Biofilm Inhibition of Cinnamaldehyde Against A Group of Intracanal Pathogens Compared to Chlorhexidine. An In Vitro Study

Soha Hany Atawia[1*], Dina Hamdy[2], Nagwa Mohammed Ali Khattab[3]

[1]Teaching Assistant of Pediatric Dentistry and Dental Public Health, Faculty of Dentistry, Ain Shams University, soha.mahmoud@dent.asu.edu.eg , https://orcid.org/0009-0004-7896-2995
[2]Associate Professor of Pediatric Dentistry and Dental Public Health, Faculty of Dentistry, Ain Shams University, dina-hamdy@dent.asu.edu.eg , https://orcid.org/0000-0002-7074-3569
[3]Professor of Pediatric Dentistry and Dental Public Health, Faculty of Dentistry, Ain Shams University, nagwakhattab@dent.asu.edu.eg , https://orcid.org/0000-0001-6327-7910

Corresponding Author, Soha Hany Atawia, soha.mahmoud@dent.asu.edu.eg
Received 01 July 2026, Revised 11 July 2026, Accepted 20 July 2026, Available Online 27 July 2026

Abstract

Aim: Evaluation of the antibiofilm effectiveness of cinnamaldehyde against three principal endodontic pathogens—*Staphylococcus aureus, Enterococcus faecalis, and Candida albicans*—and to compare its efficacy with that of chlorhexidine utilizing a microtiter plate biofilm model.

Materials and Methods: After being extracted, cinnamon oil was analyzed using gas chromatography–mass spectrometry (GC–MS). Broth dilution was used to calculate its minimal inhibitory concentration (MIC). Biofilm production was quantified via the crystal violet technique, with absorbance measured at 550 nm. One-way ANOVA with post hoc multiple comparisons was used for statistical analysis of the data; $p \leq 0.05$ was deemed statistically significant.

Results: Cinnamaldehyde produced a significant reduction in biofilm formation compared with the negative control for all tested microorganisms. Chlorhexidine also reduced biofilm formation; however, its effect against *S. aureus* did not reach statistical significance. No significant differences were detected between cinnamon oil and chlorhexidine in their activity against *E. faecalis* and *S. aureus*, whereas cinnamon oil showed better results than chlorhexidine against *C. albicans*.

Conclusions: Cinnamon oil effectively inhibits biofilms of common intracanal pathogens and may serve as a natural alternative to chlorhexidine as an intracanal irrigant. To verify its effectiveness, more clinical research is required.

Keywords: cinnamon essential oil; cinnamaldehyde; antibiofilm; intracanal pathogens; chlorhexidine.

How to cite this article: Atawia SH, Hamdy D, Khattab NMA. Biofilm Inhibition of Cinnamaldehyde Against A Group of Intracanal Pathogens Compared to Chlorhexidine: An In Vitro Study. Int J Drug Deliv Technol. 2026;16(72s): 1694-1702. DOI: 10.25258/ijddt.16.72s.190

Introduction

The main objective of root canal treatment is to eradicate intracanal microorganisms and prevent reinfection, though the complexity of canal anatomy limits complete disinfection. In primary molars, this difficulty is increased by intricate morphology, physiological root resorption, and proximity to developing permanent successors. Irrigation is therefore essential in pediatric endodontics to lubricate canals and remove necrotic tissue and debris, with commonly used agents including sodium hypochlorite, chlorhexidine, EDTA, citric acid, and hydrogen peroxide.[1]

Despite its widespread use and effectiveness, chlorhexidine (CHX) has notable limitations, such as reversible staining, taste alterations, oral burning, parotid gland enlargement, and mucosal desquamation. These issues can reduce patient compliance and limit its long-term use.[2] Additionally, microorganisms that can form biofilm have up to a thousand-fold greater resistance to CHX compared to planktonic cells.[3]

Highly ordered structures called microbial biofilms are created when sessile bacteria encase themselves in an extracellular polymeric substance (EPS) that they produce on their own. This protective matrix promotes microbial adhesion, survival, and long-term persistence in endodontic infection. Unlike planktonic microorganisms that exist as individual free-floating cells, microbes embedded within biofilms exhibit markedly increased tolerance to antimicrobial agents and host immune defenses. Consequently, this structural organization enables them to avoid immune

surveillance and decreases their susceptibility to phagocytic elimination. [4]

