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Preventive effects of an original combination of grape seed polyphenols with amine fluoride on dental biofilm formation and oxidative damage by oral bacteria

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Abstract

Aims: To investigate the preventive effects of an original combination of a grape seed extract (GSE) with an amine fluoride (Fluorinol®) on dental plaque formation and oxidative damage caused by oral bacteria.

Methods and Results: The antibacterial activity of the compounds was assessed using the broth macrodilution method, and their antiplaque activity was evaluated on a multispecies biofilm grown on saliva-coated hydroxyapatite discs. The effect on glucosyltransferases activity was analysed through reductions in the overall reaction and the quantity of insoluble glucan synthesized. The combination of 2000 µg ml⁻¹ of GSE with 10 mg ml⁻¹ of Fluorinol® significantly decreased the biofilm formation (up to 4 log₁₀ of reduction) and inhibited by 97.4% the insoluble glucan synthesis by glucosyltransferases. The antioxidant activity of this combination, alone or incorporated into a formulated mouthwash (Eludril daily®), was determined using the Trolox equivalent antioxidant capacity assay (TEAC), and both showed significantly greater antioxidant capacity than vitamin C.

Conclusions: The GSE/Fluorinol® combination showed both a significant antiplaque activity and an important antioxidant capacity in vitro, without any bactericidal effects.

Significance and Impact of the Study: This is, to our knowledge, the first report on the properties of an original combination of a polyphenolic extract with amine fluoride that could be used for the prevention of oral diseases and oxidative damage associated.

Keywords
adherence, amine fluoride, antioxidant, glucosyltransferases, Oral biofilm, polyphenols.

Introduction

Oral diseases, such as dental caries, periodontitis and oral mucosal lesions, are major public health problems worldwide, and their effects on general health and quality of life are substantial (Petersen et al. 2005). According to the World Health Organization Report 2012 worldwide, 60–90% of school children and nearly 100% of adults have dental cavities. Moreover, severe periodontal disease, which may result in tooth loss, is found in 15–20% of middle-aged (35–44 years) adults (Petersen 2003; WHO 2012). The major aetiological agent for these diseases is the dental plaque, a biofilm within micro-organisms that are closely associated and embedded in a matrix of bacterial and salivary exo-polymers (Listgarten 1999). In the healthy state, both dental biofilm and adjacent host tissues maintain a delicate balance, establishing a harmonious relationship between the two, and some oral pathologies may arise when this equilibrium is compromised and when an imbalance occurs among the indigenous bacteria (Liljemark and Bloomquist 1996).

Although tooth brushing is an effective method to mechanically remove the dental biofilm, it is not always sufficient and antiplaque chemicals are needed (Marsh...
and Bradshaw 1993). Several classes of compounds are used as antiplaque agents in dentistry. These molecules mainly include chlorhexidine gluconate, bis-biquanides, quaternary ammonium salts and iodine derivatives (Marechal 1991). Unfortunately, most of these therapeutic agents are not without long-term side effects whose primary is the imbalance of oral ecosystem, caused mainly by their bactericidal properties. It is for this reason that antiplaque agents with little or no direct bactericidal activity are sought. Numerous in vitro and in vivo studies have been conducted and have shown that polyphenols possess interesting antiplaque properties, without bactericidal activity (Daglia et al. 2002; Weiss et al. 2004; Petti and Scully 2009). In the same line of thought, it has been reported that fluoride exerts its antiplaque activity through an indirect antimicrobial activity, primarily by reducing overall acid production and by inhibiting the enamel demineralization (Shellis and Duckworth 1994; Bradshaw et al. 2002).

In addition, as microbial adherence is considered as an essential first step in dental plaque formation, it seems worthwhile to search for molecules having antiadhesive effects. The synthesis of insoluble glucans by glucosyltransferases (GTFs) from Streptococcus sp. contributes significantly to the bacterial colonization (Yamashita et al. 1993). Thus, a primary means of controlling bacterial adherence and oral biofilm formation would be to inhibit the functions of these enzymes, and precisely, these inhibitory effects on GTFs activity have been demonstrated for some polyphenolic extracts (Ooshima et al. 1994; Osawa et al. 2001; Xiao et al. 2007).

