Biofouling

The Journal of Bioadhesion and Biofilm Research

ISSN: 0892-7014 (Print) 1029-2454 (Online) Journal homepage: https://www.tandfonline.com/loi/gbif20

In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces

Patricia Milagros Maquera-Huacho, Caroline Coradi Tonon, Marilia Ferreira Correia, Renata Serignoli Francisconi, Ester Alves Ferreira Bordini, Élcio Marcantonio & Denise Madalena Palomari Spolidorio

To cite this article: Patricia Milagros Maquera-Huacho, Caroline Coradi Tonon, Marilia Ferreira Correia, Renata Serignoli Francisconi, Ester Alves Ferreira Bordini, Élcio Marcantonio & Denise Madalena Palomari Spolidorio (2018) *In vitro* antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces, Biofouling, 34:6, 699-709, DOI: 10.1080/08927014.2018.1485892

To link to this article: https://doi.org/10.1080/08927014.2018.1485892

Published online: 06 Sep 2018.

Submit your article to this journal

Article views: 136

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=gbif20

BIOFOULING, 2018
VOL. 34, NO. 6, 699–709
https://doi.org/10.1080/08927014.2018.1485892

In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces

Patricia Milagros Maquera-Huacho[a], Caroline Coradi Tonon[a], Marilia Ferreira Correia[b], Renata Serignoli Francisconi[b], Ester Alves Ferreira Bordini[c], Élcio Marcantonio[a] and Denise Madalena Palomari Spolidorio[b]

[a]Department of Oral Diagnosis and Surgery, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil; [b]Department of Physiology and Pathology, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil; [c]Department of Dental Materials and Prosthodontics, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

ABSTRACT

This study evaluated the antibacterial properties of carvacrol and terpinen-4-ol against *Porphyromonas gingivalis* and *Fusobacterium nucleatum* and its cytotoxic effects on fibroblast cells. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were examined. The minimum biofilm inhibition concentration (MBIC) was evaluated by XTT assay. Biofilm decontamination on titanium surfaces was quantified (CFU ml^{-1}), evaluated by confocal laser scanning microscopy (CLSM) and cytotoxic activity by MTT. The MIC and MBC for carvacrol were 0.007% and 0.002% for *P. gingivalis* and *F. nucleatum*, and 0.06% for terpinen-4-ol for both microorganisms. The MBIC for carvacrol was 0.03% and 0.06% for *P. gingivalis* and *F. nucleatum*, and for terpinen-4-ol was 0.06% and 0.24%. The results indicated anti-biofilm activity using carvacrol (0.26%, 0.06%) and terpinen-4-ol (0.95%, 0.24%) and showed cytotoxic activity similar to chlorohexidine (CHX). However, terpinen-4-ol (0.24%) showed higher cell viability than other treatments. Carvacrol and terpinen-4-ol showed antibacterial activity in respect of reducing biofilms. Moreover, CHX-like cytotoxicity was observed.

ARTICLE HISTORY
Received 5 February 2018
Accepted 30 May 2018

KEYWORDS
Carvacrol; terpinen-4-ol; peri-implantitis; antibacterial activity; biofilm; cytotoxic activity

Introduction

The formation of bacterial biofilms on dental implant material surfaces (titanium) may lead to the development of peri-implant diseases, influencing the long-term success of dental implants (Mahato et al. 2016). The presence of periodontopathic bacteria is the main cause of the development and progression of peri-implant diseases; the microenvironment around implants may favor the colonization of anaerobic Gram-negative bacteria (Renvert and Quirynen 2015). Thus, the microbiota associated with peri-implant disease may be described as a polymicrobial infection in which anaerobic microorganisms dominate (Mombelli and Decaillet 2011; Belibasakis 2014; Renvert and Quirynen 2015).

Various treatment modalities have been put forward for the treatment of peri-implantitis, including regenerative techniques and decontamination methods (Figuero et al. 2014; Mahato et al. 2016). However, there is no consensus on the treatment of peri-implant infections due to the heterogeneity of the studies and the variability in the types of implant surfaces (Mellado-Valero et al. 2013). Thus, the application of antimicrobial agents for removal of the surface biofilm is useful for the successful treatment of implanted patients (Lindhe and Meyle 2008; Mellado-Valero et al. 2013; Mahato et al. 2016).

Different natural products have been investigated as promising agents for surface biofilm removal and the prevention and treatment of periodontal and peri-implant diseases (Botelho et al. 2007; Ciandrini et al. 2014). Carvacrol, a monoterpene phenol (2-methyl-5-(1-methylethyl) phenol), is found in the volatile oils of *Thymus vulgaris*, *Carum copticum* and *Oreganum* spp., and it is the main natural constituent (70%) of these aromatic plants (Baser, 2008). Carvacrol has broad-spectrum antimicrobial activity against pathogenic microorganisms including drug-resistant bacteria and yeast biofilms (Dalleau et al. 2008; Ciandrini et al. 2014).

Another phytotherapeutic agent is tea tree essential oil (*Melaleuca alternifolia* – TTO), which has an

CONTACT Patricia M. Maquera Huacho patricia_mmaquera@hotmail.com
© 2018 Informa UK Limited, trading as Taylor & Francis Group

Australian origin. TTO has antibacterial, antifungal, antiviral, anticancer and anti-inflammatory proprieties (Hart et al. 2000; Catalán et al. 2008; Kwiecinski et al. 2009; Greay et al. 2010). TTO comprises ~100 compounds, and terpinen-4-ol, the major component (30–40%) has been specifically assigned antimicrobial and anti-inflammatory activities (Hart et al. 2000; Nogueira et al. 2014).

The purpose of this study was to evaluate the antibacterial properties of carvacrol and terpinen-4-ol against the peri-implant pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in a planktonic state and in single-species and dual-species *in vitro* biofilms on titanium discs. The cytotoxic effects of carvacrol and terpinen-4-ol were also evaluated on fibroblast cells.

Material and methods

Bacterial conditions

The *Porphyromonas gingivalis* (ATCC 33277) and *Fusobacterium nucleatum* (NCTC 10326) used in this study were grown on Brucella agar (Becton Dickinson, Heidelberg, Germany) supplemented with 5% sheep blood, hemin (10 mg ml^{-1}) and menadione (5 mg ml^{-1}) for 72 h at 37 °C in an anaerobic chamber (atmosphere of 85% N_2, 10% H_2 and 5% CO_2) (Don Whitley Scientific MG500, Shipley, UK). Broth cultures were prepared in brain-heart infusion (BHI) broth supplemented with 1% yeast extract, hemin (5 mg ml^{-1}) and menadione (10 mg ml^{-1}) and incubated for 48 h under anaerobic conditions.