Enterococcus faecalis, a Gram-positive facultative anaerobe, is frequently involved in failed endodontic treatment, with reported prevalence approaching 90%. Its persistence has been attributed to biofilm formation, virulence factor production, tolerance to alkaline conditions, and expression of enterococcal surface protein (Esp), which promotes adhesion and antimicrobial resistance. Its ability to survive within dentinal tubules further complicates eradication, particularly in primary teeth with complex root anatomy. [5]

Staphylococcus aureus is an opportunistic microorganism implicated in primary and persistent endodontic infections, including methicillin-resistant strains. Its pathogenicity is mediated by exotoxins and enzymes, such as coagulase and alpha toxin, that contribute to tissue damage. Biofilm formation markedly increases antimicrobial resistance by as much as 1000-fold compared with planktonic cells—thereby contributing to treatment difficulty and recurrence. [6,7]

Candida albicans has been identified in approximately 7%–9% of endodontic infections, particularly in failed cases. Its persistence is attributed to dentin adhesion, biofilm formation, and resistance to commonly used intracanal medicaments, supported by phenotypic switching, efflux pump activity, and hyphal invasion. Co-infection with bacteria in nearly 10% of cases further emphasizes the polymicrobial nature of these infections. [8]

Consequently, the persistence of the previously mentioned microorganisms within biofilms has been recognized as an indication that more effective antimicrobial and antibiofilm approaches are required to achieve thorough root canal disinfection and enhance treatment outcomes. Accordingly, there is considerable interest in identifying irrigants with potent antimicrobial activity and favorable biocompatibility. [9]

Cinnamaldehyde, one of the main components of cinnamon essential oil, revealed broad-spectrum antimicrobial action and was considered a promising natural alternative. Available evidence suggests that cinnamaldehyde is less susceptible to conventional antibiotic resistance mechanisms because it acts primarily through biofilm regulation rather than direct bactericidal activity, thereby minimizing the development of resistance.[10–12] However, its antimicrobial and antibiofilm effects against endodontic pathogens have not been well studied. Thus, the purpose of this investigation was to evaluate the antibacterial and antibiofilm efficacy of cinnamon oil against major endodontic pathogens.

According to the null hypothesis (H_0), treatment with cinnamaldehyde does not result in a measurable difference in antibiofilm activity compared with chlorhexidine against the tested microbial species (*S. aureus*, *E. faecalis*, and *C. albicans*).

Ethics approval

Ethical approval for the study protocol was granted by the Research Ethics Committee at the Faculty of Dentistry, Ain Shams University (Approval No. FDASU-Rec EM122312).

Materials and Methods

An in vitro experimental approach was adopted for the present study that follows CRIS Guidelines 2014 (Checklist for Reporting In-vitro Studies); cinnamon oil was extracted from bark by hydrodistillation, and the three microorganisms (*Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans*) were cultured from stock isolates using standard selective media and incubation conditions. Actively growing colonies were transferred to appropriate broth media to prepare fresh cultures. All suspensions were adjusted to 0.5 McFarland for use in MIC determination and biofilm assays.

Sample Size Estimation

Power Analysis and Sample Size (PASS) software (version 15.0.10) was used for sample size calculation. An alpha (α) level of 0.05 (5%), a beta (β) level of 0.20 (20%), i.e., power=80%, and after assuming that there was a large effect size difference (h=0.8) between cinnamaldehyde and chlorhexidine regarding biofilm inhibition, the predicted sample size (n) was found to be 72 specimens. . These were allocated equally into three groups of 24 specimens each, with each group further divided into three subgroups of 8 specimens according to the tested microorganism.

Sample collection

A total of 72 test specimens were included, comprising 24 experimental units for each of the microorganisms *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans*. Microbial isolates were obtained from a private microbiology laboratory and used to prepare standardized inocula for the subsequent antimicrobial and antibiofilm assays.

Sample grouping

The 72 microbial specimens were allocated to three groups (n = 24 each): Group I, cinnamon oil (study group); Group II, 2% chlorhexidine (positive control); and Group III, untreated inoculum (negative control). Each group was further stratified according to the tested microorganism into three subgroups (n = 8):

subgroup A, *Staphylococcus aureus*; subgroup B, *Enterococcus faecalis*; and subgroup C, *Candida albicans*.