On the other hand, the deposit of dental plaque is not the only mechanism involved in the development of oral diseases. Indeed, many studies have shown that the excessive production of reactive oxygen species (ROS) by oral bacteria could lead to oxidative damages and inflammation of gingival tissues, periodontal ligament and alveolar bone (Canakci et al. 2005). ROS present in periodontal pockets can come from a direct production by some dental plaque bacteria as the generation of hydrogen peroxide by viridans streptococci (Okahashi et al. 2013). However, most of ROS might be produced by polymorphonuclear leucocytes (PMN) activated by the host inflammatory cells in response to the overgrowth of oral pathogens (Canakci et al. 2005). This abnormal host response leading to oxidative stress damages and periodontal diseases development is probably due to an imbalance between ROS and antioxidants due to the both an increase in free radical production and a defect in the total antioxidant activity of saliva (Sculley and Langley-Evans 2002). Thus, the intake of molecules limiting the production of ROS by their antiplaque activity and also with significant antioxidant properties seems to be an interesting strategy to control the periodontal diseases development, and polyphenols are likely candidates (Bagchi et al. 1998; Houde et al. 2006).

In a previous work, we showed the antiplaque and antioxidant activities of a grape seed extract (GSE), a rich source of polyphenolic compounds, and tested for the first time for oral hygiene purposes (Furiga et al. 2009). GSE inhibited the biofilm formation in a dose-dependent manner until the concentration of 2000 μg ml⁻¹ (sub-MBC levels), to which GSE showed its optimal antibiofilm activity. Indeed, experiments performed with higher concentrations (4000–10000 μg ml⁻¹) in this extract led to a fall of its effectiveness on the biofilm formation. GSE presented a high Trolox equivalent antioxidant capacity (TEAC) value, proving its capacity to scavenge free radicals.

To improve these first results, we investigated in this study the combination of GSE with an amine fluoride (Fluorinol®; Pierre Fabre Laboratories, Castres, France), known for its protective properties on the development of caries (Griffin et al. 2007). For this, we examined the capacity of the combination, versus each compound tested alone, to inhibit multispecies biofilm formation and glucosyltransferases activity, and its antioxidant activity. Moreover, given that many compounds may be ineffective when incorporated into mouthrinse formulations and that polyphenols are generally unstable in oxidative conditions (Herrera et al. 2003), we finally checked the stability of the combination by analysing its antioxidant capacity in a formulated mouthwash (Eludril Daily®, Pierre Fabre Laboratories, Castres, France).

Materials and methods

Chemicals and mouthwash

Grape seed extract, 3-pyridinemethanol hydrofluoride (Fluorinol®) and Eludril Daily® mouthwash were provided from the Pierre Fabre Laboratories (Castres, France). GSE was extracted from the vine Vitis vinifera by Societé Française de distillerie (Vallon Pont d’Arc, France). It contains 97% (w/v) polyphenols, mainly catechin and epicatechin (polyphenolic contents determined by the supplier). Eludril Daily® mouthwash is composed of water, 2000 μg ml⁻¹ of GSE, 10.2 mg ml⁻¹ of Fluorinol®, xylitol, PEG-40, hydrogenated castor oil, citral, aroma (flavour), limonene, sodium hydroxide, sodium methylparaben, sodium propylparaben and sodium saccharin.

