of fruit pericarp extract. The pericarp hexane extract was chromatographed on a silica gel column and eluted with mixtures of *n*-hexane/ethyl-acetate and ethyl-acetate/ethanol of increasing polarity to obtain the compounds as previously described (Santos et al. 1999). Their structure was identified as the polyprenylated benzophenone 7-epiclusianone [(1R,5R,7R)-3-benzoyl-4-hydroxy-8,8-dimethyl-1,5,7-tris(3-methylbut-2-en-1-yl) bicyclo[3.3.1]non-3-ene-2,9-dione] using infrared, ultraviolet, and mass spectra data and nuclear magnetic resonance spectroscopy, and their molecular geometries established using single crystal X-ray diffraction (XRD) analysis (Santos et al. 1999). The purity level was > 99.85% as determined by HPLC (Almeida et al. 2008). Sodium fluoride (NaF) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

The 7-epiclusianone (250 µg ml⁻¹), with or without fluoride (125 ppmF), was prepared in 2.5 mM phosphate buffer (pH 7.2) containing 15% (v/v) ethanol. The positive (250 ppmF) and negative controls were also prepared in the same buffer containing 15% (v/v) ethanol. The final pH value of all the treatment solutions was 6.5. All the solutions were prepared just prior to carrying out the assays.

Biofilm assays

Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs (0.05" diameter × 0.04"–0.06" thickness; surface area of 2.7 ± 0.2 cm² from Clarkson Chromatography Products Inc., Williamsport, PA) placed in a vertical position in batch cultures at 37°C and 5% CO₂ (Koo et al. 2005, Duarte et al. 2008). Biofilms of *S. mutans* were formed in ultrafiltered (Amicon 10 kDa molecular weight cut-off membrane; Millipore Co., MA, USA) tryptone–yeast extract broth with addition of 30 mM sucrose (Koo et al. 2005). The biofilms were grown undisturbed for 24 h to allow initial biofilm formation. At this point (24 h-old), the biofilms were treated twice daily (10 am and 4 pm) with a 60 s exposure each time until the fifth day of the experimental period (120 h-old biofilm) with one of the following: 7-epi (250 µg ml⁻¹), 7-epi + 125 ppm fluoride (F), 250 ppm F, and a vehicle-control (15% ethanol, v/v). The concentration of 7-epiclusianone was selected based on data from the authors' previously published response to dose study on inhibition of specific virulence attributes of *S. mutans* (eg GtfB and GtfC activity) and biofilm formation (Murata et al. 2008). Fluoride at 125 ppm F was not included because it is devoid of any significant effects on biofilm biomass (see Supplementary data [Supplementary material is available *via* a multimedia link on the online article webpage]). Fluoride at 250 ppm is a clinically proven anticaries agent and this is the concentration found in most of the currently available fluoride-based mouth rinses (Zero 2006). Treatments with 15% (v/v) ethanol allowed continued biofilm formation, and did not affect the biochemical composition or cell viability when compared to biofilms treated with saline solution. The biofilms were exposed to the treatments for 60 s, double-dip rinsed in sterile saline solution and transferred to fresh

culture medium. The treatments and rinsing procedures were repeated 6 h later. The culture medium was replaced daily. Each biofilm was exposed to the respective treatment a total of eight times.

Biofilm analyses

At the end of the experimental period, the biofilms were removed and subjected to sonication using three 30-s pulses at an output of 7W (Branson Sonifier 150; Branson Ultrasonics, Danbury, Conn., USA) (Koo et al. 2003); the sonication procedure provided the maximum recoverable viable counts. The homogenized suspension was analyzed for biomass (dry weight), bacterial viability (cfu per mg of biofilm dry weight), and polysaccharide composition. The extracellular water soluble and water insoluble polysaccharides, and intracellular iodophilic polysaccharides (IPS) were extracted and quantified by colorimetric assays as detailed by Koo et al. (2003) and Duarte et al. (2008); the exopolysaccharides were quantified by the phenolsulfuric method (Dubois et al. 1956) using glucose as standard whereas IPS was quantified using 0.2% I₂/2% KI solution and glycogen as standard, as described by DiPersio et al. (1974).

