International Journal of Dental Sciences
www.dentaljournal.in
ISSN: 2663-4708
Received: 26-09-2026, Accepted: 26-10-2026, Published: 10-11-2026
Volume 8, Issue 4, 2026, Page No. 45-50

Antimicrobial effect of ultrasonic activation of methylene blue during photodynamic therapy on a multispecies biofilm in root canals: an *ex vivo* study

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DOI: https://doi.org/10.66856/ijds.2026.8.4.8153

Abstract
Objetive: To evaluate and compare the antimicrobial efficacy of photodynamic therapy with and without ultrasonic activation of methylene blue against a multispecies biofilm developed in the root canals of human single-rooted teeth.
Methods: Eighty human single-rooted teeth were randomly allocated into four groups (n = 20): untreated multispecies biofilm (G1), conventional endodontic treatment (G2), photodynamic therapy (G3), and photodynamic therapy with ultrasonic activation of methylene blue (G4). The biofilm consisted of *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*, and *Candida albicans* and was cultured for 14 days. In G3, 0.005% methylene blue was used with a 5-minute pre-irradiation period, followed by irradiation with a 660-nm, 100-mW diode laser for 90 seconds. In G4, the photosensitizer was ultrasonically activated for 20 seconds before irradiation. Residual microbial load was quantified as colony-forming units per milliliter (CFU/ml). Overall differences were analyzed using the Kruskal-Wallis test, and pairwise comparisons were performed using the Mann-Whitney U test with Holm-Bonferroni adjustment.
Results: Median microbial loads were 2.93×10^6 CFU/ml in G1, 0 CFU/ml in G2, 1.65×10^5 CFU/ml in G3, and 2.89×10^4 CFU/ml in G4. Relative reduction compared with the untreated control group (G1) was 100.00% for G2, 94.37% for G3, and 99.01% for G4. Highly significant overall differences were observed among the groups ($H [3] = 69.94$; $p < 0.001$; $\varepsilon^2 = 0.885$). G4 showed a significantly lower microbial load than G3 ($p < 0.001$), with a large effect size ($r = 0.80$) and an estimated difference of 0.70 log units.
Conclusion: Ultrasonic activation of methylene blue significantly enhanced the antimicrobial efficacy of photodynamic therapy against intracanal multispecies biofilm. Under the experimental conditions evaluated, this strategy may enhance PDT as an adjunctive procedure for endodontic disinfection.

Keywords: Biofilm, endodontics, methylene blue, photodynamic therapy, ultrasonics

Introduction

Infection control is one of the main objectives of endodontic treatment and depends on the reduction of microorganisms and their by-products through chemomechanical preparation (Tonini *et al*., 2022; Das U *et al*., 2025) [8, 23]. However, the anatomical complexity of the root canal system and the organization of microorganisms within biofilms may limit their complete elimination (Siqueira & Rôças, 2022) [21]. Biofilms, particularly multispecies biofilms, exhibit an extracellular matrix and diverse microbial interactions that promote their persistence and tolerance to antimicrobial agents (Flemming *et al*., 2010; Joshi *et al*., 2021) [10, 15]. Although sodium hypochlorite remains one of the irrigants of choice because of its antimicrobial activity and ability to dissolve organic tissue, its action may be limited in difficult-to-reach areas, which has encouraged the development of complementary disinfection strategies (Tonini *et al*., 2022; Boutsioukis *et al*., 2022 [5, 23].

Among these strategies, photodynamic therapy (PDT) is based on the interaction between a photosensitizer, a light source, and molecular oxygen, resulting in the generation of reactive species capable of inducing oxidative damage and inactivating microorganisms (Plotino *et al*., 2019; Hamblin *et al*., 2016) [14, 19]. Its efficacy may be influenced by photosensitizer distribution, pre-irradiation time, irradiation parameters, and biofilm characteristics (Hamblin *et al*., 2016) [14]. Ultrasonic activation of the photosensitizer has been proposed as an alternative to improve its penetration and distribution within the root canal system, and experimental studies have reported greater microbial reduction when it is combined with PDT (Niavarzi *et al*., 2016; Alves-Silva *et al*., 2025) [3].