Cinnamon oil extraction

Dried cinnamon bark was purchased from the market and coarsely crushed using an electrical grinder to obtain 350 g of powder. Essential oil extraction was performed using a Clevenger-type apparatus (Witeg Labor Technik GmbH, Germany). For each run, powdered bark was mixed with distilled water and subjected to hydrodistillation for 3 h. The extraction produced 6.73 g of oil, corresponding to 1.92% (w/w) of the original dry material. Residual moisture was removed with anhydrous sodium sulfate (1–5% w/w) (ACG-EGYPT). All procedures were conducted at the Pharmaceutical Laboratory, Department of Medicinal and Aromatic Plants Research, National Research Centre. The oil was stored in a deep freezer until (GC–MS) analysis. [13]

GC–MS Analysis

GC–MS analysis was performed according to previously published methods. [14,15] using a TRACE GC Ultra (THERMO Scientific, USA) coupled with an ISQ Single Quadrupole Mass Spectrometer at the National Research Center. The system utilized a TG-WAX MS column (30 m × 0.25 mm, 0.25 µm film thickness) with helium as the carrier gas (1.0 mL/min, split ratio 1:10). The temperature program started at 60°C for 1 minute, then increased by 3°C/min to 240°C, where it was maintained for 1 minute. Both the injector and detector were set to 240°C. A 1 µL sample (1:100 in hexane) was injected. Spectra were recorded via electron ionization (70 eV, m/z 40–450). Compound identification was based on comparison with authentic standards and spectral libraries (Wiley, NIST).

The extracted oil GC–MS analysis identified cinnamaldehyde as the predominant constituent (79.48%). Other detected compounds included α-cubebene (6.82%), δ-cadinene (3.52%), à-muurolene (3.17%), and hydrocinnamaldehyde (0.93%). These findings confirm that the extracted oil was rich in cinnamaldehyde (Figure 1; Table 1).

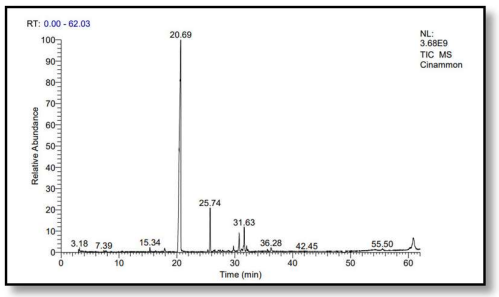

Figure 1: Chromatogram of extracted cinnamon oil

Table (1) GC-MS analysis of the essential oil's chemical components

R.T.*	Area %	Compounds
15.34	**0.93**	Hydrocinnamaldehyde
17.88	**0.66**	trans-Cinnamaldehyde
20.67	**79.48**	Cinnamaldehyde
25.35	**0.35**	α-Gurjunene
25.74	**6.82**	α-Cubebene
26.55	**0.29**	AROMADENDRENE
28.87	**0.28**	Humulene
29.78	**0.72**	γ-Muurolene
30.76	**3.17**	à-Muurolene
31.15	**0.51**	Cinnamaldehyde, o-methoxy-
31.63	**3.52**	δ-Cadinene
32.04	**0.84**	CADINA-1,4-DIENE
32.25	**0.28**	α-Calacorene
35.66	**0.46**	Cubenol
36.27	**0.81**	tau.-Muurolol

microbial suspensions were adjusted to the 0.5 McFarland standard and used for MIC determination and biofilm inhibition tests.

Cinnamon oil minimal inhibitory concentration

The MIC of cinnamon oil was determined using broth dilution. [16] A series of two-fold serial dilutions of cinnamon oil was prepared using brain–heart infusion broth, yielding concentrations equivalent to 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 of the original stock solution. The first dilution was obtained by combining 0.25 mL of the stock preparation with an equal volume (0.25 mL) of sterile broth. Successive

Cultivation of Microorganisms

Three microorganisms often linked with endodontic infections—*S. aureus*, *E. faecalis*, and *C. albicans*—were obtained as stock isolates. The bacterial isolates were subcultured on blood agar (Liofilchem, Italy) and incubated at 37°C for 24 hours. *C. albicans* was subcultured on Sabouraud dextrose agar (Oxoid Ltd., Cambridge, UK) and incubated at 30-37 degrees Celsius for 24-48 hours. Fresh, actively growing cultures were obtained by inoculating well-isolated colonies into brain-heart infusion broth or Sabouraud dextrose broth (HiMedia, India), as required. Before each experiment,

* Retention Time (R.T): The time a compound takes to travel through the GC column to the detector, which is characteristic under fixed conditions

nm utilizing a microplate reader (Thermo Labsystems; Waltham, MA).