Animal study

The animal experiment was reviewed and approved by the Ethical Committee on Animal Research at the University of Campinas, Campinas, SP, Brazil – UNICAMP (Protocol # 963-1) and was performed according to methods described previously (Bowen et al. 1988; Koo et al. 1999). A total of 48 SPF female pups from 7 litters of SPF Wistar rats were provided by CEMIB (UNICAMP). At weaning, pups of the rats aged 21 days were infected with *S. mutans* UA159, and randomly placed into four groups of 12 animals. From this point, the molar teeth of the animals were treated topically by means of a camel hair brush twice daily, as follows: 7-epi (250 µg ml⁻¹), 7-epi + 125 ppm F, vehicle control (15% ethanol, v/v), and 250 ppm F (positive control). The authors have previously shown that twice daily treatment with 125 ppm F is significantly less effective (cariostatic) than 250 ppm F both on smooth- and sulcal-carries *in vivo* (Koo et al. manuscript submitted; also see Supplementary data [Supplementary material is available *via* a multimedia link on the online article webpage]), thus, 125 ppm F was not included in this study. Each group of 12 animals was provided with diet 2000 (which contains 56% sucrose) and 5% sucrose water *ad libitum* (Bowen et al. 1988). The animals were weighed weekly, and their behavior and physical appearance was noted daily. The experiment proceeded for 5 weeks, at the end of which the animals were euthanized by CO₂ asphyxiation. The lower left jaw was aseptically dissected, suspended in 5.0 ml of sterile saline solution, and sonicated (three 10 s pulses at 5 s intervals, at 30 W, Vibracell, Sonics & Material Inc.; this procedure provides the maximum recoverable viable counts). The suspension was plated on mitis salivarius agar plus bacitracin to estimate the *S. mutans* populations and on blood agar to determine the total cultivable microbiota (Bowen et al. 1988). The smooth and sulcal surfaces caries and their severity (Ds, dentin exposed; Dm, 3/4 of the dentin affected; Dx, all dentin affected) were evaluated according to Larson's modification of Keyes' system (Larson 1981). The determination of the caries score was blinded by codification of the jaws and was done by one calibrated examiner.

Histopathological evaluation

Histopathological evaluations were performed for all rats. Tissue samples obtained from the heart, lung, pancreas, spleen, liver, stomach, intestine, kidney, ovary, brain, eyes, tongue, palatal tissue, and submandibular glands of euthanized rats were fixed with 12% formalin (Lillie 1965). Thereafter, the tissue samples were dehydrated in a graded alcohol series and embedded in paraffin, sectioned at ~5 µm, stained with hematoxylin and eosin to be examined under a light microscope (Carl Zeiss, Germany) (Lillie 1965).

Statistical analyses

For the *in vitro* studies, the data were analyzed by ANOVA, and the *F*-test was used to detect difference between and among the groups. When significant differences were detected, pair wise comparisons were made among all the groups by Tukey's method to adjust for multiple comparisons. The data from animal study were subjected to ANOVA and Tukey–Kramer Honest Standard Deviation (HSD) test for all pairs. Statistical software JMP version 3 (SAS Institute 1990) was used to perform the analyses. The level of significance was set at 5% for both studies.

Results and discussion

The discovery of novel chemotherapeutic agents, other than microbiocides, that disrupt the establishment and virulence of biofilms is a promising route to prevent or reduce infectious diseases, eg dental caries (Cegelski et al. 2008; Koo and Jeon 2009). Furthermore, such agents could be used to enhance the effectiveness of the protective effects of fluoride, which (despite some antibacterial effects) does not address effectively the infectious character of the disease. Natural products are still major sources of innovative therapeutic agents for infectious diseases (Newman 2008). Exploration of biodiversity from rich environments, such as Brazil, has led to the discovery of 7-epiclusianone (7-epi), a novel pharmacologically active compound (Almeida et al. 2008; Murata et al. 2010). Here, the authors expand further on their previous *in vitro* findings (Murata et al. 2008) by showing that 7-epi reduces the development of dental caries disease *in vivo*. Furthermore, this agent could also reduce the concentration of fluoride required for therapy.