Antimicrobial preparation

Serial dilutions of carvacrol (0.26–0.0005% v/v) (Sigma-Aldrich, St Louis, MO, USA) with 2% dimethylsulfoxide (DMSO) (Sigma-Aldrich) (Botelho et al. 2007) and terpinen-4-ol (3.8–0.001% v/v) with 0.4% DMSO, were prepared in BHI medium (Nogueira et al. 2014). Chlorhexidine stock solution (CHX 2%) was used as a positive control and was prepared at 0.2%.

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to the CLSI M11-A8 (2012). Serial doubling dilutions of carvacrol and terpinen-4-ol were prepared and distributed in 96-well plates. The final inoculum concentration (200 µl) of each species was 1.0×10^5 CFU ml^{-1} per well. A bacterial suspension without carvacrol or terpinen-4-ol served as a positive control, and BHI medium served as a blank and negative control.

After incubation under anaerobic conditions at 37 °C for 48 h (Ciandrini et al. 2014), bacterial growth was monitored by absorbance at 600 nm using a micro plate reader (Synergy H1 Multi-Mode Reader – BioTek, Winooski, VT). The MIC was defined as the lowest carvacrol and terpinen-4-ol concentration without bacterial growth. MBC was obtained after plating 10 µl aliquots from each well on Brucella agar and defined as the lowest concentration of carvacrol and terpinen-4-ol at which 99.9% of the bacteria were killed (Kwiecinski et al. 2009). The plates were incubated at 37 °C for 72 h under anaerobic conditions.

Biofilm assays

Biofilm metabolic activity assessment using the XTT

Single-species biofilms formed by each bacterial species (*P. gingivalis* and *F. nucleatum*) were grown in 96-well plates as described by Ciandrini et al. (2014) with some modifications. Unstimulated human saliva (CAAE 49473115.0.0000.5416) was collected in the morning from a healthy individual without active caries, lesions or periodontal diseases (Moura et al. 2006). For the formation of a salivary pellicle, 50 µl of sterile human saliva were added to each well and incubated for 4 h with gentle shaking at 37 °C (Ciandrini et al. 2014). After this, the saliva was removed, and the wells were washed with phosphate buffered saline (PBS). Then the wells were inoculated with 200 µl of each bacterial suspension (1.0×10^7 CFU ml^{-1}). The plates were incubated at 37 °C for 48 h. After this time, each well was washed with PBS, dilutions of carvacrol and terpinen-4-ol were added (200 µl) and the plates were incubated for 24 h under anaerobic conditions. BHI medium was used as a blank and in the untreated control group. Bacterial metabolic activity following the exposure to carvacrol and terpinen-4-ol was measured by a XTT reduction assay (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide – Sigma-Aldrich), in which the cells metabolically reduce sodium to a soluble formazan product (Banzi et al. 2014). The plates were incubated at 37 °C for 3 h and the absorbance of the supernatant was measured at 492 nm using an ELISA reader (Synergy H1 Multi-Mode Reader – BioTek). The minimum biofilm inhibition concentration (MBIC) was defined as

the lowest concentration tested that inhibited biofilm formation by 50% (Miladi et al. 2016).

Biofilm formation on specimens

Single- and dual-species biofilms were grown on sterile titanium discs with an SLA (Sand-balsted, Large-grit, Acid-etched) surface produced using TiO_2 microparticles for blasting and subsequent acid conditioning (5 mm; thickness 2 mm) kindly provided by Implacil De Bortoli (São Paulo, Brazil), as described by Ciandrini et al. (2014) with modifications. Subsequently, the titanium discs were placed in sterile 24-well plates, and the salivary pellicle was pre-formed as described for the single biofilm. Titanium discs were placed in sterile 24-well plates, and 1 ml of each bacterial species was inoculated for single biofilms or 500 µl of each oral bacterial suspension (1.0×10^7 CFU ml^{-1}) were inoculated for dual-species biofilms. Biofilms were incubated at 37 °C for five days in BHI supplemented as described, and the medium was changed every 24 h. After five days, the discs were transferred to new wells containing 1 ml of carvacrol (0.26% or 0.06%) or terpinen-4-ol (0.95% or 0.24%). These concentrations were the lowest tested concentrations for MBIC, which indicated antimicrobial effectiveness and prevention of *P. gingivalis* and *F. nucleatum* biofilm formation. The discs were incubated under anaerobic conditions at 37 °C for 5 min, according to the standard clinical protocols (Schou et al. 2003; Ungvári et al. 2010). Then, all discs were washed with PBS and transferred to sterile Eppendorf tubes with 1 ml of physiological saline. Then, titanium discs were sonicated for 20 min, scraped carefully for 1 min and harvested by vigorous vortexing for 1 min. The resulting material (adherent bacteria) was diluted and plated on Brucella agar for 48–72 h at 37 °C under anaerobic conditions for further quantitative analysis of CFU ml^{-1}.

Confocal laser scanning microscopy (CLSM)

For CSLM images, titanium discs were first washed gently with sterile PBS to remove unattached bacteria. The bacteria were then labeled with 0.01 mM of Syto-9 and 0.06 mM of propidium iodide (PI), Live/Dead stain BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA) for 15 min, according to the manufacturer's instructions. The excitation/emission wavelengths were 488/500 nm for SYTO 9 and 488/635 nm for propidium iodide. Fluorescence from the stained cells was viewed by CLSM (LSM 780 inverted, Zeiss, Germany). CLSM images were acquired using software (ZEN 2012, Zeiss) at a resolution of 1,024 by 1,024 pixels. Images from a single focal plane of the biofilm were captured by the system using a 20× magnification lens. The areas selected for analysis of the biofilm were defined randomly and away from the edge of the specimens.

L929 fibroblast culture and cytotoxicity analysis

L929 mouse fibroblast cells were seeded at a density of 1.0×10^4 cells $well^{-1}$ into 96-well plates (100 µl) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Gibco, Grand Island, NY, USA), and incubated at 37 °C in a humidified incubator with 5% CO_2 for 24 h. Thereafter, the culture medium of the confluent cell monolayers was replaced by terpinen-4-ol (0.95%, 0.24%), carvacrol (0.26%, 0.06%), DMSO (0.4%, 2%), CHX (0.2%) or DMEM alone (negative control group), and the plates were incubated for 5 min, 1 h and 24 h. After each period, the cytotoxic effects were evaluated using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – Sigma-Aldrich) (Basso et al. 2013). Afterwards, the culture medium was aspirated and replaced by 100 µl of MTT solution (5 mg ml^{-1}) and incubated at 37 °C for 4 h. Then, the culture medium with the MTT solution was replaced by 100 µl of acidified isopropanol solution (0.04 N HCl) to dissolve the violet formazan crystals, producing a homogenous bluish solution resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells. Cell viability was evaluated by spectrophotometry (570 nm) with an ELISA plate reader (Synergy H1 Multi-Mode Reader – BioTek).