Bacterial strains and growth conditions

The micro-organisms tested in this study were Streptococcus mutans ATCC 25175, Streptococcus sobrinus ATCC
33478, Lactobacillus rhamnosus ATCC 7469, Actinomyces viscosus ATCC 15987, Porphyromonas gingivalis ATCC 33277 and Fusobacterium nucleatum ATCC 10953. All strains were obtained from the Institut Pasteur collection (Paris, France) and were cryo-preserved at −80°C. Before each experiment, two subcultures were prepared in Trip-Tory Broth (Difco, Sparks, MD, USA) for Actinomyces and Streptococcus, in MRS medium (Merck, Darmstadt, Germany) for the lactobacilli and in Wilkins Chalgren Anaerobe broth (Oxoid, Dardilly, France) for the anaerobes and incubated at 37°C for 24 h. Streptococcus mutans, Strep. sobrinus, Act. viscosus and Lactobacillus rhamnosus are closely associated with the caries diseases, and P. gingivalis and Fus. nucleatum are major putative pathogenic bacteria that occur in moderate to severe chronic or aggressive periodontitis.

Antimicrobial susceptibility testing

The antibacterial activity of GSE (250–8000 µg ml⁻¹) and Fluorinol® (2.5–10.2 mg ml⁻¹) for the six reference strains was examined by determining the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) using the broth macrodilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2009).

For the six bacterial strains, an overnight culture of 5 × 10⁵ CFU ml⁻¹ was inoculated into tubes containing twofold dilutions of the test compounds or ultrapure water (control). The MIC was determined as the lowest concentration of test compound that will inhibit the visible growth of bacteria after 24-h incubation at 37°C. For MBC determination, an aliquot (30 µl) from tubes containing no visible growth was diluted 100-fold in ultrapure water and then subcultured onto specific media. The plates were incubated anaerobically at 37°C for 48–72 h. The MBC was defined as the lowest concentration of test compound that did not permit any visible growth on the appropriate agar plate after the incubation period (99.9% killed). Each concentration of the test compounds was tested in triplicate.

Inhibitory effect on multispecies biofilm

Saliva collection

Whole unstimulated saliva was collected into a sterile chilled tube, via a spitting method, from 15 healthy adult volunteers, nonsmoking and not taking antibiotic treatment for at least 3 months. The volunteers had refrained from eating, drinking and brushing teeth for at least 1–5 h prior to collection. Saliva samples were pooled in sterile tubes and centrifuged at 12,000 g for 30 min at 4°C to remove cellular debris. The resulting supernatant was then pasteurized at 65°C for 30 min and re-centrifuged in sterile tubes. The resulting supernatant was immediately dispensed into sterile 50-ml polypropylene tubes and stored at −20°C. The efficacy of pasteurization was assessed by plating 100 µl of processed saliva samples onto Columbia Blood agar plates and no CFU (i.e. detection limit of 10 CFU ml⁻¹) observed after 72 h at 37°C on either aerobically or anaerobically incubated plates.

Biofilm assay

The biofilm was assayed according to the model developed by Guggenheim et al. (2001) with few modifications as previously described (Furiga et al. 2008b).

Briefly, hydroxyapatite (HA) discs (Clarkson Chromatography Products Inc., Williamson, PA, USA) were placed in a well of a sterile 24-well cell culture plate and incubated with pasteurized saliva (800 µl) for 4 h at room temperature with gently shaken to allow for formation of a salivary pellicle. Then, saliva was aspirated from each well and replaced with a mixture of 800 µl of pasteurized saliva, 800 µl of fluid universal medium (FUM) containing 0.15% (w/v) glucose and 0.15% (w/v) sucrose and 200 µl of bacterial inoculum prepared by combining 1 ml of overnight precultures (OD₅₅₀nm = 1.0 ± 0.02) of each of the six strains previously cited. The inoculum contained reproducibly between 10⁷ and 10⁸ CFU of each species per ml. After anaerobic incubation for 16 h at 37°C, HA discs were 1 min exposed to GSE (2000 µg ml⁻¹), or Fluorinol® (6.8 and 10.2 mg ml⁻¹), or the combination of the two compounds at these same concentrations, or ultrapure water (control), rinsed and then replaced in their wells. This treatment was repeated after four and 8 h. Between each exposition, plate was incubated anaerobically at 37°C. After 16-h incubation, discs were again threefold exposed to test compounds at 4-h intervals. Incubation media were replaced following the first and fourth 1-min exposure.