7-Epi reduces development of dental caries *in vivo*

The present study has shown that topical applications of 7-epi significantly reduced the formation of biofilms by *S. mutans* on a saliva-coated apatitic surface and effectively disrupted the amounts of exopolysaccharides in the extracellular matrix (50–70% reduction; vs the vehicle-control, $p < 0.05$) (Table 1) confirming previous findings (Murata et al. 2008). Thus, a study was carried out to determine whether the antibiofilm activities of 7-epi would be translated into cariostatic effects *in vivo*, using a well-established rodent model of dental caries (Bowen et al. 1988; Koo et al. 2005). Rats harbor a complex and mixed oral flora even though in the present study they were infected by *S. mutans* UA159, a proven virulent (cariogenic) bacterium (Ajdic et al. 2002). As shown in Tables 2 and 3, animals treated with 7-epi resulted in 70–80% less severe smooth-surface lesions and 50–70% less severe sulcal-surface lesions than the vehicle control treatment (at Dm, 3/4 of the dentin was affected, and Dx, the whole dentin was affected, levels; $p < 0.05$). These effects in reducing development of carious lesions clearly indicate that the active compound is available at an efficacious concentration (despite brief exposure and clearance in the oral cavity) when a sucrose-rich diet is ingested by the animals (vs the vehicle-control, $p < 0.05$). The persistence of a therapeutic effect of a topically applied agent in the oral cavity is a highly desirable property in developing novel chemotherapeutic approaches against biofilm-related oral diseases, such as dental caries (Brecx 1997). It is noteworthy that the total and the *S. mutans* UA159 viable counts recovered from the animals' plaque were not significantly affected by treatments with 7-epi when compared to the vehicle-control treatments ($p > 0.05$), which agrees well with its lack of bactericidal activity against biofilms formed *in vitro* (Table 1).

These observations indicate that the anticaries mechanisms of 7-epi may be related to combined inhibitory effects on several virulence attributes of *S. mutans*, including exopolysaccharides synthesis, and the acidogenic and aciduric properties of this pathogen (Murata et al. 2008). The test agent is a potent noncompetitive/uncompetitive inhibitor of enzymatic activity of GtfB (which synthesizes primarily insoluble glucans rich in alpha 1,3-

linkages) and GtfC (which synthesizes soluble and insoluble glucans containing alpha 1,6- and alpha 1,3-linkages) (Murata et al. 2008). The inhibition of these specific Gtfs is critical because of their distinct roles in biofilm formation by *S. mutans* (Koo et al. 2010). GtfC is primarily localized on the apatitic surface forming glucans *in situ* that serve as bacterial binding sites promoting adherence and accumulation of *S. mutans* on the surface (Vacca-Smith and Bowen 1998; Koo et al. 2010). On the other hand, GtfB binds with greater avidity to the *S. mutans* cell-surface (and to other oral bacteria) than do the other enzymes, and more importantly in an enzymatically active form (Vacca-Smith and Bowen 1998). The presence of surface-bound GtfB-glucans enhances the binding of bacterial cells to each other promoting the formation of highly structured microcolonies, and further accumulation of biofilms on the apatitic surface (Koo et al. 2010). In addition, Gtf enzymes are proven virulence factors associated with development of caries disease *in vivo* (Yamashita et al. 1993). Thus, the effective inhibition of GtfB and GtfC would significantly reduce the synthesis of insoluble exopolysaccharides, and thereby disrupt the establishment of the extracellular matrix and biofilm biomass without necessarily impacting the vitality of the bacterial cells.

Furthermore, 7-epi also affects the glycolytic activity and acidurance of *S. mutans* by causing disturbances of the net membrane permeability to protons, and consequently acidification of the cytoplasm (Murata et al. 2008). The combination of a weak-acid effect and partial inhibition of *S. mutans* membrane-associated F-ATPase likely lowered the ΔpH across the cell membrane (Murata et al. 2008), which could disrupt the activity of pH-sensitive enolase and other enzymes of the glycolytic pathway (Belli et al. 1995). These activities resulted in less acidogenic biofilms of *S. mutans* when treated with 7-epi twice daily (Murata et al. 2008). Nevertheless, it is also possible that the overall inhibitory effects on bacterial metabolism could affect the physiological responses and growth rate of this organism in biofilms. The data not only validate previous *in vitro* studies (Murata et al. 2008) but also support the hypothesis that is possible to prevent or reduce caries disease by disrupting the virulence attributes and establishment of *S. mutans* within biofilms.

The overall toxicity of 7-epi was investigated through histological evaluation of several organs obtained from all rats. External examination of the rats revealed that the status of nutrition and development was normal, without any signal of disease and with no significant differences in the weight gains among the groups (data not shown). The skin and natural orifices showed no morphologic alterations. Macroscopic and microscopic observation of heart, lung, pancreas, spleen, liver, stomach, intestine, kidney, ovary, brain, eyes, tongue, palatal tissue, and submandibular glands demonstrated no difference among the experimental groups and there were no detectable pathological signs.

Treatments with 7-epi alone clearly reduced the development of carious lesions *in vivo* although the anticaries effects were not as effective as the positive control (250 ppm F, Tables 2 and 3).