Therefore, the aim of the present study was to evaluate and compare the antimicrobial efficacy of photodynamic therapy with and without ultrasonic activation of methylene blue against a multispecies biofilm developed in the root canals of human single-rooted teeth.

Materials and Methods

Experimental design

An *ex vivo* study was conducted at the General Microbiology Laboratory of Universidad Hemisferios, with approval from the institution's Bioethics Committee (CEUHE25-180). Eighty human single-rooted teeth were randomly allocated into four experimental groups of 20 specimens each: untreated multispecies biofilm (G1), conventional endodontic treatment (G2), photodynamic therapy (G3), and photodynamic therapy with ultrasonic activation of methylene blue (G4). Additional non-inoculated teeth were used as sterility controls.

Sample selection and preparation

Permanent human single-rooted teeth with a single root canal, a completely formed apex, and no previous endodontic treatment were selected. Teeth with fractures, resorptions, cracks, or root curvature greater than 20° according to Schneider's method were excluded, as reported by Schneider (1971) [20].

The crowns were sectioned to standardize the root length to 16 mm. Working length was determined using a #10 K-file, and the apical foramen was sealed with epoxy resin. The specimens were sterilized in an autoclave at 121 °C for 15 minutes at 15 psi.

Preparation of the multispecies biofilm

The biofilm consisted of *S. aureus* (clinical isolate), *E. faecalis* ATCC 29212, *S. mutans* ATCC 25175, and *C. albicans* ATCC 20231. *S. aureus*, *E. faecalis*, and *C. albicans* were incubated aerobically at 37 °C for 24 hours, whereas *S. mutans* was incubated anaerobically at 37 °C for 48 hours.

Suspensions of *S. aureus*, *E. faecalis*, and *C. albicans* were adjusted to 0.5 McFarland, and *S. mutans* to 1.0 McFarland. The four suspensions were then combined in equal proportions and mixed with Brain Heart Infusion (BHI) broth.

Each root canal was inoculated with 10 µl of the multispecies suspension using a NaviTip endodontic tip. The specimens were incubated anaerobically at 37 °C for 14 days, with approximately 10 µl of BHI replenished every 48 hours. The presence of microorganisms within the biofilm was verified by analyzing the samples using biochemical tests and phenotypic characteristics of the colonies.

Root canal disinfection protocols

In G1, corresponding to the untreated biofilm group, the root canals received no antimicrobial treatment and were rinsed with 5 ml of sterile saline solution.

In G2, corresponding to the conventional endodontic treatment group, the root canals were instrumented with WaveOne Gold Medium 35/.06 (Dentsply Sirona, Ballaigues, Switzerland) using 10 ml of 5.25% NaOCl. The irrigant was activated for 30 seconds using an Irrisonic® tip (Helse Dental, Brazil) coupled to an Endo 3 unit (Woodpecker, China). Subsequently, 1 ml of 17% EDTA was applied for 1 minute, followed by a final rinse with 5 ml of sterile saline solution.

In G3, treated with photodynamic therapy (PDT), the root canals were initially irrigated with 5 ml of sterile saline solution for 1 minute. Subsequently, 10 µl of 0.005% methylene blue was applied, with a pre-irradiation time of 5 minutes. Irradiation was performed using a 660-nm, 100-mW diode laser for 90 seconds with a Whitening Premium device (DMC Equipamentos, Brazil). The optical fiber was inserted to the working length and moved in a helical motion from the apical toward the cervical region. Finally, the root canals were rinsed with 5 ml of sterile saline solution.

In G4, treated with photodynamic therapy with ultrasonic activation, the conditions described for G3 were reproduced; however, during the pre-irradiation period, 0.005% methylene blue was ultrasonically activated for 20 seconds using a smooth flexible polymer tip coupled to an Endo 3 unit (Woodpecker, China). Laser irradiation was subsequently performed using the same parameters described for G3.