Statistical analysis

SPSS 17.0 software (IBM) was used to perform the statistical analysis. The antimicrobial activity and the comparison of bacterial quantification (log_{10}) after antimicrobial treatments for single biofilms formed on titanium discs were statistically analyzed by Kruskall–Wallis non-parametric test complemented by Mann–Whitney tests. Biofilm inhibition and bacterial quantification for dual-species biofilms on titanium discs were evaluated using an ANOVA and Tukey tests. The absorbance values of the cytotoxicity analysis were compared using a two-way ANOVA. All the statistical inferences were considered at the

level of significance of 5%. All experiments were repeated three times on different days.

Results

Antibacterial activity and biofilm inhibition

The bacteriostatic, bactericidal and biofilm inhibition effectiveness of different concentration ranges of carvacrol (from 0.0005% to 0.26%) and terpinen-4-ol (0.001% to 3.8%) were tested in planktonic cultures and on biofilms of *P. gingivalis* and *F. nucleatum*. The MIC, MBC and MBIC values of the antimicrobial agents against *P. gingivalis* and *F. nucleatum* are shown in Table 1. *P. gingivalis* and *F. nucleatum* were sensitive to carvacrol with MIC values of 0.007% and 0.002%, respectively and terpinen-4-ol with MICs values of 0.06% to both bacterial species. The results of the MIC and MBC experiments were consistent. The MBIC values of carvacrol were 0.03% and 0.06% and those of terpinen-4-ol were 0.06% and 0.24% for *P. gingivalis* and *F. nucleatum*, respectively.

Effect of carvacrol and terpinen-4-ol on biofilm viability

The inhibitory activities of carvacrol and terpinen-4-ol were determined against single- and dual-species biofilms of *P. gingivalis* and *F. nucleatum* on titanium surfaces (Figures 1, 2 and 3). Terpinen-4-ol (0.95% and 0.24%) and carvacrol (0.26% and 0.06%) significantly reduced *P. gingivalis* viability compared with that of an untreated *P. gingivalis* biofilm ($p < 0.0001$) (Figure 1a). Figures 2a and 3a show that the antimicrobial activity of terpinen-4-ol (0.95%) and carvacrol (0.26 and 0.06%) are similar to that of CHX 0.2% and significantly different from that of the control biofilm of *F. nucleatum* and the dual-species biofilm ($p < 0.0001$). *F. nucleatum* biofilms and dual-species biofilms treated with terpinen-4-ol 0.24% were not significantly reduced compared with those of the control groups ($p > 0.05$). This terpinen-4-ol concentration showed similar results to those of CHX 0.2% on dual-species biofilms of *P. gingivalis* and *F. nucleatum.*

CLSM images (Figures 1b, 2b and 3b) revealed the presence of microorganisms throughout the substratum. Biofilm thickness after cultivation for five days ranged from 25–30 µm under these experimental conditions. In the images, the live cells in the biofilms were stained and emitted a green fluorescence signal, while the dead cells were stained and produced red fluorescence. The untreated control biofilm emitted green fluorescence in all biofilms. *P. gingivalis* biofilms treated with CHX (0.2%) emitted obvious red fluorescence and inconspicuous green fluorescence and almost complete red fluorescence after treatment with terpinen-4-ol (0.95% and 0.24%) and carvacrol (0.26% and 0.06%) (Figure 1b). Compared to the control, CHX (0.2%) and carvacrol (0.06%) had a significant impact on the viability of the *F. nucleatum* biofilms. After the treatment with terpinen-4-ol (0.95% and 0.24%), *F. nucleatum* biofilms exhibited extensive green fluorescence, indicating that many cells survived. In contrast, treatment with carvacrol (0.26%) almost completely killed the bacterial cells within single-species biofilms formed on titanium discs by *F. nucleatum* (Figure 2b). Furthermore, the treatment with CHX (0.2%) and carvacrol (0.06%) had minimal effect on dual-species biofilms. However, the treatment with terpinen-4-ol (0.95% and 0.24%) led to a significant increase in the red fluorescence of the dual-species biofilms. Carvacrol (0.26%) was also able to effectively kill the majority of cells in the dual-species biofilm (Figure 3b).

Cell viability analysis (MTT assay)

The viability of the L929 mouse fibroblast cells under different treatments is summarized in Figure 4. The number of cells in the control group was set at 100%. The number of cells progressively increased after 5 min (93.75 ± 16.42%), 1 h (95.67 ± 19.12%) and 24 h (121.87 ± 10.95%). DMSO (2%) and DMSO (0.4%) did not significantly change the relative growth of fibroblast cells in comparison with that of the control group after exposure for 5 min or 1 h ($p > 0.05$). However, DMSO (2% and 0.4%) caused a statistically significant reduction in fibroblast cells after exposure for 24 h in comparison with that of the control group ($p < 0.0001$). Chlorhexidine (0.2%) caused a decrease in the number of cells compared with the control group after 5 min, 1 h, and 24 h ($p < 0.0001$).

Table 1. Antibacterial activity and inhibition of biofilm development by carvacrol and terpinen-4-ol against *Porphyromonas gingivalis* and *Fusobacterium nucleatum.*

Bacterial strains	Carvacrol			Terpinen-4-ol		
	MIC[a] (%)	MBC[b] (%)	MBIC[c] (%)	MIC[a] (%)	MBC[b] (%)	MBIC[c] (%)
P. gingivalis	0.007	0.007	0.03	0.06	0.06	0.06
F. nucleatum	0.002	0.002	0.06	0.06	0.06	0.24

[a]Minimum inhibitory concentration.
[b]Minimum bactericidal concentration.
[c]Minimum biofilm inhibition concentration.