The obtained optical density values reflected the extent of biofilm inhibition induced by cinnamon oil and chlorhexidine compared to untreated controls, where an inverse relationship exists between biofilm inhibition and the measured optical density values.

Statistical Analysis

All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) version 20. Numerical data were summarized as mean, standard deviation, median, range, and 95% confidence interval. Data distribution was assessed for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests. ANOVA with Bonferroni's post hoc test was used for intergroup comparisons. All p-values are two-sided. P-values ≤0.05 were considered significant.

Results

One-way ANOVA revealed a significant difference in mean optical density values among the three groups—cinnamon oil (Group I), chlorhexidine (Group II), and negative control (Group III) ($p \leq 0.001$). Mean ± SD values are presented in Table 2 and Figure 2.

Table 2. Mean ± SD optical density values (OD_{550}) of tested microorganisms among study groups.

Group / Subgroup	Cinnamon oil (I)	Chlorhexidine (II)	Negative control (III)	p-value
S. aureus (A)	0.197± 0.076 (a)	0.279±0.096 (a,b)	0.354± 0.032 (b)	0.001*
E. Faecalis (B)	0.288± 0.057 (a)	0.267±0.052 (a)	0.384± 0.030 (b)	0.000*
Candida (C)	0.176± 0.066 (a)	0.247±0.069 (b)	0.348± 0.044 (c)	0.000*

Significance level: $p \leq 0.05$; *significant; ns, non-significant. Within each row, mean ± standard deviation (SD) values marked with the same superscript letter were not significantly different from one another according to the post hoc multiple-comparison analysis.

dilutions were then generated by transferring 0.25 mL from each preceding tube into 0.25 mL of fresh sterile broth, resulting in six dilution levels. Following dilution preparation, 0.25 mL of a standardized microbial inoculum was added to each tube, bringing the final volume to 0.5 mL. The inoculated tubes were incubated at 37 °C for 24–48 hours.

As the natural opacity and turbidity of the essential oil impeded direct visual assessment of microbial growth in broth, aliquots of 10 µL were aseptically taken from each tube and streaked onto blood agar plates. The plates were subsequently incubated at 37 °C for 24 hours to confirm microbial viability. The MIC was determined as the lowest dilution that showed complete absence of growth following subculture. Based on these findings, the MIC was identified as the 1/16 dilution, which was subsequently selected for the biofilm inhibition experiments

Microtiter Plate-Based Biofilm Assay

The antibiofilm activity of cinnamon oil and chlorhexidine on *Staph. aureus*, *E. faecalis*, and *Candida* were evaluated via the microtiter plate crystal violet assay as described by O'Toole. [17] Standardized microbial suspensions (0.5 McFarland) were inoculated into sterile, flat-bottom 96-well polystyrene microplates (GenFollower Biotech CO., LTD, China) (100 µL per well).

For each microorganism, wells were assigned to three experimental conditions: Group I, cinnamon oil at the MIC; Group II, 2% chlorhexidine; and Group III, untreated control. The tested agent was added to the inoculated wells at the start of the experiment to allow biofilm formation in the agent's presence. Control wells received inoculum without any antimicrobial agent. Eight replicate wells were included for each experimental condition.