Root canal sampling and microbiological analysis

After each protocol, the root canal walls were scraped using a sterile #25 Hedström file, followed by insertion of three sterile #15 absorbent paper points. The samples were transferred to microtubes containing 1,000 µl of phosphate-buffered saline and homogenized using a vortex mixer for 1 minute.

Serial dilutions were performed, and 100 µl of each sample was spread onto Mueller-Hinton agar supplemented with 5% sheep blood. The plates were incubated for 24 hours, after which colony-forming units per milliliter (CFU/ml) were counted. Sterility controls remained incubated under the same conditions to rule out contamination.

Statistical analysis

Data were organized in Microsoft Excel and analyzed using Python 3.12. Quantitative variables were described using mean, standard deviation, median, interquartile range, and minimum and maximum values.

Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using the Levene and Brown-Forsythe tests. Because the assumptions for parametric testing were not met, overall differences among the four groups were assessed using the Kruskal-Wallis H test. Pairwise comparisons were performed using the Mann-Whitney U test with Holm-Bonferroni adjustment. Dunn's test was used as a complementary analysis, and rank-biserial correlation was calculated as a measure of effect size. A p value < 0.05 was considered statistically significant.

Results

A total of 80 teeth were analyzed, with 20 specimens included in each of the four experimental groups. Residual microbial load differed markedly among the treatments. The median values were 2.93×10^6 CFU/ml in G1, 0 CFU/ml in G2, 1.65×10^5 CFU/ml in G3, and 2.89×10^4 CFU/ml in G4. Among the two groups treated with photodynamic therapy, G4 showed a lower residual microbial load than G3. Because of the wide range of counts and the presence of zero values, the data were represented using the $\log_{10}(\text{CFU/ml} + 1)$ transformation (Figure 1).

Overall analysis using the Kruskal-Wallis test showed statistically significant differences among the four groups ($H(3) = 69.94$; $p < 0.001$), with a large effect size ($\varepsilon^2 = 0.885$).

Pairwise comparisons using the Mann-Whitney U test with Holm-Bonferroni adjustment showed highly significant differences among the groups. G1 had a higher microbial load than G2, G3, and G4 ($p < 0.001$ for all comparisons). Likewise, G2 had a lower microbial load than G3 and G4 ($p < 0.001$ for both comparisons).

