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Original Article

Antibiofilm activities of denture cleaning tablets against *Streptococcus anginosus* - Cytotoxic effects on human gingival fibroblast cells

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ABSTRACT

Background and Aims: *Streptococcus anginosus* and *Candida albicans* exert negative effects on oral health and can cause biofilm formation on tooth surfaces. Partial or total denture surfaces used by people who have lost their teeth due to various reasons are sensitive to microorganism invasion and biofilm formation, similar to tooth surfaces. Therefore, this study was conducted to investigate the antibiofilm activities of various denture cleaning tablets (DCTs) used to disinfect dentures against *S. anginosus* clinical isolates and *C. albicans* standard strain and their cytotoxic effects against oral epithelial cells.

Methods: The biofilm-forming abilities of strains were determined using the crystal violet assay. The modified time-killing curve (TKC) method was used to evaluate the dynamic bactericidal or fungicidal activities of DCTs against biofilms of *S. anginosus* isolates and *C. albicans* ATCC 10231. In vitro cytotoxicity experiments of DCTs on human gingival fibroblast cell lines (HGF-1) were also conducted using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining method.

Results: Overall, 18.18% and 36.36% of *S. anginosus* isolates were strong and moderate biofilm formers, respectively. *C. albicans* ATCC 10231 also formed a strong biofilm. The TKC analysis revealed that all the examined DCTs inhibited almost all living cells in mature biofilms at every time point. The cytotoxic activities of DCTs against HGF-1 cell lines were in the range of 93%–95% at their direct usage concentrations.

Conclusion: DCTs exhibit rapid and strong activity against biofilms, which is extremely important for biofilm-related infections. Nevertheless, it is necessary to consider the cytotoxic effects of DCTs on HGF-1 cells for consumers' oral health.

Keywords: Biofilm, denture cleaning tablet, cytotoxic effects, *Streptococcus anginosus*

INTRODUCTION

Streptococci are generally the most prevalent and dominant bacteria among oral or dental pathogens, and the majority of studies focus on *Streptococcus mutans* or *Streptococcus mitis* species. Conversely, several studies have also demonstrated that *S. anginosus* is a major colonizer in the early stages of natural oral biofilm formation. *S. anginosus* not only contributes to the early colonization of teeth but also forms the biofilm structure that potentially exerts adverse effects on oral health (Heller et al., 2016).

S. anginosus, similar to all streptococci, possess strong and wide-ranging adherence properties, including binding to human tissue components, epithelial cells, and other bacteria (Jenkinson, 1994). In the oral cavity, streptococci produce specific adhesion proteins such as α-amylase-binding protein A, antigen I/II, SspA/SspB, and surface lectins (Nobbs, Lamont, & Jenkinson, 2009). These proteins bind to the pellicles on tooth surfaces, allowing the bacterial adhesion on teeth and maturing the plaque formation that increases the number of other bacteria involved in biofilm formation (Ritz, 1967). Consequently, *Streptococcus* species can form aggregates both within the same or among different species, and these characteristics play a crucial role in biofilm formation on teeth (Li et al., 2004; Ruhl et al., 2014; Heller et al., 2016).

When mechanical or chemical dental cleaning is not performed adequately, biofilm formation, a well-understood process, commonly occurs on tooth surfaces with the participation of especially *Streptococcus* spp, yeasts such as *Candida albicans*, and other microorganisms. Partial or total denture surfaces, which are used by people who have lost their teeth due to various reasons, to continue their normal chewing, speaking, and other functions, are also susceptible to microorganism in-

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vasion and biofilm formation, similar to tooth surfaces. Some studies indicate that inadequate or inappropriate care of prostheses results in inflammation (stomatitis) in the prosthetic area (Gendreau and Zg, 2011; Ramage et al., 2018), with the occurrence of bacterial, fungal, and polymicrobial infections (Srinivasan and Gulabani, 2010). In addition to insufficient prosthesis cleaning, factors such as salivary pH, smoking, and consumption of sugary foods are crucial for predisposition to oral and dental infections (Martori et al., 2014; Ramage et al., 2005). Because of the bacteria that adhere to the surfaces of dentures, providing an appropriate environment for microbial plaque formation and biofilms, studies have emphasized the importance of cleansers that are effective against microorganisms and possess noncorrosive properties for teeth or prostheses (Emami et al., 2014).

