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ORIGINAL ARTICLE

Could a chelant improve the effect of curcumin-mediated photodynamic antimicrobial chemotherapy against dental intact biofilms?

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Abstract

To our knowledge, there is still no evidence in relation to the combination of curcumin with chelants to improve the effects of antimicrobial photodynamic therapy (aPDT) on complex dental caries biofilms. Therefore, the aim of this study was to evaluate the antimicrobial effect of curcumin-ethylenediaminetetraacetic acid (EDTA)-mediated aPDT on the vitality of intact biofilms of dentin caries microcosms. Biofilms were grown on glass slabs in McBain medium plus 1% sucrose in microaerophily at 37 °C for 5 days. Then, biofilms were treated with associations of 600 μmol L^{-1} curcumin combined or not with 1% EDTA and 37.5 or 75 J cm^{-2} LED (455 nm). The vitality was determined by a confocal laser scanning microscopy (CLSM) after staining biofilms with a mixture of 2.5 g L^{-1} fluorescein diacetate and 0.25 g L^{-1} ethidium bromide. Statistical analysis was conducted by Kruskal-Wallis and post hoc Dunn's test ($P < 0.05$). Three treatments were able to reduce the vitality of overall biofilms: curcumin + 75 J cm^{-2} LED, curcumin-EDTA + 37.5 J cm^{-2} LED, and curcumin-EDTA + 75 J cm^{-2} LED. Also, the vitality of inner layers of biofilms was significantly reduced only after the combination of aPDT with EDTA. Therefore, the association of curcumin and EDTA improved the antimicrobial effect of aPDT on dentin caries microcosms, considering the application of lower light densities and deeper layers of biofilms.

Keywords Curcumin · Ethylenediaminetetraacetic acid · Photochemotherapy · Biofilm · Dental caries

Introduction

Curcumin is a phenolic compound, extracted from the root of *Curcuma longa*, commonly used as a yellow-orange seasoning [1]. It has a molecular weight equal to 368.38 g moL^{-1}, being insoluble in water; hence, different solvents are employed for its dissolution, such as dimethylsulfoxide (DMSO) [1], n-glucamine [2–4], and ethanol [5]. In recent years, curcumin has been applied with good results as an antitumor, antioxidant, anti-inflammatory, antifungal, antibacterial, and anticarcinogenic agent [6, 7].

Previous studies have demonstrated that curcumin can be used as a photosensitizing agent in antimicrobial photodynamic therapy (aPDT) to reduce the viability of cariogenic microorganisms [2, 3, 5, 8, 9]. aPDT is a two-step therapy that involves the application of a photosensitizing agent (Ps), which is absorbed by microbial cells, and the irradiation of a complementary light source, with a broad band absorption spectrum with maximum absorbance peak at a wavelength ~425 nm [10]. This interaction increases the molecular energy of Ps to an excited triplet state, producing free radicals (reaction type I) or reactive oxygen species, such as singlet oxygen (reaction type II) [11]. Both reactions can occur simultaneously, causing death and/or damage to cells, affecting cell membrane, mitochondria, lysosomes, or cellular DNA [12–14].

However, aPDT may not have a satisfactory effect on oral biofilms. They are characterized by a complex three-dimensional structure organized in multiple microbial groups, which increases their pathogenicity and virulence. Also,

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microorganisms are found embedded and protected by an extracellular matrix and dead cell debris, predisposing biofilms to a greater resistance against antimicrobial agents when compared to planktonic cells [15–19].

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent able to dissolve the inorganic portion of smear layer, widely used in endodontics [20] as an adjunct to intracanal irrigation solutions [21]. It favors the permeability of cell membrane which might contribute to the action of Ps in aPDT [22]. To our knowledge, this hypothesis has not yet been tested on dental caries biofilms.

The aim of this study was to evaluate the effect of aPDT mediated by the combination of curcumin and EDTA on the vitality of intact dentin caries microcosms. The null hypotheses were that curcumin-EDTA-mediated aPDT would have no effect on the vitality of intact biofilms, considering their entire depth (*H0*) and their distinct layers (*H0'*).

Materials and methods

Ethical standards and inclusion criteria

This study was approved by the Committee for Ethics in Human Research of the Bauru School of Dentistry (CAAE: 34559314.6.0000.5417), following the ethical standards of Declaration of Helsinki. Three 7–11-year old children were recruited in the track suppressed for blinded review. The inclusion criteria considered the detection of at least one dentin caries lesion in primary molars, without pulp exposure. Children diagnosed with systemic diseases or syndromes, or who made systemic antibiotic usage within 90 days before dentin collection were excluded.