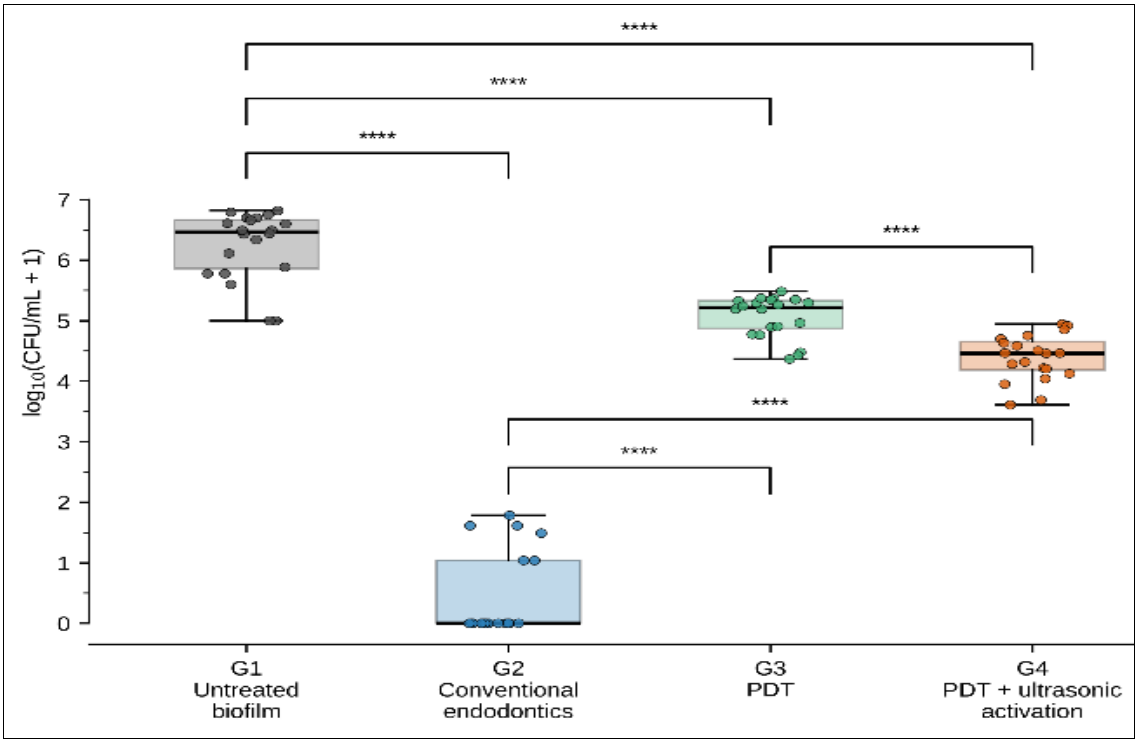


Fig 1: Antimicrobial efficacy of photodynamic therapy with and without ultrasonic activation in the disinfection of multispecies biofilm in root canals.

Note: G1 (untreated biofilm) showed the highest microbial load, whereas G2 (conventional endodontic treatment) showed the lowest residual microbial load. G3 received photodynamic therapy (PDT) only, without previous endodontic treatment, and G4 received PDT with ultrasonic activation, also without previous endodontic treatment. Between these two groups, G4 showed lower counts than G3, indicating a greater antimicrobial effect associated with ultrasonic activation of the photosensitizer. Brackets indicate pairwise comparisons using the Mann–Whitney U test with Holm-Bonferroni adjustment. **** $p < 0.001$.

In the main comparison of the study, G4 showed a significantly lower microbial load than G3 (U = 360; adjusted $p < 0.001$; r = 0.80). On the transformed scale, G4 showed 4.386 ± 0.378 $\log_{10}$(CFU/ml + 1), compared with 5.065 ± 0.346 $\log_{10}$(CFU/ml + 1) in G3. The estimated difference between the two groups was 0.70 log units, with a large effect size.

When the treatments were compared with the untreated biofilm group, the relative reduction calculated from the medians was 100.00% for G2, 94.37% for G3, and 99.01% for G4. Although G2 had a median of 0 CFU/ml, six of the 20 samples showed residual microbial growth; therefore, the 100% value corresponds to the reduction calculated from the median and does not imply the absence of cultivable microorganisms in all G2 specimens (Figure 2).