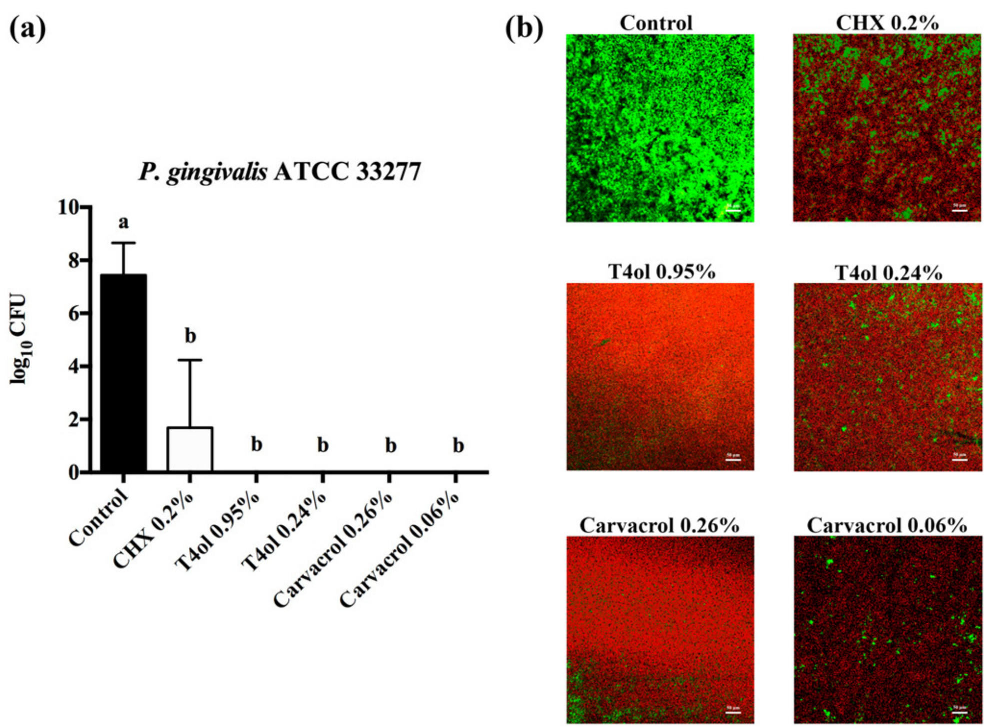

Figure 1. (a) The effect of carvacrol (0.26% and 0.06%) and terpinen-4-ol (0.95% and 0.24%) in comparison to CHX (0.2%) against *P. gingivalis* single-species biofilms (on titanium surfaces after incubation for five days). Statistical analysis was performed using Kruskall–Wallis non-parametric test complemented by Mann–Whitney tests ($p < 0.05$). (b) Representative CLSM images of an untreated *P. gingivalis* biofilm (control), *P. gingivalis* biofilm treated with 0.2% chlorhexidine (CHX), terpinen-4-ol 0.95%, terpinen-4-ol 0.24%, carvacrol 0.26% and carvacrol 0.06%. Dead cells are stained red; live cells are stained green.

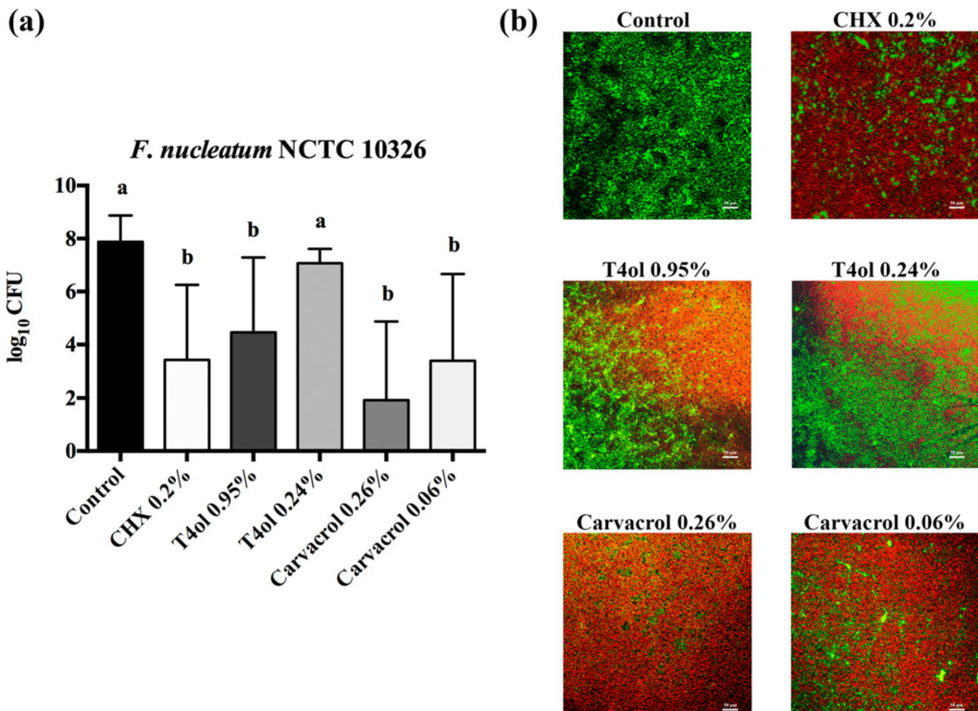

Figure 2. (a) The effect of carvacrol (0.26% and 0.06%) and terpinen-4-ol (0.95% and 0.24%), in comparison to CHX (0.2%) against *F. nucleatum* single-species biofilms (on titanium surfaces after incubation for five days). Statistical analysis was performed using Kruskall–Wallis non-parametric test complemented by Mann–Whitney tests ($p < 0.05$). (b) Representative CLSM images of an untreated *F. nucleatum* biofilm (control), *F. nucleatum* biofilm treated with 0.2% chlorhexidine (CHX), terpinen-4-ol 0.95%, terpinen-4-ol 0.24%, carvacrol 0.26% and carvacrol 0.06%. Dead cells are stained red; live cells are stained green.

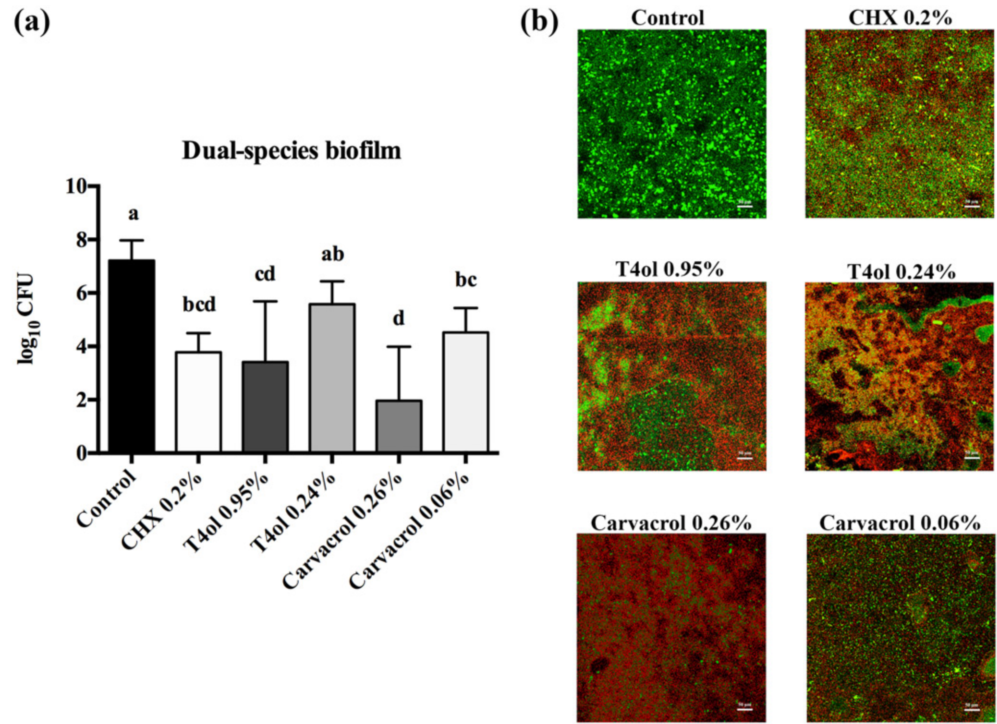

Figure 3. (a) The effect of carvacrol (0.26% and 0.06%) and terpinen-4-ol (0.95% and 0.24%), in comparison to CHX (0.2%) against dual-species biofilms of *P. gingivalis* and *F. nucleatum* (on titanium surfaces after incubation for five days). Statistical analysis was performed using ANOVA and Tukey tests ($p < 0.05$). (b) Representative CLSM images of a dual-species biofilm of *P. gingivalis* and *F. nucleatum* (control), dual-species biofilm treated with 0.2% chlorhexidine (CHX), terpinen-4-ol 0.95%, terpinen-4-ol 0.24%, carvacrol 0.26% and carvacrol 0.06%. Dead cells are stained red; live cells are stained green.