Although various cleaning agents are used for oral and prosthesis health, most of the users perform prosthesis cleaning through brushing with toothpaste, but this method can cause surface abrasions and make it easier for microbial adhesion (Verran et al., 2014; Orgini et al., 2012). It has been suggested that regional use of prosthesis cleaners can facilitate the accumulation of mature prosthesis biofilms (Apratim et al., 2013). Conversely, although some cleaning agents used in combating biofilms are effective against planktonic forms of oral microorganisms, they exert limited effects on mature biofilms (Jose et al., 2010). These previous studies have highlighted the difficulty in oral hygiene and the challenge in combating biofilms.

The present study was conducted to investigate the antibiofilm activities of various denture cleaning tablets (DCTs) used for disinfecting prostheses against *S. anginosus* isolates and a *C. albicans* standard strain. In addition, their cytotoxic properties against oral epithelial cells were evaluated.

MATERIALS AND METHODS

DCTs

Five DCTs, used for the cleaning of removable dental prostheses in Turkiye, were selected and purchased commercially for this study. Table 1 shows the detailed information regarding the ingredients of these effervescing tablet products. Before each test, all DCTs were disintegrated in 100 ml of tap water at 25°C–30°C for 5 min, according to the instructions for their use.

Bacterial strains

A total of 11 *S. anginosus* isolates obtained from specimens submitted to the routine Clinical Microbiology Laboratories of Marmara University Pendik Training and Research Hospital, Cerrahpaşa Medical Faculty Hospital, and Istanbul Başakşehir Çam and Sakura City Hospital were used in this study. All clinical isolates were typed at the species level by MALDI-TOF MS. Furthermore, *S. pneumoniae* ATCC 49619 and *C. albicans* ATCC 10231 standard strains were used in this study.

Media

Brain heart infusion (BHI) broth medium containing 2.5%–5% lysed horse blood (Difco Laboratories) and Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) buffered to pH 7.0 with morpholine propane sulfonic acid (Sigma) were used to determine the antibiofilm activities against bacteria and fungi, respectively.

Biofilm formation

S. anginosus isolates were cultured in BHI broth for 24 h at 5% CO_2 and 37°C, adjusted for 0.5 McFarland turbidity, and diluted in fresh media, resulting in a final concentration of 1×10^6 CFU/mL. Next, a 200-µL sample of this suspension was added to the wells of 96-well tissue culture microtiter plates (Greiner Bio-One, Kremsmuenster, Austria) and incubated for 24 h at 37°C in the presence of 5% CO_2. After incubation, the residual media was aspirated gently, and wells were washed three times with 200 µL physiological buffered saline (PBS) solution. Then 200 µL of 99% methanol was added to the wells for chemical fixation for 15 min and aspirated, after which the plates were allowed to air-dry. For biofilm staining, 200 µL of 0.1% crystal violet was added to each well for 5 min, and after removing the dye, the plates were washed with tap water, and the bound crystal violet was solubilized by adding 95% ethanol for 30 min. The optical density (OD) was measured at 600 nm. Tests were repeated three times. Standard *S. pneumoniae* ATCC 49619 and *C. albicans* ATCC 10231 strains were used as the positive control, and BHI broth or RPMI-1640 medium was used as the negative control. Biofilm formation was interpreted as follows:

OD (isolate) ≤ OD (negative control) = negative biofilm formation; OD (negative control) ≤ OD (isolate) ≤ 2× OD (negative control) = weak biofilm formation; 2× OD (negative control) ≤ OD (isolate) ≤ 4× OD (negative control) = moderate biofilm formation; 4× OD (negative control) ≤ OD (isolate) = strong biofilm formation (Nirwati et al., 2019)