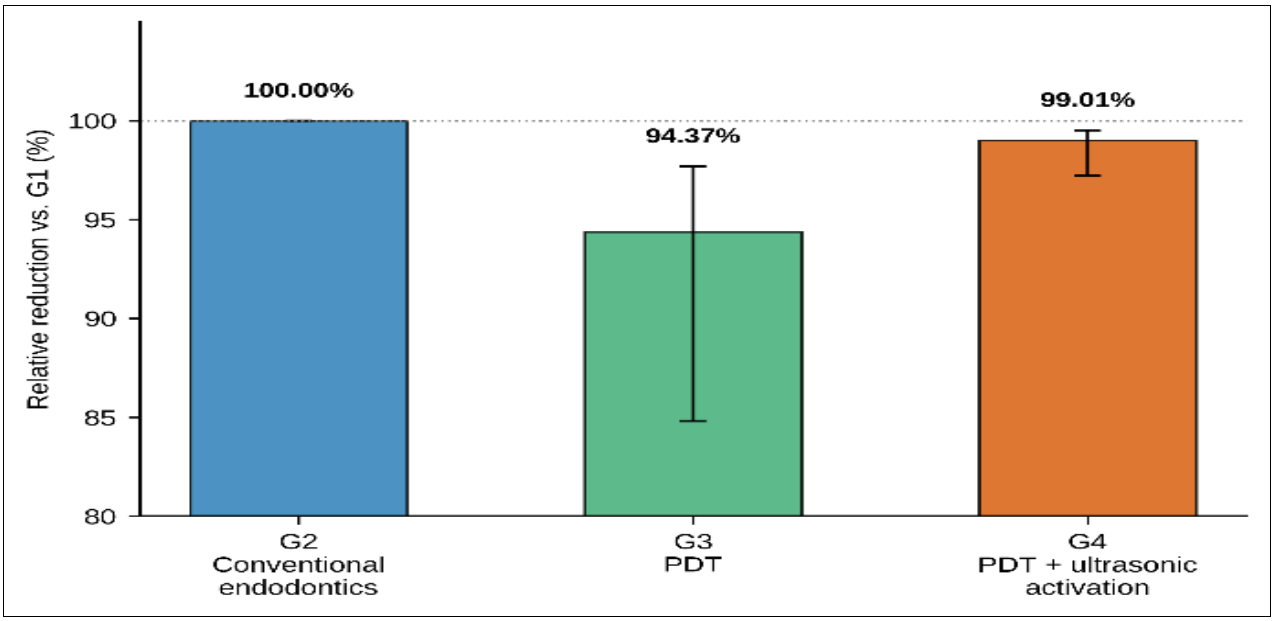


Fig 2: Relative reduction in microbial counts in dental specimens after conventional endodontic treatment, photodynamic therapy, and photodynamic therapy with ultrasonic activation compared with the control group (multispecies biofilm).

The confidence intervals obtained using percentile bootstrap with 10,000 resamples showed median-based reductions of 99.99-100% for G2, 84.81-97.63% for G3, and 97.21-99.53% for G4. The greatest reduction among the photodynamic therapy protocols was observed when methylene blue underwent ultrasonic activation.

Discussion

The main finding of the present study was that ultrasonic activation of methylene blue increased the antimicrobial activity of photodynamic therapy against an intracanal multispecies biofilm. PDT without activation achieved a relative reduction of 94.37%, whereas the addition of ultrasonic activation increased this reduction to 99.01%. In addition, G4 showed a significantly lower microbial load than G3, with a large effect size, demonstrating that the incorporation of ultrasound produced an additional effect under the experimental conditions evaluated.

The reduction achieved with PDT without activation is consistent with the wide variability reported for this technique. Other studies, like Chrepa *et al*. (2014) and Fimple *et al*. (2008) [7, 9], have reported bacterial reductions ranging from 91.3% to 100%, whereas reductions close to 80% have been observed in polymicrobial models treated with methylene blue. These differences may be related to biofilm composition and maturity, photosensitizer concentration and distribution, pre-irradiation time, and irradiation parameters, as previously reported by Plotino *et al*. (2019), Chrepa *et al*. (2014), and Fimple *et al*. (2008) [7, 9, 19].

The additional benefit observed with ultrasonic activation has also been described experimentally. Niavarzi *et al*. (2019) [18] mentioned that the incorporation of ultrasound during PDT has increased the reduction of *E. faecalis* and has been associated with greater photosensitizer penetration depth. Likewise, lower residual contamination has been observed when photodynamic therapy is combined with ultrasonic activation, as reported by Ghinzelli *et al*. (2014) [11]. In multispecies models treated with 0.005% methylene blue and 20 seconds of activation, as in the study by Alves-Silva *et al*. (2025) [3], an additional antimicrobial effect of the combined protocol has also been identified. That is to say, the findings of Niavarzi *et al*. (2019), Ghinzelli *et al*. (2014), and Alves-Silva *et al*. (2025) [3, 11, 18] support the possibility that ultrasonic agitation may enhance photosensitizer distribution and penetration and increase its contact with the biofilm.