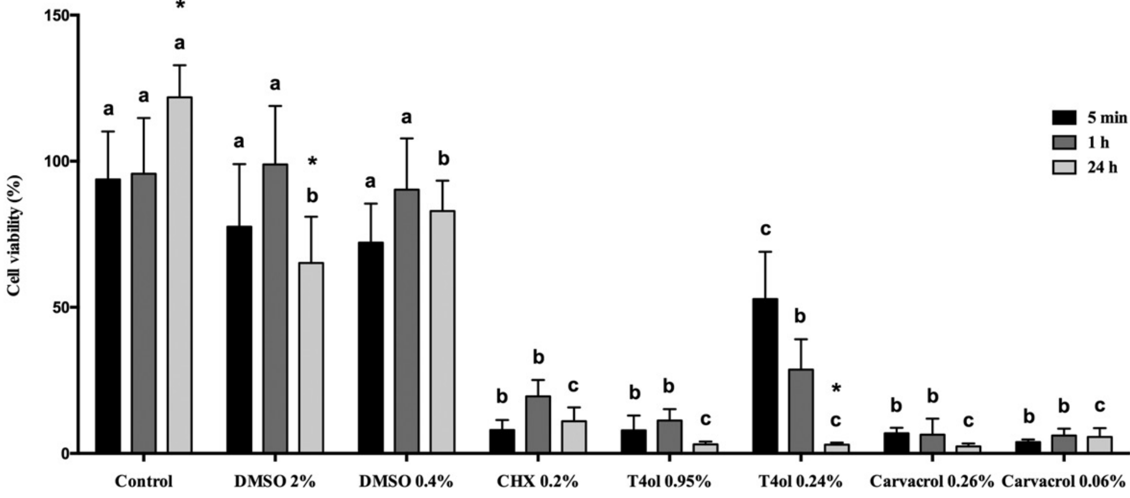

Figure 4. Cell viability (% of control) of fibroblasts L929 following treatment with carvacrol and terpinen-4-ol for different periods of time. Asterisks indicate comparisons among the times for the same group and lower case letters indicate comparisons among the groups for the same time. Groups represented by different letters are significantly different. (Two-way ANOVA, $p < 0.05$.)

Terpinen-4-ol (0.95% and 0.24%) caused a significant decrease in the number of cells after 5 min (7.89 ± 5.07% and 52.8 ± 16.19%, respectively), 1 h (11.23 ± 3.9% and 28.66 ± 10.40%), and 24 h (3.09 ± 0.92% and 2.97 ± 0.75%); the differences were significant compared with those of the control group ($p < 0.0001$). However, the fibroblast cells showed high viability after exposure for 5 min to terpinen-4-ol 0.24% compared to that with CHX (0.2%); the difference was significant: 52.80 ± 16.19% *vs* 7.99 ± 3.44% ($p < 0.0001$). Furthermore, after exposure for 5 min, 1 h, and 24 h to carvacrol (0.26%) (6.83 ± 1.90%, 6.36 ± 5.50%, and 2.32 ± 1.04%, respectively) and carvacrol (0.06%) (3.85 ± 0.90%, 6.10 ± 2.37%, and

5.62 ± 3.03%), cell viability showed a significant decrease compared with that of the control groups ($p < 0.0001$).

Discussion

The present study showed the antibacterial effect of carvacrol and terpinen-4-ol against the peri-implant pathogens *P. gingivalis* and *F. nucleatum*. There are few studies on the antimicrobial effect of carvacrol on periodontopathic bacteria. The MIC and MBC in the present study resulted (Table 1) in values lower than those reported by Ciandrini et al. (2014) in tests of carvacrol on *P. gingivalis* and *F. nucleatum* (MIC of 0.25% and MBC of 0.50%). There are no reported studies that have examined the antibacterial activity of terpinen-4-ol on *P. gingivalis* and *F. nucleatum*. Terpinen-4-ol has been shown to be the main active component of *Melaleuca alternifolia* (TTO) (Hart et al. 2000; Nogueira et al. 2014), and several studies have reported on the antimicrobial activity of TTO, promoting bactericidal and bacteriostatic effects at low concentrations against *P. gingivalis* W83 (Graziano et al. 2016) and ATCC 33277 (Takarada et al. 2004) and against *F. nucleatum* and *P. gingivalis* isolates from the oral cavity (Hammer et al. 2003).

Salivary mucoproteins form a pellicle on teeth and implant surfaces, and this event is crucial for bacterial adhesion (Hahnel et al. 2008; Busscher et al. 2010). Thus, in the current study, unstimulated human saliva was used for biofilm formation to simulate clinical conditions. A preliminary assessment of MBIC using an XTT assay was carried out to evaluate the lowest concentration tested, which inhibited biofilm formation by 50% (Miladi et al. 2016) for each species. Although XTT is a semi quantitative colorimetric assay, it has been demonstrated to correlate well with other quantitative techniques such as ATP and CFU assays (Ramage et al. 2002; Jin et al. 2004). In the presence of carvacrol and terpinen-4-ol, the metabolic activity of cells in biofilms was reduced after incubation for 24 h as a 48 h pre-formed biofilm (Table 1). The results showed that the two reference strains were more susceptible to carvacrol at lower concentrations than they were to terpinen-4-ol. The MBIC of *P. gingivalis* and *F. nucleatum* varied between 0.03 to 0.06% (carvacrol) and 0.06 to 0.24% (terpinen-4-ol), suggesting the ability of these compounds to prevent biofilm formation. These results indicate that both the carvacrol and terpinen-4-ol used in this study had an effect on the metabolic activity of the biofilm. In contrast, Ciandrini et al. (2014) showed a biofilm inhibition concentration (BIC) value of 1% for carvacrol on single-species biofilm of *P. gingivalis* and *F. nucleatum*.