Time-killing curve analyses

The modified time-killing curve (TKC) method was used to determine the dynamic bactericidal or fungicidal activities of DCTs against the biofilms of six moderate or strong biofilm former *S. anginosus* isolates and *C. albicans* ATCC 10231 (Dosler & Karaaslan, 2014). For this purpose, 24–48 h biofilms of *S. anginosus* and *C. albicans* were prepared using BHI broth medium in 24-well tissue culture microtiter plates. DCT solutions were added to each corresponding well, and the plates were incubated for 0, 2, 4, 6, and 24 h at 37°C in the presence of

Table 1. The active ingredients of examined DCTs

DCTs	Ingredients
1	Potassium Caroate, Sodium Bicarbonate, Sodium Carbonate, Citric Acid, Sorbitol, VP/VA Copolymer, Sodium Lauryl Sulfate, Sodium Lauryl Sulfoacetate, Aroma, CI 73015.
2	Potassium Caroate, Sodium Bicarbonate, Sodium Carbonate, Citric Acid, Sorbitol, VP/VA Copolymer, Sodium Lauryl Sulfate, Sodium Lauryl Sulfoacetate, Aroma, CI 73015
3	Sodium Bicarbonate, Citric Acid, Potassium Caroate (potassium monopersulfate), Sodium Carbonate, Sodium Carbonate Peroxide, TAED, Sodium Benzoate, PEG-180, Sodium Lauryl Sulfate, VP/VA Copolymer, Aroma, Subtilisin, Cellulose Gum, CI 42090, CI 73015.
4	Sodium Bicarbonate, Citric Acid, Potassium Caroate (potassium monopersulfate), Sodium Carbonate, Peroxide Sodium Carbonate, TAED, sodium benzoate, PEG-180, Sodium Lauryl Sulfate, Aroma, VP/VA Copolymer, Cellulose Gum, CI 42090, CI 73015.
5	Potassium Caroate, Sodium Bicarbonate, Sodium Carbonate, Sodium Carbonate Peroxide, Sodium Sulfate, Malic Acid, PEG-150, Citric Acid, Sodium C10-13 PEG 90, Aroma, TAED, Potassium Persulfate, Alkyl Benzenesulfonate, Aqua, Sodium Chloride, Cl 42090, Cl 28440

5% CO_2. After each incubation period, wells were washed two times with sterile PBS, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole, assay was performed to evaluate the viability of live microorganisms in biofilms. The MTT solution purchased as a powder from Sigma-Aldrich Chemical A.Ş. (St. Louis, MO, USA) was prepared using the PBS solution. Biofilms were incubated with 0.5 mg/mL of MTT solution for 10 min at 37°C. After washing, the purple formazan crystals that formed inside the living cells were dissolved with dimethyl sulfoxide (DMSO), and then the OD values were measured at 570 nm.

Cytotoxicity assay

In vitro cytotoxicity experiments of DCTs on human gingival fibroblast cell lines (HGF-1) (ATCC CRL-2014) were conducted using the MTT staining method. Cells were grown in Eagle's minimum essential medium (Gibco) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin G under a humidified, 5% CO_2 atmosphere at 37°C. The cells were seeded at a density of 1×10^4 cells per well in 96-well tissue culture microplates and kept for 24 h to ensure cell attachment. This was followed by incubation in the absence or presence of two-fold serial dilutions of DCTs between direct usage concentration and five dilutions less (1–1/32-fold) for 24 h at 37°C under 5% CO_2 atmosphere. Cell viability was determined using the MTT assay according to the manufacturer's protocol. The microplates were covered with foil to protect from light and incubated for 3 h at 37°C in a 5% CO_2 environment. After incubation, the MTT solution was removed from the wells, DMSO solution (Sigma, 100 µL) was added to dissolve the formazan crystals, and the plates were placed on a shaker for 15 min to completely dissolve the dye. The OD values were measured at 570 nm using a microplate reader (EON-BioTek Instruments, Winooski, VT, USA) (Andrighetti et al., 2003). In each assay, three replicates were used for each concentration, and the process was repeated three times. The cytotoxic effects of each compound were obtained as % cytotoxicity compared with the control.