The incubation of microplates was conducted at 37 °C for 24–48 hours to promote biofilm formation in the presence of the tested compounds. Planktonic cells were carefully aspirated, and wells were gently washed with sterile saline to remove non-adherent microorganisms. Subsequently, 125 µL of a 0.1% (w/v) crystal violet solution (Vion Biosciences, New York, USA) was added to each well for biofilm staining, followed by an incubation period of 15 minutes at room temperature. The removal of excess dye and the elimination of unbound stain were achieved by thoroughly washing the wells with sterile distilled water. After air-drying, the bound crystal violet was solubilized through the application of 33% acetic acid. The quantification of biofilm biomass was performed by measuring the absorbance at 550

including efficacy against resistant organisms such as *Enterococcus faecalis*. Lower concentrations (0.12%–1%) are generally less potent and have shorter substantivity. This concentration is widely used in intracanal disinfection protocols and is consistent with recommendations in pediatric endodontic practice. [22,23]

This study used clinical isolates rather than reference strains to better reflect the microbiological conditions encountered in endodontic infections. Clinical isolates capture natural genetic variability, virulence expression, and antimicrobial resistance patterns present in real-world disease, thereby enhancing the clinical relevance of the findings.[24]

Assessment of antibiofilm activity is often more clinically relevant than microbial counting alone. Conventional colony-forming unit (CFU) or planktonic counts primarily quantify free-floating cells and may underestimate microbial persistence because cells within biofilms possess unique phenotypic properties, exhibit lower metabolic rates, and show higher ability to resist antimicrobial agents than free-floating microbial cells. [25,26]

Moreover, a reduction in CFU does not necessarily indicate disruption of the biofilm matrix or loss of adherent biomass that contributes to chronic infection and reinfection. Accordingly, biofilm-based assays provide a more clinically relevant estimate of irrigant performance against the structured microbial communities found in infected root canals.[25–27]

Quantification of biofilm accumulation was performed through optical density measurements at 550 nm obtained with a microplate-reading system. Optical density reflects light absorption and scattering by the stained biofilm biomass and is proportional to its extent. In this assay, lower OD_{550} values indicated reduced biofilm formation and, therefore, greater antibiofilm efficacy. This method is widely acknowledged as a quick, easy, and affordable approach for assessing the impact of antimicrobial drugs on the development of biofilms and microbial growth. [28]

The findings demonstrated that cinnamon oil significantly inhibited biofilm development in all tested microorganisms when compared with the untreated control group, and its effectiveness was found to be comparable to that of chlorhexidine. The antibiofilm activity observed could be due to the high level of cinnamaldehyde, which is the main active component of cinnamon oil. Cinnamaldehyde has been found to have broad-spectrum antibacterial and antifungal properties, which

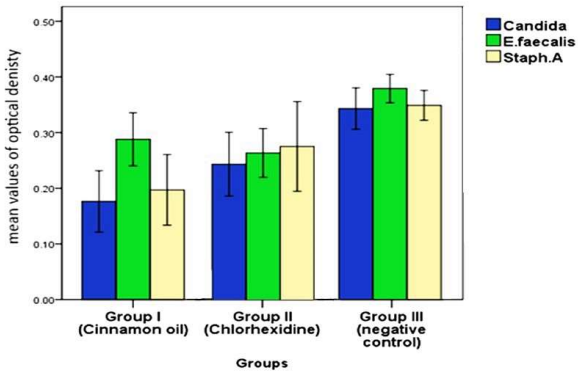

Figure 2: Bar chart showing mean values of optical density of tested microbes among the tested groups.

Bonferroni post hoc analysis demonstrated significant differences in biofilm inhibition among the groups across the three microbial subgroups. The cinnamon oil group (Group I) showed significantly lower biofilm formation than the negative control group (Group III) for all tested microorganisms—*C. albicans*, *E. faecalis*, and *S. aureus* (all $p < 0.001$). In contrast, the chlorhexidine group (Group II) showed significant biofilm reduction compared with the control group only for *E. faecalis* ($p < 0.001$) and *C. albicans* ($p = 0.004$), whereas the reduction against *S. aureus* was not statistically significant ($p = 0.056$). Direct comparison between the two treatment groups showed no significant difference for *S. aureus* ($p = 0.080$) or *E. faecalis* ($p = 0.464$). However, cinnamon oil demonstrated significantly greater biofilm suppression than chlorhexidine against *C. albicans* ($p = 0.049$).

Discussion

The present in vitro study investigated the antibiofilm efficacy of cinnamon oil against three major endodontic pathogens—*Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans*—[18–20]and compared its effect with that of chlorhexidine utilizing a microtiter plate-based biofilm assay. The study was carried out in compliance with the CRIS guidelines to enhance the quality, transparency, and reproducibility of in vitro dental research.