Harvesting the biofilm

At the end of the experiment (age of biofilm of 64 h), HA discs were washed with physiological saline and placed in a sterile plastic Petri dish, and their surface was scraped with a sterile dental root curette. The surface of the scraped disc and the Petri plate were then rinsed with 1 ml of physiological saline. Aliquots of harvested biofilm were diluted and spiral-plated onto Mitis Salivarius agar + tellurite (Difco, Sparks, MD, USA) for Streptococcus, MRS agar (Difco) for Lactobacillus, Trypticase Soy agar (Difco) for Actinomyces or Wilkins and Chalgren Anaerobe agar supplemented with blood and GN supplement (Oxoid, Dardilly, France) for Fusobacterium and Porphyromonas. After 48–72 h of incubation at 37°C, Gram staining was performed to confirm the identity of
species on each medium. The CFU per population for triplicate discs were averaged and subjected to logarithmic transformation.

Confocal laser scanning microscopy
Noninvasive confocal imaging of the biofilms (t = 64 h) was accomplished with a Confocal Visible Leica DMR TCS SP2 AOBS fitted with water-immersion dipping lenses (×63). Specimens were stained with LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Cergy-Pontoise, France) for microscopy according to the manufacturer’s instructions. An excitation wavelength of 488 nm was used, and all light rays emitted above 500 nm were collected. Biofilm structure was analysed in duplicate by taking a series of horizontal sections, each with a 1-μm thickness. Digital images were processed using Leica Confocal Software Lite (Leica Microsystems, Wetzlar, Germany).

Effect on glucosyltransferases activity
Crude cell-free GTF preparation was obtained from a culture of Strep. sobrinus ATCC 33478 by ammonium sulfate precipitation as previously described (Furiga et al. 2008a).

For inhibition assays, test compounds, dissolved in ultrapure water, were added to the reaction mixture composed of the crude GTF preparation (0·1 U ml⁻¹), 65 mmol l⁻¹ K₂HPO₄ buffer (pH 6·5), 50 g l⁻¹ sucrose, 0·1 g l⁻¹ NaN₃ and 2 g l⁻¹ dextran T10. Incubation was carried out at 37°C, and the reaction was stopped by heating the samples at 100°C for 10 min.

The initial reaction rate was determined by measuring the reducing sugar concentration increase over the three first hours of reaction by dinitrosalicylic acid assay, with fructose as a standard (Sumner and Howell 1935). One unit of total GTF activity (U) was defined as the amount of enzyme that catalysed the release of reducing sugars equivalent to 1 μmol of fructose per min under standard conditions. Appropriate substrate and enzyme blanks were included to correct for any free reducing group not emanating from sucrose transformation.

To determine the amount of insoluble glucan synthesized, the heated samples were centrifuged (17 600 g, 30 min, 4°C) after 24-h incubation. The insoluble glucan was recovered from the pellet, washed three times with ultrapure water, dried at 65°C for 24 h and then weighed.

Trolox equivalent antioxidant capacity assay
The method is based on the capacity of a sample to scavenge the ABTS [2,2′-azinobis(3-ethylenothiazoline-6-sulfonic acid) diammonium salt] radical cation (ABTS⁺), as compared to standard antioxidant Trolox [6-hydroxy-2,5,7,8-tetramethyloxigen-2-carboxylic acid]. All chemical reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

ABTS⁺ solution was generated from ABTS as previously described (Re et al. 1999). GSE, Fluorinol®, ascorbic acid (as control) and Trolox (as standard) were dissolved in phosphate-buffered saline (PBS). Then, 20 μl of each test compound solution, or Eludril Daily® mouthwash, was added to the ABTS⁺ solution (1980 μl). The absorbance at 734 nm was read exactly 6 min after initial mixing. Appropriate solvent blanks were run in each assay.