RESULTS

Biofilm formation assay

Of the 11 *S. anginosus* isolates obtained from various clinical specimens, 18.18% (2 isolates) formed strong biofilms, 36.36% (4 isolates) formed moderate biofilms, and 45.46% (5 isolates) formed weak biofilms. *C. albicans* ATCC 10231 strain also formed strong biofilms.

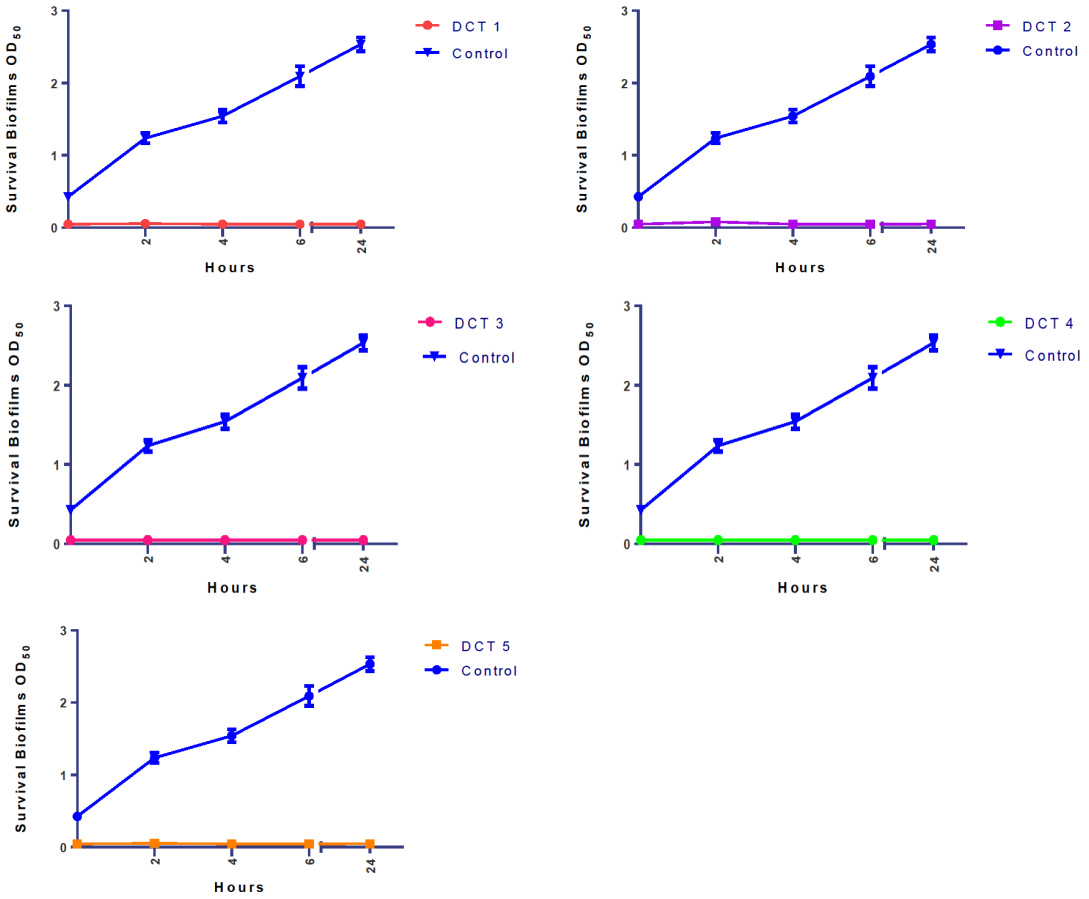

Figure 1. Time killing curves of the studied 5 DCTs against *S. anginosus* isoiates. X-axis represents time, and Y-axis represents the ODs' of biofilm mass. The control shows the result for bacteria have not treated with any product. The results are shown as the averages of two experiments against 6 different isolates.