7-Epi and fluoride as an alternative anticaries combination therapy

Enhancement of the protective effects of fluoride by including substances in the preparation which affect the virulence of cariogenic bacteria and/or enhance the antibacterial effects of fluoride clearly has clinical potential to reduce the prevalence of dental caries without increasing (and possibly decreasing) the concentration of fluoride exposure (NIH 2001;Koo 2008). 7-Epi may enhance the cariostatic properties of fluoride because of their interconnected biological activities against *S. mutans*, which could also reduce the concentration of fluoride required for therapy. Fluoride, at the levels found in plaque, disrupts the proton permeability of the *S. mutans* cells membrane, affecting its glycolytic activity and production–secretion of Gtfs (Marquis et al. 2003;Koo et al. 2006). The aim

here was to demonstrate whether the combination of 7-epi with 125 ppm F can be as cariostatic as (or better than) 250 ppm F (a clinically proven anti-caries agent).

In this study, it is shown that 7-epi in combination with a lower concentration of fluoride (125 ppm F) was highly effective in reducing the biomass and total amounts of protein, extracellular, and intracellular polysaccharides (IPS) of *S. mutans* biofilms *in vitro* (40–70% reduction) when compared with the vehicle control ($p < 0.05$; Table 1). In contrast, 7-epi alone did not inhibit the accumulation of IPS in the treated biofilms whereas fluoride alone (at 125 or 250 ppm F) was unable to reduce the exopolysaccharide accumulation in the *in vitro* biofilm model. Fluoride greatly reduces the ATP pools in biofilm cells, and the net result is a marked reduction in IPS synthesis (Marquis et al. 2003; Koo et al. 2005). The IPS, a glycogen-like storage polymer, can be metabolized when exogenous fermentable substrates have been depleted in the oral cavity. As a result, IPS can promote the formation of dental caries by prolonging the exposure of tooth surfaces to organic acids with a concomitant lower fasting pH in the matrix of the plaque (Tanzer et al. 1976; Spatafora et al. 1995).

The combination of agents also effectively reduced the incidence and severity of smooth-surface and sulcal caries compared with the vehicle control group ($p < 0.05$; Tables 2 and 3). The total cultivable microflora and *S. mutans* populations were unaffected by the treatments ($p > 0.05$; Table 4). More importantly, the combination of agents was as effective as the positive control (250 ppm F) in reducing the development of both smooth- and sulcal-caries. It is noteworthy that addition of 125 ppm F to epi-7 preparations had a comparatively larger cariostatic effect on the severity of sulcal surface lesions than on a smooth surface. Although 125 ppm F has negligible effects on biofilm biomass, EPS or the incidence of sulcal-caries, it does reduce (i) IPS accumulation by *S. mutans* biofilm cells and (ii) the severity of sulcal carious lesions (albeit not as effectively as 250 ppm F) (see Supplementary data [Supplementary material is available via a multimedia link on the online article webpage]). Thus, the *in vitro* and *in vivo* data suggest that 7-epi acting in concert with 125 ppm F could result in enhanced cariostatic activity by simultaneously disrupting insoluble-exopolysaccharides and IPS synthesis by *S. mutans* within biofilms. This would affect *in situ* plaque-matrix formation, acid production, and bacterial metabolism/physiology modulating the formation of pathogenic biofilms related to caries disease. At the same time, the presence of fluoride reduces the demineralization and enhances the remineralization process of enamel/dentin at the biofilm-tooth interface (ten Cate 1999).

Collectively, the data show that 7-epi reduces caries development by targeting the major virulence factors of *S. mutans* and could be used in combination with a lower concentration of fluoride without reducing its cariostatic effectiveness. The combination of 7-epi with fluoride may represent a potentially useful alternative to the current chemotherapeutic strategies to prevent dental caries disease. Additional studies are warranted to optimize the 7-epi and fluoride combinations (both in terms of concentration of agents and treatment regimen), and to elucidate the molecular mechanisms of action of these agents. This would provide a guide for enhancing further the effectiveness of this anticaries chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Effects of 7-epiclusianone (7-Epi), fluoride (F) and associations on the composition of *S. mutans* UA159 biofilm.