Three different dilutions of the compound under investigation were selected, which produced absorbance values in the most linear region of the Trolox–response curve (20–80% inhibition of the blank value). All determinations were carried out at least three times, and the three dilutions were analysed in triplicate. The dose–response curves obtained with the test compound and Trolox were plotted as the percentage of absorbance decrease against the amount of antioxidants expressed as μg ml⁻¹ (samples) or in micromolar units (Trolox). The TEAC of the test compound was calculated as the ratio between the slopes of the dose–response curves of the test compound and Trolox.

Statistical analysis
Student’s t-test was used to calculate the significance of the difference between the mean effects of a given compound compared with the control group. For each assay, all determinations were carried out in triplicate, from three independent experiments. Statistically significant values were defined as P < 0·05, P < 0·01 or P < 0·001.

Results
Effects on planktonic cells
The antibacterial activity of GSE against the six oral bacterial strains tested showed high MIC and MBC values (Table 1). The MBC was always found to be two- to fourfold higher than MIC value. No bactericidal activity was obtained for Fluorinol® at the highest tested concentration of 10·2 mg ml⁻¹ (data not shown).

Antibiofilm activity
Grape seed extract (2000 μg ml⁻¹), Fluorinol® (6·8–10·2 mg ml⁻¹) and the combination of both were tested at sub-MBC levels for their ability to impair the multispecies biofilm formation (Fig. 1).

Each compound tested alone showed a significant inhibition of the biofilm formation. For each bacterium of
the biofilm, the antibiofilm activity of the combination of 2000 μg ml⁻¹ of GSE with 10-2 mg ml⁻¹ of Fluorinol® was greater than that of each compound tested alone, resulting in a significant inhibition of 1.37 log₁₀ for Lactobacillus to 4.76 log₁₀ for Porphyromonas. In addition, the inhibitory effect on biofilm formation of the combination was higher when the concentration of Fluorinol® increased.

After 64 h of incubation, the control biofilm observation by CLSM (Fig. 2a) showed a densely populated biofilm containing a large number of microcolonies, within which the occasional small lacuna was seen. Three-dimensional (3D) image of the control biofilm (Fig. 2a’) shows the presence of a uniformly spread biofilm with a thickness of 33.6 ± 2.1 μm. CLSM observations of biofilms incubated with 10-2 mg ml⁻¹ of Fluorinol® (Fig. 2b,b’) or 2000 μg ml⁻¹ of GSE (Fig. 2c,c’) revealed that a decrease in the number of microcolonies and thickness of these biofilms of 21.6 ± 0.8 and 17.9 ± 3.2 μm, respectively, were significantly (P < 0.05) smaller compared with the control biofilm. For the biofilm incubated with GSE (2000 μg ml⁻¹) and Fluorinol® (10-2 mg ml⁻¹), a very few low-density aggregates having a thickness of 10.3 ± 2.5 μm with many unstained regions between them, and some single cells were observed on the surface of the HA discs (Fig. 2d,d’). Thickness of biofilm treated with the combination was significantly (P < 0.01) thinner than that of control biofilm. Moreover, the LIVE/DEAD® staining indicated a very low level of cell damage, less than one per cent, both for control and for biofilm treated with the combination.

**Glucosyltransferases inhibition**

The effects on GTFs activity of GSE (2000 μg ml⁻¹), Fluorinol® (10-2 mg ml⁻¹) and their combination were analysed through the initial overall reaction rate determined by measuring the amount of reducing sugars released and through the quantity of insoluble glucan (IG) synthesized (Fig. 3). GSE and the combination significantly inhibited both the initial overall reaction rate and the IG synthesis. The combination led to an increased inhibitory activity of GSE, visible on the amount of reducing sugars released, with an inhibition of 43-9% for GSE and 65-7% for the combination. Concerning the reduction in the quantity of IG synthesized after 24 h, the increase in inhibitory activity was not visible.