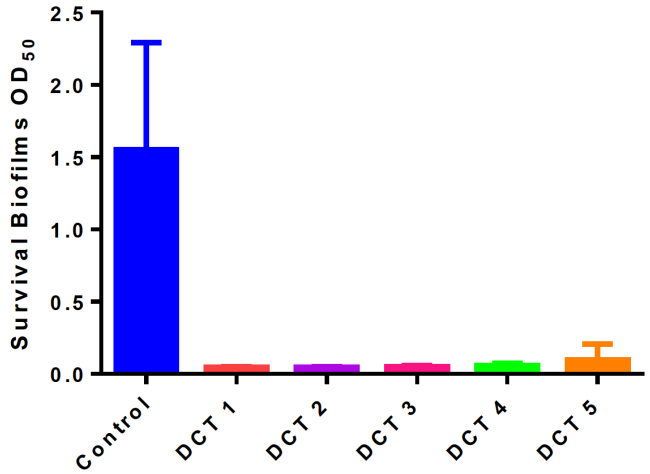

Figure 2. Antibiofilm activities of 5 DCTs against *C. albicans* ATCC 10231. These results were obtained by TKC analysis at the 1st hour and remained the same until the 24th hour. X-axis represents different DCTs, and Y-axis represents the ODs' of biofilm mass. The control shows the result for *C. albicans* not treated with any product. The results are shown as the averages of two experiments.

TKC analyses

The modified TKC analyses revealed that all the examined DCTs exhibited >3-log10 killing ability against six moderate and strong biofilms of *S. anginosus* isolates and *C. albicans* ATCC 10231 within 1 h. All DCTs also inhibited almost all living cells in mature biofilms at every time point (Figures 1 and 2, respectively).

Cytotoxicity assay

The cytotoxicity assay revealed that DCTs exhibited 93%–95% cytotoxicity for HGF-1 cell lines at their direct usage concentrations. These cytotoxic effects continued to be observed at 2.93%–10.26% even at their 1/32 dilutions. These findings demonstrated that the DCTs possess no cell proliferative activity but they exert severe cytotoxic side effects at the usage concentrations and below (Figure 3).