Cinnamon was selected because of its well-documented antibacterial and antifungal properties.[11] Bark was used for oil extraction because it is rich in cinnamaldehyde and trans-cinnamaldehyde, the principal compounds responsible for antimicrobial activity [21], whereas leaves and fruits contain higher concentrations of phenolic compounds such as eugenol, which may exhibit comparatively weaker antibacterial effects. [10]

Chlorhexidine at a concentration of 2% was selected as the positive control because of its strong and sustained antimicrobial activity,

effectiveness than chlorhexidine, particularly against *E. faecalis*. One possible explanation is that **Marcoux et al.** used reference strains, whereas the present study used clinical isolates. Additional differences in test concentrations, oil purity, biofilm maturation conditions, and methodology may also have contributed to the discrepant findings.

This study provides clinically relevant evidence regarding the antimicrobial potential of cinnamon oil against *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans*. The use of clinical isolates strengthens the applicability of the findings, and standardized protocols for inoculum preparation, incubation, and biofilm formation support reliability and reproducibility.

Because cinnamon oil demonstrated efficacy comparable to that of chlorhexidine, the null hypothesis could not be rejected. These results underscore its potential as a natural option for intracanal disinfection, especially considering the negative effects linked to chlorhexidine, including tooth staining, taste changes, and mucosal irritation.

This study has several limitations, including the absence of evaluation within the complex root canal anatomy, lack of cytotoxicity and anti-inflammatory assessment, and the use of single-species biofilms. Additional in vivo and clinical studies are needed to confirm these findings and to determine the safety and therapeutic applicability of cinnamon oil under clinically relevant conditions.

Conclusion

Within the constraints of this in vitro investigation, cinnamon essential oil demonstrated significant antibiofilm activity against *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans*, highlighting its potential as a natural antimicrobial adjunct in pediatric endodontic irrigation protocols.

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are mediated through multiple mechanisms, including interference with biofilm formation, disruption of mature biofilm structures, inhibition of quorum-sensing and cyclic di-GMP signaling pathways, and enhancement of microbial cell membrane permeability. [29]

The notable reduction in optical density values suggests that cinnamon oil was effective against biofilm-associated growth, which is generally more resistant to conventional antimicrobial agents. This finding highlights its potential as an antibiofilm agent against clinically challenging endodontic pathogens.[30] Chlorhexidine also reduced biofilm formation but did not achieve statistical significance against *S. aureus*, possibly because of the organism's robust biofilm architecture, reduced metabolic activity, and the presence of persister cells that limit disinfectant penetration.[31]

The observed outcomes correspond with findings described in previous literature. **Ali et al.**[32] and **Hu et al.** [33] found that *E. faecalis* biofilm formation was inhibited by cinnamaldehyde through quorum sensing disruption and reduction of virulence factor expression. Similarly, **Ferro et al.** [34] reported bactericidal activity against *S. aureus* and *E. faecalis*. **Borges-Grisi et al.** [35] demonstrated that cinnamaldehyde and α-terpineol significantly reduced cell viability and phospholipase activity in *C. albicans* and *E. faecalis* biofilms. In addition, **Jia et al.** [36] confirmed a potent antibiofilm action of cinnamaldehyde against methicillin-resistant *S. aureus* (MRSA).

The present findings also agree with those of **Tartor et al.**[37], who showed that cinnamon oil has strong antifungal and antibiofilm activity against *Candida albicans*, including inhibition of growth, biofilm development, as well as disruption of biofilm architecture and modulation of virulence-associated genes.

In contrast, **Tavafi** [38] reported a minimum biofilm inhibitory concentration (MBIC) of 125 µg/mL and a minimum biofilm eradication concentration (MBEC) of 250 µg/mL for chlorhexidine against *S. aureus*, indicating effective inhibition and eradication at relatively low concentrations. Likewise, **Abdallah et al** [39] described strong time- and concentration-dependent antibiofilm effects of chlorhexidine and sodium hypochlorite against *S. aureus* biofilms. The discrepancy from the present findings may be explained by differences in isolate source, strain-specific biofilm-forming capacity, disinfectant concentration or formulation, and experimental methods for biofilm growth and quantification.

However, **Marcoux et al.**[40] reported different findings stating that cinnamon oil had greater

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