Biofilms are organized and structured microbial communities embedded in polymeric matrices that release planktonic cells to colonize surfaces (Belibasakis 2014). Implant surfaces have been modified in various ways to increase bone formation around the implant and to increase the healing capacity of the bone tissue. Surface sandblasting with titanium oxide showed a regular pattern with peaks produced by blasting, with microporosity obtained by chemical treatment with acids, leading to a roughness value of 0.699 ± 0.056 μm and contact angle values between 82° and 78° (Gehrke et al. 2014). These characteristics can generate high osteoconductivity and good bone formation (Gehrke et al. 2016). In this way, this type of surface benefits the osseointegration process. However, the physico-chemical properties (higher free energy) and texture characteristics (higher roughness) of the implant surfaces may favor bacterial colonization. John et al. (2015) showed that SLA has higher plaque accumulation than that of machined-modified acid-etched surfaces. Thus, this surface type can be a disadvantage for the removal of the mineralized and mature plaque in patients. In the present *in vitro* study, the detachment of biofilms was difficult to evaluate due to the characteristics of the surface and the maturity of the developed biofilms on the titanium surface.

As observed from CLSM and CFU quantification, the results showed a significant difference between carvacrol (0.26% and 0.06%), terpinen-4-ol (0.95%) and CHX 0.2% on biofilm viability in single- and dual-species mature biofilms of *P. gingivalis* and *F. nucleatum* compared to those of the control (Figures 1, 2 and 3). In contrast to the results shown by Ciandrini et al. (2014), where carvacrol was most effective on immature biofilms, the results in this study showed that carvacrol and terpinen-4-ol were effective on mature five-day biofilms. In addition, single-species biofilms of *P. gingivalis* seem to be more susceptible to carvacrol and terpinen-4-ol than to CHX. Although the results in this study do not show statistically significant differences between the test groups and CHX, carvacrol (0.26%) showed a greater biofilm inhibition effect on mature biofilms of *F. nucleatum* and two pathogenic bacterial species. This significant effect can be explained by its mechanism of action. Monoterpenes, such as carvacrol and terpinen-4-ol, due to their lipophilic character, inhibit peptidoglycan synthesis, damage microbial membrane structures, inhibit cellular respiration (Cox et al.

2000) and modify bacterial membrane surface hydrophobicity, increasing membrane fluidity and permeability (Lambert et al. 2001). Due to the expansion of the membrane phospholipids by increasing fluidity, ion leakage might occur and consequently attenuate the permeable barrier role of the cell membrane (Turi et al. 1997). In addition, monoterpenes interrupt synthesis of genetic material, induce coagulation of cytoplasmic constituents, form toxic compounds, interrupt normal cell communication (quorum sensing) and reduce the pH inside the cell (Radulović et al. 2013). In contrast to monoterpenes, chlorhexidine causes membrane disruption and inhibition of proteolytic and glycosidic enzymes, inhibiting and reducing the viability of microorganisms (Ready et al. 2015). Furthermore, CHX appears to have a limited distribution in the extracellular polymer matrix and therefore does not have total efficacy in eradicating biofilms (Corbin et al. 2011; Brindle et al. 2011), which corroborates the results of this study.

It is necessary to determine the possible toxic effects of carvacrol and terpinen-4-ol on cells to evaluate their potential clinical applications. In this study, the cells were exposed for different contact times to the test substances and regardless of the concentration, the results revealed a concentration-and-time-dependent decrease at all the concentrations assayed for carvacrol, terpinen-4-ol and CHX (Figure 4). The monoterpenes carvacrol and terpinen-4-ol are isomeric. Despite having the same molecular formula, these compounds exhibit different levels of cytotoxic activity against cell lines. Thus, unlike the other concentrations evaluated, after 5 min contact time, terpinen-4-ol (0.24%) showed mild-grade cytotoxicity compared with the control group (93.75%) according to the ISO 10993-5 2009 Standard: Biological Evaluation of Medical Devices: In vitro Method. This study evaluated the toxic effects of carvacrol and terpinen-4-ol on fibroblasts since these cells are involved in the re-osseointegration and osseointegration processes.

The results show the cytotoxic effect of carvacrol on fibroblasts based on the concentrations with an anti-biofilm effect. Nevertheless, few studies have reported on the cytotoxicity of carvacrol on fibroblasts. Melo et al. (2014) reported an IC_{50} value (the concentration that inhibited 50% of cell growth) of 62.5 $\mu g\ ml^{-1}$ for carvacrol in the MRC5 (normal human fibroblast). In contrast, Houdkova et al. (2017) showed that the lowest cytotoxicity for carvacrol was an IC_{50} value of 5.09 $\mu g\ ml^{-1}$ using the same cell line as used by Melo et al. (2014). On the other hand, several studies reported cytotoxic effects of carvacrol on different cells such as HepG2 (Yin et al. 2012), SiHa, HeLa (Mehdi et al. 2011), Caco 2 cells (Dusan et al. 2006; Llana-Ruiz-Cabello et al. 2014), human oral cancer cells (Liang and Lu 2012), human metastatic breast cancer cells (Arunasree 2010) and HL-60 promyelocytic and Jurkat T lymphoma cells (Bhakkiyalakshmi et al. 2016). There are no studies that suggest that terpinen-4-ol has cytotoxic effects on fibroblasts. However, this compound exhibited cytotoxic effects on murine AE17 mesothelioma and B16 melanoma tumor cell lines (Greay et al. 2010) and on Jurkat cells (Döll-Boscardin et al. 2012).

It is difficult to compare the results of this study with other monoterpene studies because some factors such as pH, bacterial strains, incubation time, temperature, concentration tests and other factors can influence the results. A limitation of this study was the use of only two pathogenic oral species to develop single- and dual-species biofilms. However, this is a preliminary study, and further studies with multi-species oral biofilms will be necessary in order to mimic complex peri-implant microbiology. Although some clinical and animal studies reported the efficacy of carvacrol (0.02% and 0.5%) in the treatment of periodontitis, these studies are limited to the total reduction of the biofilm and do not differentiate the effect of carvacrol on each of the bacteria present in this complex biofilm (Botelho et al. 2009; Shahab et al. 2011). Furthermore, considering the limited number of studies of cytotoxic effects of carvacrol and terpinen-4-ol on fibroblasts, this paper may also open the way for future development of therapeutic opportunities against peri-implantitis.

Conclusion

Carvacrol and terpinen-4-ol showed antimicrobial activity, promoting bactericidal and bacteriostatic effects at low concentrations. Due to their antimicrobial and anti-biofilm properties, both monoterpenes might represent a promising approach for the prevention and treatment of peri-implant diseases. Additionally, considering the higher concentration of antimicrobial and anti-biofilm activity of carvacrol and terpinen-4-ol, chlorhexidine-like cytotoxicity was observed. Nevertheless, the results suggest that terpinen-4-ol (0.24%) has a lower cytotoxicity profile than CHX.

Acknowledgements

The authors would like to thank the following companies: Implacil De Bortoli (São Paulo, Brazil) and CTR/IPEN-

CNEN/SP Brazil for collaboration and donation of equipment for the development of this research.

Disclosure statement

The authors declare that they have no conflict of interest.