However, the magnitude of microbial reduction is not directly comparable across all studies. Differences in microbial composition, biofilm maturation time, initial microbial density, pre-irradiation time, activation duration, and irradiation parameters may influence the antimicrobial response, as described by Abdelkarim-Elafifi *et al*. (2021), Ali *et al*. (2020), and Vendramini *et al*. (2020) [1, 2, 25]. Other methods aimed at improving the distribution of antimicrobial agents have also shown favorable results, as reported by Tennert *et al*. (2015). Guity *et al*. (2025) [13, 22] observed that the combination of photodynamic therapy with SWEEPS-assisted photoacoustic irrigation increased antimicrobial activity against biofilms, while Torres *et al*. (2026) [24] described that the combined use of 5.25% NaOCl with 660-nm diode laser irradiation produced greater bacterial reduction than the irrigant alone in *ex vivo* models. Although the physical mechanisms differ, these findings support the use of complementary strategies to improve disinfection in difficult-to-reach areas.

Conventional endodontic treatment showed the lowest microbial load in the study, with a median of 0 CFU/ml. However, six of the 20 samples showed cultivable microbial growth. The high reduction observed may be related to the antimicrobial activity of 5.25% NaOCl used during chemomechanical preparation, as demonstrated by Mohmmed *et al*. (2017) [17]. High concentrations of NaOCl have demonstrated considerable antimicrobial capacity to reduce or eliminate cultivable microorganisms, although bacterial persistence may still be observed in some samples even after endodontic preparation, as described by Konadu *et al*. (2024) and Aveiro *et al*. (2020) [4, 16].

The efficacy of irrigation may be influenced by needle insertion depth and design, irrigant flow and volume, and root canal anatomy, as reported by Boutsioukis and Arias-Moliz (2022) and Gomes *et al*. (2023) [5, 12]. Likewise, variations in the effectiveness of endodontic procedures related to the technique and the operator have been reported by Boutsioukis *et al*. (2007) [6], particularly regarding delivered volume, duration, and irrigation flow. These factors may contribute to the heterogeneity observed among specimens subjected to conventional treatment.

The median value of 0 CFU/ml observed in G2 should be interpreted as the absence of detectable growth under the culture conditions used and not necessarily as sterility of the root canal system. Residual microorganisms may remain below the detection limit of the method or persist in anatomical regions inaccessible to the sampling procedure. Therefore, the microbiological reduction obtained by culture should be interpreted considering the inherent limitations of the method and the experimental model, as described by Konadu *et al*. (2024) and Aveiro *et al*. (2020) [4, 16].

Overall, the results support ultrasonic activation as a complementary strategy capable of increasing the antimicrobial effect of PDT. However, photodynamic therapy should not be considered a substitute for conventional chemomechanical preparation. Its main potential role lies in complementing disinfection through improved distribution of the photosensitizer in regions where the action of conventional procedures may be limited, as observed by Niavarzi *et al*. (2019), Ghinzelli *et al*. (2014), and Alves-Silva *et al*. (2025) [3, 11, 18].

The limitations of the present study include its *ex vivo* design, the use of an experimental biofilm composed of four microorganisms, and the inability to fully reproduce the microbiological, anatomical, and biological complexity of a clinical endodontic infection. In addition, previous studies by Abdelkarim-Elafifi *et al*. (2021), Ali *et al*. (2020), and Vendramini *et al*. (2020) [1, 2, 25] have highlighted the heterogeneity of published protocols, which limits direct comparisons among studies. Future studies should standardize photosensitizer concentration, pre-irradiation and activation times, irradiation parameters, and biofilm characteristics, as well as evaluate the protocol under clinical conditions.

Conclusion

Under the experimental conditions of the present study, photodynamic therapy showed antimicrobial activity against intracanal multispecies biofilm. Ultrasonic activation of

methylene blue significantly increased its efficacy, increasing microbial reduction from 94.37% with non-activated PDT to 99.01% with the activated protocol.

Residual microbial load was significantly lower when the photosensitizer underwent ultrasonic activation, with a large effect size. These findings support ultrasonic activation as a complementary strategy to enhance photodynamic therapy; however, further studies are required before establishing a definitive clinical protocol.

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