### Table 1

<table>
<thead>
<tr>
<th>Oral Bacteria</th>
<th>MIC* (μg ml⁻¹)</th>
<th>MBC† (μg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus mutans</td>
<td>1000</td>
<td>4000</td>
</tr>
<tr>
<td>Streptococcus sobrinus</td>
<td>2000</td>
<td>4000</td>
</tr>
<tr>
<td>Actinomyces viscosus</td>
<td>1000</td>
<td>4000</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus</td>
<td>4000</td>
<td>8000</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>4000</td>
<td>8000</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>2000</td>
<td>8000</td>
</tr>
</tbody>
</table>

*Minimal inhibitory concentration: concentration at which no bacterial growth was visible after 24-h incubation at 37°C.
†Minimal bactericidal concentration: concentration at which the CFU number was reduced by 99.9%.
Figure 2 Scanning confocal laser microscopy images of biofilms (t = 64 h) stained with the LIVE/DEAD® BacLight™ bacterial viability kit. Biofilm treated with (a) ultrapure water (control), (b) Fluorinol® 10·2 mg ml⁻¹, (c) GSE 2000 µg ml⁻¹, (d) GSE 2000 µg ml⁻¹ and Fluorinol® 10·2 mg ml⁻¹. Three-dimensional images of biofilms depicting biofilm thickness are shown in the right panels.
samples. Fluorinol® showed no antioxidant effect. Therefore, the TEAC for the combination and for Eludril Daily® was determined relative to the concentration of the active agent (i.e. GSE) present in these solutions. GSE and Eludril Daily® presented a high TEAC value, significantly ($P < 0.001$) greater than those of ascorbic acid, proving their capacity to scavenge the ABTS°• radical cation. No statistical difference ($P < 0.001$) was obtained between the TEAC values of GSE, the combination and Eludril daily®.

**Discussion**

Dental plaque control is a key factor in the prevention of dental caries and periodontal diseases. Many antiplaque agents exist, the gold standard being chlorhexidine. To overcome the long-term side effects of such antiseptics, such as teeth staining and the assignment of the biological equilibrium in the oral cavity, previous studies have investigated the effects of polyphenols for oral hygiene purposes (Song et al. 2006; Xiao et al. 2007). In this context, we focused on the study of biological capacities of the combination of a grape seed extract, known for its antiplaque and antioxidant activities (Furiga et al. 2009), with an amine fluoride limiting the enamel demineralization (Griffin et al. 2007).

In a first step, we analysed the antimicrobial activity of GSE and Fluorinol® to check the absence of bactericidal effects that may have an adverse impact on the oral ecosystem. Unlike previous studies which showed that amine fluoride had bactericidal activity (Rosin-Grete and Linzir 1995), Fluorinol® exhibited no effect against the six bacterial strains at the highest tested concentration of 10.2 mg ml$^{-1}$, corresponding to 1500 ppm of fluoride, that is, the maximum concentration permissible in an over-the-counter product in the European Union. Perhaps, this difference between our results can be explained by the fact that most previous studies are clinical studies, and thus, the anticaries effects observed, related to a direct antibacterial activity against oral bacteria, would be actually consequence of many other mechanisms.

Concerning GSE, there is no restrictive concentration in the use of these polyphenolic extracts because they are natural raw materials used in food industry, beverage, health food and cosmetics and their lack of toxicity has been reported (Yamakoshi et al. 2002). The MIC values obtained for GSE were high (1000–4000 µg ml$^{-1}$) compared with those of chlorhexidine ($<0.5–15.6$ µg ml$^{-1}$) against the same oral bacteria (Furiga et al. 2008b). Compared with other polyphenolic extracts, these MIC values were similar to those previously obtained with Polygonum cuspidatum extract (Song et al. 2006) and Nidus vespar extract (Xiao et al. 2007), and much higher than those

![Figure 3 Effect of grape seed extract (GSE, 2000 µg ml$^{-1}$), Fluorinol® (10.2 mg ml$^{-1}$) and the combination of GSE (2000 µg ml$^{-1}$) and Fluorinol® (10.2 mg ml$^{-1}$) on glucosyltransferases (GTFs) activity. Enzymatic activity (U ml$^{-1}$) was analysed through the initial overall reaction rate determined by measuring the amount of reducing sugars released, and through the quantity of insoluble glucan (IG) synthesized. Results are presented as percentage of enzymatic activity with respect to control (without any compound). Results are expressed as means and standard deviations of triplicate experiments. Statistical differences ($***P < 0.001$) between test compound and control (*), (□), Reducing sugars; (●), IG.](image-url)
obtained with seeds and skin of wine grape extracts (Toukairin et al. 1991).