Funding

This work was supported by the Coordination of Improvement of Higher Education Personnel (CAPES) and São Paulo Research Foundation (FAPESP) [grants: 2015/08742-1; 2009/54035-4].

ORCID

Patricia Milagros Maquera-Huacho http://orcid.org/0000-0002-2193-9979
Denise Madalena Palomari Spolidorio http://orcid.org/0000-0003-2376-1024

References

Arunasree KM. 2010. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB231. Phytomedicine. 17:581–588. doi:10.1016/j.phymed.2009.12.008

Banzi ÉC, Costa AR, Puppin-Rontani RM, Babu J, García-Godoy F. 2014. Inhibitory effects of a cured antibacterial bonding system on viability and metabolic activity of oral bacteria. Dent Mater. 30:238–244. doi:10.1016/j.dental.2014.04.007

Baser KH. 2008. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Curr Pharm Des. 14:3106–3119. doi:10.2174/138161208786404227

Basso FG, Oliveira CF, Kurachi C, Hebling J, de Souza Costa CA. 2013. Biostimulatory effect of low-level laser therapy on keratinocytes in vitro. Lasers Med Sci. 28:367–374. doi:10.1007/s10103-012-1057-8

Belibasakis GN. 2014. Microbiological and immuno-pathological aspects of peri-implant diseases. Arch Oral Biol. 59:66–72. doi:10.1016/j.archoralbio.2013.09.013

Bhakkiyalakshmi E, Suganya N, Sireesh D, Krishnamurthi K, Saravana Devi S, Rajaguru P, Ramkumar KM. 2016. Carvacrol induces mitochondria-mediated apoptosis in HL-60 promyelocytic and Jurkat T lymphoma cells. Eur J Pharmacol. 772:92–98. doi:10.1016/j.ejphar.2015.12.046

Botelho MA, Nogueira NA, Bastos GM, Fonseca SG, Lemos TL, Matos FJ, Montenegro D, Heukelbach J, Rao VS, Brito GA. 2007. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. Braz J Med Biol Res. 40:349–356. doi:10.1590/S0100-879X2007000300010

Botelho MA, Martins JG, Ruela RS, I R, Santos JA, Soares JB, França MC, Montenegro D, Ruela WS, Barros LP et al. 2009. Protective effect of locally applied carvacrol gel on ligature-induced periodontitis in rats: a tapping mode AFM study. Phytother Res. 23:1439–1448. doi:10.1002/ptr.2798

Brindle ER, Miller DA, Stewart PS. 2011. Hydrodynamic deformation and removal of *Staphylococcus epidermidis* biofilms treated with urea, chlorhexidine, iron chloride, or DispersinB. Biotechnol Bioeng. 108:2968–2977. doi:10.1002/bit.23245

Busscher HJ, Rinastiti M, Siswomihardjo W, van der Mei HC. 2010. Biofilm formation on dental restorative and implant materials. J Dent Res. 89:657–665. doi:10.1177/0022034510368644

Catalán A, Pacheco JG, Martínez A, Mondaca MA. 2008. In vitro and in vivo activity of *Melaleuca alternifolia* mixed with tissue conditioner on *Candida albicans*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 105:327–332. doi:10.1016/j.tripleo.2007.08.025

Ciandrini E, Campana R, Federici S, Manti A, Battistelli M, Falcieri E, Papa S, Baffone W. 2014. In vitro activity of Carvacrol against titanium-adherent oral biofilms and planktonic cultures. Clin Oral Investig. 18:2001–2013. doi:10.1007/s00784-013-1179-9

Corbin A, Pitts B, Parker A, Stewart PS. 2011. Antimicrobial penetration and efficacy in an in vitro oral biofilm model. Antimicrob Agents Chemother. 55:3338–3344. doi:10.1128/AAC.00206-11

Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR, Wyllie SG. 2000. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). J Appl Microbiol. 88:170–175.

Dalleau S, Cateau E, Bergès T, Berjeaud JM, Imbert C. 2008. In vitro activity of terpenes against *Candida* biofilms. Int J Antimicrob Agents. 31:572–576. doi:10.1016/j.ijantimicag.2008.01.028

Döll-Boscardin PM, Sartoratto A, Sales Maia BH, Padilha de Paula J, Nakashima T, Farago PV, Kanunfre CC. 2012. In vitro cytotoxic potential of essential oils of *Eucalyptus benthamii* and its related terpenes on tumor cell lines. Evid Based Complement Alternat Med. 2012:342652. doi:10.1155/2012/342652

Dusan F, Marián S, Katarína D, Dobroslava B. 2006. Essential oils-their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability. Toxicol in Vitro. 20:1435–1445. doi:10.1016/j.tiv.2006.06.012

Figuero E, Graziani F, Sanz I, Herrera D, Sanz M. 2014. Management of peri-implant mucositis and peri-implantitis. Periodontol 2000. 66:255–273. doi:10.1111/prd.12049

Gehrke SA, Zizzari VL, Iaculli F, Mortellaro C, Tetè S, Piattelli A. 2014. Relationship between the surface energy and the histologic results of different titanium surfaces. J Craniofac Surg. 25:863–867. doi:10.1097/SCS.0000000000000873

Gehrke SA, Ramírez-Fernandez MP, Granero Marín JM, Barbosa Salles M, Del Fabbro M, Calvo Guirado JL. 2016. A comparative evaluation between aluminium and titanium dioxide microparticles for blasting the surface titanium dental implants: an experimental study in rabbits. Clin Oral Implants Res. [2016 Sept 24]; [1–6]. doi: doi:10.1111/clr.12973.

Graziano TS, Calil CM, Sartoratto A, Franco GC, Groppo FC, Cogo-Müller K. 2016. In vitro effects of *Melaleuca alternifolia* essential oil on growth and production of volatile sulphur compounds by oral bacteria. J Appl Oral Sci. 24:582–589. doi:10.1590/1678-775720160044

Greay SJ, Ireland DJ, Kissick HTA, Levy MW, Beilharz TV, Riley CF, Carson CF. 2010. Induction of necrosis and cell cycle arrest in murine cancer cell lines by *Melaleuca alternifolia* (tea tree) oil and terpinen-4-ol. Cancer Chemother Pharmacol. 65:877–888. doi:10.1007/s00280-009-1093-7

Hahnel S, Rosentritt M, Handel G, Burgers R. 2008. Influence of saliva substitute films on initial *Streptococcus mutans* adhesion to enamel and dental substrata. J Dent. 36:977–983. doi:10.1016/j.jdent.2008.08.004

Hart PH, Brand C, Carson CF, Riley TV, Prager RH, Finlay-Jones JJ. 2000. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. Inflamm Res. 49:619–626. doi:10.1007/s000110050639

Hammer KA, Dry L, Johnson M, Michalak EM, Carson CF, Riley TV. 2003. Susceptibility of oral bacteria to *Melaleuca alternifolia* (tea tree) oil in vitro. Oral Microbiol Immunol. 18:389–392. doi:10.1046/j.0902-0055.2003.00105.x