Treatments*	Dry weight (mg)	Insoluble polysaccharide (mg)	Soluble polysaccharide (μg)	Iodophilic polysaccharide (μg)	cfu (×10 ⁶)
Vehicle control	5.0 (0.1) ^a	1.6 (0.2)	186.2 (29.5) ^a	135.6 (16.2) ^a	1.3 (0.3) ^a
7-Epi 250 μg ml ⁻¹	2.2 (0.2) ^b	0.8 (0.1) ^b	92.3 (8.8) ^b	139.7 (28.5) ^a	1.2 (0.2) ^a
7-Epi 250 μg ml ⁻¹ + 125 ppm F	2.2 (0.2) ^b	0.9 (0.1) ^b	90.2 (23.2) ^b	86.6 (18.6) ^b	1.0 (0.4) ^a
Positive control (250 ppm F)	4.6 (0.2) ^a	1.7 (0.2) ^a	196.3 (15.1) ^a	85.3 (13.2) ^b	0.9 (0.3) ^a

Values (SD, *n* = 12), for each parameter, followed by the same superscripts are not significantly different from each other (*p* > 0.05, ANOVA, comparison for all pairs using Tukey test).

All results were expressed in dry weight by biofilm disc.

* Twice daily with 1-min exposure for each treatment.

Table 2

Effects of 7-epiclusianone (7-Epi), fluoride (F) and associations on caries development (smooth-surface and dentin severity) in rats, after a 5-week experiment.

Treatments*	Smooth surface			
	Total	Severity		
		Ds	Dm	Dx
Vehicle control	76.2 (10.1) ^a	42.5 (11.8) ^a	18.3 (7.4) ^a	6.4 (7.2) ^a
7-Epi 250 µg ml ⁻¹	62.9 (7.4) ^b	25.5 (7.8) ^b	3.3 (4.8) ^b	1.9 (3.5) ^b
7-Epi 250 µg ml ⁻¹ + 125 ppm F	33.2 (2.6) ^c	21.0 (7.7) ^b	0.3 (0.5) ^b	n.d.
Positive control (250 ppm F)	34.2 (4.5) ^c	22.5 (6.5) ^b	0.5 (0.8) ^b	n.d.

Values denote means with SD in parentheses ($n = 12$) of Keyes' scores modified by Larson. Values, for each parameter, followed by the same superscripts are not significantly different from each other ($p > 0.05$; ANOVA; comparison for all pairs using the Tukey–Kramer HSD).

Ds, dentin exposed; Dm, 3/4 of the dentin affected; Dx, whole dentin affected; n.d., non-detected.

* Twice daily with 1-min exposure for each treatment.

Table 3

Effects of 7-epiclusianone (7-Epi), fluoride (F) and associations on caries development (sulcal and dentin severity) in rats, after a 5-week experiment.

Treatments*	Sulcal surface			
	Total	Severity		
		Ds	Dm	Dx
Vehicle control	46.5 (4.3) ^a	38.2 (2.8) ^a	30.8 (5.1) ^a	21.0 (7.7) ^a
7-Epi 250 µg ml ⁻¹	39.7 (5.6) ^b	24.8 (5.3) ^b	14.7 (5.2) ^b	5.3 (4.5) ^b
7-Epi 250 µg ml ⁻¹ + 125 ppm F	25.7 (3.0) ^c	13.4 (4.7) ^c	2.2 (1.9) ^c	0.6 (1.0) ^c
Positive control (250 ppm F)	28.5 (3.1) ^c	15.7 (3.6) ^c	2.7 (1.5) ^c	0.3 (0.8) ^c

Values denote means with SD in parentheses ($n = 12$) of Keyes' scores modified by Larson. Values, for each parameter, followed by the same superscripts are not significantly different from each other ($p > 0.05$; ANOVA; comparison for all pairs using the Tukey–Kramer HSD).

Ds = dentin exposed; Dm = 3/4 of the dentin affected; Dx = whole dentin affected.

* Twice daily with 1-min exposure for each treatment.

Table 4

Effects of treatments on total and *S. mutans* UA159 viable populations recovered from the plaque of the animals.

Treatments*	<i>S. mutans</i> UA159 (cfu $\times 10^6$)	Total microflora (cfu $\times 10^6$)
Vehicle control	2.0 (1.6) ^a	3.0 (1.7) ^a
7-Epi 250 $\mu\text{g ml}^{-1}$	1.2 (0.4) ^a	1.7 (0.7) ^a
7-Epi 250 $\mu\text{g ml}^{-1}$ + 125 ppm F	1.7 (0.8) ^a	3.0 (1.1) ^a
Positive control (250 ppm F)	1.2 (0.3) ^a	2.0 (0.6) ^a

Values denote means with SD in parentheses ($n = 12$). Values, for each parameter, followed by the same superscripts are not significantly different from each other ($p > 0.05$; ANOVA; comparison for all pairs using the Tukey–Kramer HSD).

* Twice daily with 1-min exposure for each treatment.