The antiplaque effect of GSE and Fluorinol®, and their combination, was then tested on a multispecies biofilm. This step is needed to evaluate potential antiplaque molecules because bacteria within a biofilm possess different properties than planktonic bacteria, including an increased protection against antimicrobial agents (Leung et al. 2005). The two compounds tested alone showed a significant inhibition of the biofilm formation. As previously described, the most successful concentration for GSE is 2000 μg ml⁻¹ and higher concentrations in this extract led to a fall of its effectiveness, principally caused by its poor dissolution in water (Furiga et al. 2009). The inhibitory effects of Fluorinol® on biofilm formation were dose-dependent, and the optimum antibiofilm activity was obtained at the permissible concentration of 10-2 mg ml⁻¹. In addition, CLSM observation of biofilm treated with Fluorinol® showed a low level of cell damages with only a few visible dead cells, confirming that this compound exerts its antibiofilm activity by other mechanisms than just a direct antibacterial effect. The combination of these two compounds led to a synergistic effect on biofilm formation with an inhibition >3 log₁₀ on streptococci, Actinomyces and Porphyromonas, for the most effective combination of 2000 μg ml⁻¹ of GSE and 10-2 mg ml⁻¹ of Fluorinol®.

Our results can be related to those of Shapiro et al. (2002), who studied the effectiveness of twelve mouthwashes using the same biofilm system with few modifications, mainly changes in bacterial composition. As mouthrinses containing plant extracts tested in their study, our combination showed an antibiofilm activity lower than that of mouthwashes containing chlorhexidine, which led to a total biofilm eradication linked to its bactericidal effects. In addition, the level of bacteria cells was high even after treatment with our combination although values were clearly statistically significant. Same observations and a median reduction in CFU (>3 log₁₀) equivalent to those of our combination were noted with Listerine®, the most active mouthrinse containing plant extracts. However, numerous clinical studies have confirmed the significant plaque inhibitory properties of Listerine® that suppressed de novo plaque formation after four (Riep et al. 1999) and 21 days (Brecc et al. 1990) in the absence of mechanical toothcleaning. Thus, it seems that a significant reduction of cells using this in vitro model, even if the number of CFU still high, lead to a significant biological effectiveness in relevant clinical trials. Moreover, our combination showed high inhibition on streptococci and Actinomyces. These species have been identified as the major initial colonizers of the tooth surface, and the interactions between them and their substrata help establish the early biofilm community (Kolenbrander 2000). These observations and the strong decrease of 4.8 log₁₀ of Porphyromonas observed within the treated biofilm indicate that our combination achieved the expected benefits of such therapy, which is to control the biofilm development, especially with preventive effect on bacterial adhesion and the early stages of the biofilm formation leading to inhibition of the binding and proliferation of secondary colonizers involved in the pathogenicity of oral diseases, and not to a total biofilm removal. Finally, CLSM observations showed a much higher density of recovery of the surface for the control biofilm compared with treated biofilms, which indicate the presence of a large amount of exopolysaccharides in the control and the fact that the antibiofilm activity of the tested compounds corresponds to a significant decrease in the number of CFUs, but could also include destruction or reduction in the production of these polymers.