Houdkova M, Rondevaldova J, Doskocil I, Kokoska L. 2017. Evaluation of antibacterial potential and toxicity of plant volatile compounds using new broth microdilution volatilization method and modified MTT assay. Fitoterapia. 118:56–62. doi:10.1016/j.fitote.2017.02.008

Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. 2004. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. Arch Oral Biol. 49:789–798. doi:10.1016/j.archoralbio.2004.04.011

John G, Becker J, Schwarz F. 2015. Modified implant surface with slower and less initial biofilm formation. Clin Implant Dent Relat Res. 17:461–468. doi:10.1111/cid.12140

Kwiecinski J, Eick S, Wójcik K. 2009. Effects of tea tree (*Melaleuca alternifolia*) oil on *Staphylococcus aureus* in biofilms and stationary growth phase. Int J Antimicrob Agents. 33:343–347. doi:10.1016/j.ijantimicag.2008.08.028

Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. 2001. A study of the mínimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 91:453–462. doi:10.1046/j.1365-2672.2001.01428.x

Liang WZ, Lu CH. 2012. Carvacrol-induced [Ca2+] rise and apoptosis in human glioblastoma cells. Life Sci. 90:703–711. doi:10.1016/j.lfs.2012.03.027

Lindhe J, Meyle J. 2008. Peri-implant diseases: consensus report of the sixth European workshop on periodontology. J Clin Periodontol. 35:282–285. doi:10.1111/j.1600-051X.2008.01283.x

Llana-Ruiz-Cabello M, Gutiérrez-Praena D, Pichardo S, Moreno FJ, Bermúdez JM, Aucejo S, Cameán AM. 2014. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem Toxicol. 64:281–290. doi:10.1016/j.fct.2013.12.005

Mahato N, Wu X, Wang L. 2016. Management of peri-implantitis: a systematic review, 2010-2015. Springerplus. 5:105. doi:10.1186/s40064-016-1735-2

Mehdi SJ, Ahmad A, Irshad M, Manzoor N, Rizvi MMA. 2011. Cytotoxic effect of carvacrol on human cervical cancer cells. Biol Med. 3:307–312.

Mellado-Valero A, Buitrago-Vera P, Solá-Ruiz MF, Ferrer-García JC. 2013. Decontamination of dental implant surface in peri-implantitis treatment: a literature review. Med Oral Patol Oral Cir Bucal. 18:869–876.

Melo JO, Fachin AL, Rizo WF, Jesus HC, Arrigoni-Blank MF, Alves PB, Marins MA, França SC, Blank AF. 2014. Cytotoxic effects of essential oils from three *Lippia gracilis* Schauer genotypes on HeLa, B16, and MCF-7 cells and normal human fibroblasts. Genet Mol Res. 13:2691–2697. doi:10.4238/2014.April.8.12

Miladi H, Mili D, Ben Slama R, Zouari S, Ammar E, Bakhrouf A. 2016. Antibiofilm formation and anti-adhesive property of three mediterranean essential oils against a foodborne pathogen *Salmonella* strain. Microb Pathog. 93:22–31. doi:10.1016/j.micpath.2016.01.017

Mombelli A, Decaillet F. 2011. The characteristics of biofilms in peri-implant disease. J Clin Periodontol. 38:203–213. doi:10.1111/j.1600-051X.2010.01666.x

Moura JS, da Silva WP, Pereira T, Del Bel Cury AA, Garcia RCR. 2006. A comparison of the antibacterial efficacies of essential oils against oral pathogens. J Prosthet Dent. 96:205–211. doi:10.1016/j.prosdent.2006.07.004

Nogueira MN, Aquino SG, Rossa Junior C, Spolidorio DM. 2014. Terpinen-4-ol and alpha-terpineol (tea tree oil components) inhibit the production of IL-1β, IL-6 and IL-10 on human macrophages. Inflamm Res. 63:769–778. doi:10.1007/s00011-014-0749-x

Radulović NS, Blagojević PD, Stojanović-Radić ZZ, Stojanović NM. 2013. Antimicrobial plant metabolites: structural diversity and mechanism of action. Curr Med Chem. 20:932–952.

Ramage G, Vande Walle K, Bachmann SP, Wickes BL, López-Ribot JL. 2002. In vitro pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. J. Biomed. Mater. Res. Part B Appl. Biomater 46:3634–3636. doi:10.1128/AAC.46.11.3634-3636.2002

Ready D, Theodoridis G, Green I, Ciric L, Pratten J, Tay W, McDonald A. 2015. In vitro evaluation of the antibiofilm properties of chlorhexidine and delmopinol on dental implant surfaces. Int J Antimicrob Agents. 45:662–666. doi:10.1016/j.ijantimicag.2015.01.020

Renvert S, Quirynen M. 2015. Risk indicators for peri-implantitis. A narrative review. Clin Oral Implants Res. 26:15–44. doi:10.1111/clr.12636

Schou S, Holmstrup P, Jørgensen T, Skovgaard LT, Stoltze K, Hjørting-Hansen E, Wenzel A. 2003. Implant surface preparation in the surgical treatment of experimental peri-implantitis with autogenous bone graft and ePTFE membrane in cynomolgus monkeys. Clin Oral Implants Res. 14:412–422. doi:10.1034/j.1600-0501.2003.00912.x

Shahab A, Haghighati F, Baeeri M, Jamalifar H, Abdollahi M. 2011. A clinical, microbiological and immunological comparison between subgingival irrigation with Dentol and chlorhexidine in advanced periodontitis. Arch Med Sci. 7:154–160. doi:10.5114/aoms.2011.20622

Takarada K, Kimizuka R, Takahashi N, Honma K, Okuda K, Kato T. 2004. A comparision of the antibacterial efficacies of essential oils against oral pathogens. Oral Microbiol Immunol. 19:61–64. doi:10.1046/j.0902-0055.2003.00111.x

Turi M, Turi E, Koljalg S, Mikelsaar M. 1997. Influence of aqueous extracts of medicinal plants on surface hydrophobicity of *Escherichia coli* strains of different

origin. APMIS. 105:956–962. doi:10.1111/j.1699-0463.1997.tb05107.x

Ungvári K, Pelsöczi IK, Kormos B, Oszkó A, Rakonczay Z, Kemény L, Radnai M, Nagy K, Fazekas A, Turzó K. 2010. Effects on titanium implant surfaces of chemical agents used for the treatment of peri-implantitis. J Biomed Mater Res B Appl Biomater. 94:222–229. doi:10.1002/jbm.b.31644

Yin Q, Yan F, Zu XY, Wu Y, Wu X, Liao M, Deng S, Yin L, Zhuang Y. 2012. Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. Cytotechnology. 64:43–51. doi:10.1007/s10616-011-9389-y