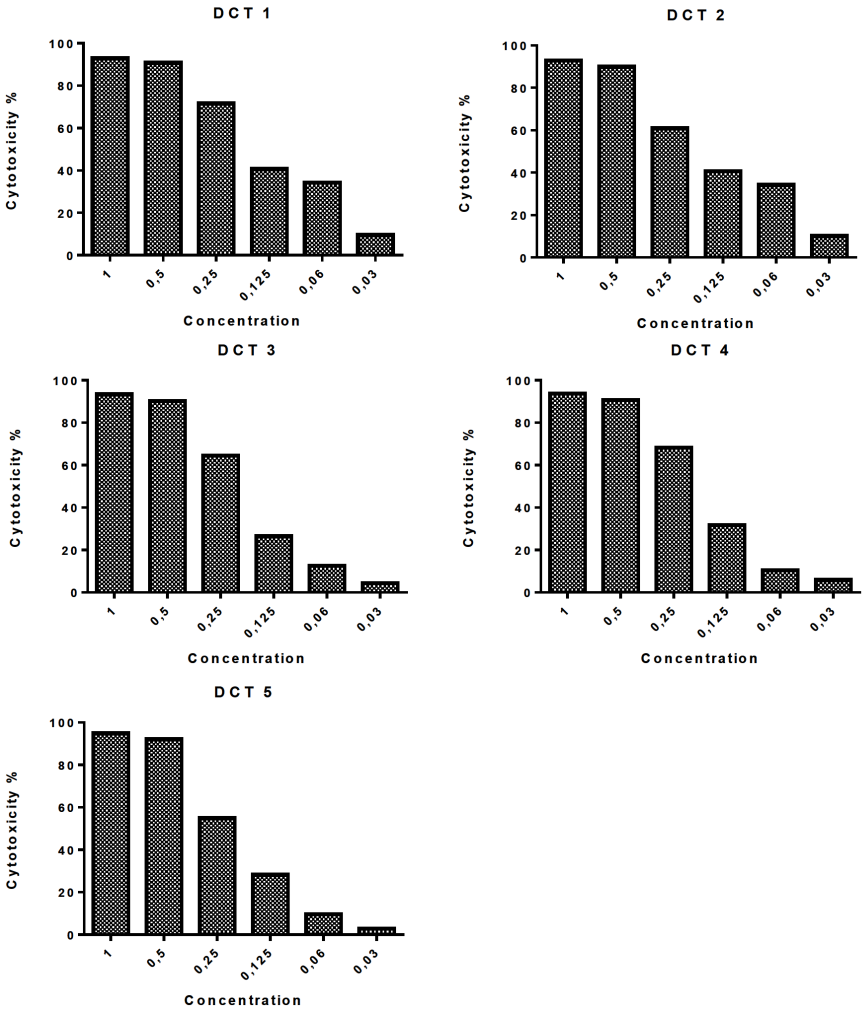

Figure 3. Cytotoxic effects of the 5 studied DCTs against HGF-1 cell line. X-axis represents the diluted product concentrations (direct-1/32). Y-axis show the % cytotoxicity values calculated by comparing with the untreated control cells.

DISCUSSION

Teeth are the major organs of chewing function and are also extremely important for phonetics and aesthetics. Although oral and dental health is an indicator for individual and social health, sometimes tooth losses may occur due to various reasons. In such cases, removable total or partial dentures are widely used for the treatment of missing teeth, especially in elderly people, and the oral health of these people is extremely important for their general systemic health and the quality of their life. Because dental prostheses often function as structures that facilitate biofilm formation for various microorganisms (Takamiya et al., 2011, Shankar et al, 2017), removing and cleaning the dentures after meals is extremely essential for health, and this process should disrupt the biofilm structure. Although several mechanical and chemical methods are used for denture cleaning, chemical cleaners are preferred to facilitate this process, especially for elderly people who have weakened motor coordination. For this purpose, several denture cleaners are available on the market, which contain alkaline peroxides, disinfectants, enzymes, and diluted acids as active ingredients (Budtz-Jorgensen et al., 1972). In practice, the simplest denture cleaning can be achieved by placing one of the DCTs in a glass of warm water and waiting for 3 min, although it is necessary to wait for at least 15 min for deep cleaning. Moreover, the prosthesis can be left overnight to achieve the best cleaning and disinfection, after which it must be rinsed with clean water before use.

In this study, we investigated the antibiofilm activities of five commercially available denture cleaning products against the mature biofilms of strong biofilm former *S. anginosus* isolates and *C. albicans* standard strain. The TKC analyses revealed that all the tested DCTs were highly effective against the biofilms, starting at the 15th min and continuing for 24 h. As all the examined DCT formulations, antibiofilm activities, and contact times were extremely similar to each other, no significant differences were observed between their effectiveness. In the

continuation of our study, we intend to determine the effectiveness of DCTs by generating biofilms on acrylic surfaces to imitate their usage areas.

Because DCTs are used externally and rinsed products, there is no legal specific limitation concerning their toxic effects on body cells. Although these products might exhibit extremely rapid and strong activity against planktonic cells or biofilms of microorganisms, it also implies that they contain high levels of antimicrobial substances and must be rinsed extremely well before use. For this purpose, although the product instructions mention that they should be rinsed thoroughly, it is always necessary to be aware of their cytotoxic effects in cases where everyone cannot read and follow those instructions. In this study, the cytotoxic properties of the products that were effective against the biofilms of *S. anginosus* isolates were investigated on HGF-1 cell lines. Results showed that the cytotoxic effects of the substances were extremely high (>90%) at their usage concentrations, with a cytotoxicity of ≤10% achieved by a dilution of only 1/32, which can be considered relatively safe. These data suggested that rinsing and washing are extremely critical steps to eliminate the toxic effects during the use of the product after the cleaning process, and they must be performed correctly.

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