The inhibition of adhesion is an attractive concept for the development of new therapies in the prevention of bacterial dental infections. The adhesion of bacteria on the teeth is strongly influenced by the synthesis of glucans by glucosyltransferases. Therefore, we next examined the inhibitory activity of the compounds on these enzymes. The inhibition of insoluble glucan synthesis >90% obtained with GSE was in agreement with results described for another grape seed extract (Toukairin et al. 1991) and for other polyphenolic extracts (Otake et al. 1991; Nakahara et al. 1993). Numerous studies have identified oligomeric proanthocyanidins (or condensed tannins) as most active in the GTFs inhibition, and one characteristic of the grape seeds is to be rich in these molecules, which could explain the observed results (Nakahara et al. 1993).

On the other hand, the adhesive insoluble glucan synthesis and the formation of dental plaque are due to the cooperative action of GTF-S (soluble glucans synthesis), GTF-I (insoluble glucans synthesis) and/or GTF-SI (soluble and insoluble glucans synthesis, Strep. mutans specific). Without this cooperative action, it would not be possible for micro-organisms to have any degree of adhesion. Indeed, it has been shown that the synthetic product of each purified enzyme alone is unable to adhere to the glass of a test tube (Koga et al. 1983). It is thus interesting to search for compounds inhibiting this combined action of GTFs. However, enzymatic inhibitors have often been studied on highly purified and poorly representative GTF preparations (Wunder and Bowen 1999). Therefore, test compounds have been evaluated in this study on a crude GTF preparation previously characterized containing a mixture of at least four different glucosyltransferases (GTF-S₁, GTF-S₂, GTF-S₃ and GTF-I), highly representative of the Strep. sobrinus GTF complex, and leading to
the synthesis of a strongly adhesive insoluble glucan (Furiga et al. 2008a). Thus, the inhibition observed by GSE of both the initial overall reaction rate and the IG synthesis by this GTF preparation is representative of the inhibition of the cooperative action of GTFs. Indeed, the inhibition by GSE of the release of reducing sugars indicated that this compound prevents cleavage of the sucrose into fructose and glucose and therefore the binding of the enzyme glucosyl residue and the synthesis of soluble glucan by GTF-S, the latter serving as a primer for the GTF-I for the insoluble glucan synthesis (Gilmore et al. 1993). This dual inhibition has also been shown for a grape juice containing 3·1 mg ml\(^{-1}\) of polyphenols (Kashket et al. 1983).

As Shani et al. (2000), we obtained a low inhibitory activity of amine fluoride on GTFs activity. The same experiment was performed with sodium fluoride at the concentration of 0·33%, corresponding to 1500 ppm of fluoride, and an inhibitory activity of 3·0 ± 1·3% of the amount of reducing sugars released was obtained, suggesting that it is the amine component of the amine fluoride that is responsible for the observed effect on GTFs (data not shown). However, this low inhibitory activity was sufficient to explain the observed increase in the inhibitory effect of GSE by the addition of Fluorinol\(^{\circledR}\). Furthermore, these enzymes are very important in the process of biofilm formation (Banas and Vickerman 2003); therefore, this potentiation of the inhibitory effect could certainly explain some of the increase in antibiofilm activity observed with the combination on multispecies biofilm.

Periodontal diseases are in part caused by an overgrowth of pathogenic bacteria, gram-negative anaerobes such as P. gingivalis and F. nucleatum. The accumulation and persistence of these bacteria initiate periodontal tissue destruction either directly by the release of specific products such as bacterial lipopolysaccharide (LPS) or indirectly through the activation of immune defence systems of the host, resulting in the excessive production of reactive oxygen species (Ozmeric 2004) leading to oxidative damage associated. However, the biofilm system used in this study was limited compared with the natural oral biofilm and the mouth ecosystem (e.g. artificial media use, biofilm composed only by reference bacteria, static model) and so, additional experiments such as tests in a flowing system and on human samples of dental plaque should be performed to confirm the results. Moreover, given that various in vivo dynamic factors such as systemic conditions, salivary flow, diet and dental anatomy could interact with the test compound effectiveness, these promising in vitro effects should be confirmed in a clinical study conducted with Eludril Daily\(^{\circledR}\) mouthwash.

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Conflict of interest

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