Dentin collection

After anesthesia and adaptation of rubber dam, infected dentin samples were collected from caries lesions with sterile curettes and immediately transferred to cryotubes containing Brain Heart Infusion broth (BHI, 37 g of Brain Heart Infusion, pH 7.2) with 20% glycerol, and stored in freezer at − 80 °C until the moment of use. Then, the tooth was restored with glass ionomer cement (Ketac Fil Plus® 3MEspe, Maplewood, USA).

Biofilm growth

To avoid the natural green fluorescence of dentin tissue in confocal laser scanning microscopy (CLSM), which makes the interpretation of signals emitted by vital microbial cells difficult [23], sterile non-fluorescent glass slabs (4 × 4 × 1 mm) with similar dental surface roughness (1200 grit) (Menzel, Braunschweig, Germany) were used to grow dentin biofilms [24].

All collected samples of dentin caries were thawed at room temperature and sonicated at 40 mW for 15 s (Single Ultra-Sonic Cell Disruptor, Merse, Campinas, Brazil). A volume of 1.5 mL of each sample was mixed and grown under anaerobic conditions at 37 °C overnight. Subsequently, 400 μL of this microbiological pool was transferred to 10 mL of modified McBain medium [25], consisting of 2.5 g L^{-1} mucin (Sigma Chemical Co., St. Louis, USA), 2.0 g L^{-1} peptone, 2.0 g L^{-1} casein peptone, 1.0 g L^{-1} yeast extract (Becton, Dickinson & Co., Sparks, USA), 0.35 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 0.2 g L^{-1} $CaCl_2$, 0.001 g L^{-1} hemin, 0.0002 g L^{-1} vitamin K1, 0.2% sucrose, and 50 mmol L^{-1} PIPES buffer (Sigma Chemical Co., St. Louis, USA), adjusted in pH 7.0. Subsequently, glass slabs were positioned into each well of a 24-well microtiter plate, and aliquots of 1 mL of microbial pool were equally distributed into the wells. This set was transferred to microaerophilic conditions (5% CO_2) at 37 °C for 24 h. After each 24 h, medium was refreshed until complete 5 days of biofilm growth.

Photosensitizer and light source

Curcumin (Sigma-Aldrich, S. Louis, USA), EDTA (Sigma-Aldrich, S. Louis, USA), and Biotable® RGB (Institute of Physics of São Carlos, São Carlos, Brazil) were used as photosensitizing agents and light source, respectively. EDTA was diluted in sterile deionized water at a concentration of 1% and adjusted to pH 8.0 (EDTA 1%). The solutions containing curcumin were prepared immediately prior to their use, by dilution in sterile deionized water with 0.2% DMSO or 1% EDTA. Both solutions were prepared in dark conditions. Biotable® RGB was used in blue light mode (455 ± 30 nm). An optical power meter (1916-C Optical Power Meter, Newport, Irvine, USA) was used to adjust the irradiation time.

Experimental groups

Glass slabs containing biofilms were randomly distributed in 10 different groups: L-C− (no treatment, control), L-C+ (curcumin alone), EDTA (EDTA alone), L-C + EDTA (curcumin + EDTA), L37.5 + C− (37.5 J cm^{-2} LED alone); L75 + C− (75 J cm^{-2} LED alone), L37.5C+ (37.5 J cm^{-2} LED + curcumin), L75 + C+ (75 J cm^{-2} LED + curcumin), L37.5 + C + EDTA (37.5 J cm^{-2} LED + curcumin + EDTA), L75 + C + EDTA (75 J cm^{-2} LED + curcumin + EDTA). Each group was composed of 2 glass slabs and the experiments were made in duplicate ($n = 4$/group).

Antimicrobial photodynamic therapy (aPDT)

Prior to treatment, biofilms were washed twice in CPW medium (5 g of yeast extract, 1 g of peptone, 8.5 g of NaCl, and 0.5 g of L-cysteine HCl, deionized water per L, pH 7.3) to remove not adhered cells. Subsequently, biofilms were incubated in wells containing curcumin, EDTA, curcumin-EDTA solutions, or deionized water for 2 min in dark conditions. Biofilms were irradiated with LED or not depending on those groups. The parameters for irradiation are described as follows: LED 455 ± 30 nm, distance of 25 mm, irradiance of 40 mW cm^{-2}, and two irradiation times (961 or 1923 s), corresponding to 37.5 and 75 J cm^{-2}, respectively. Subsequently, biofilms were transferred to a new well of a 24-well microtiter plate containing 1.5 mL of CPW medium. Although critical for the achievement of densities of energy, the decrease of distance between the light source and biofilms was not achieved because of the application of Biotable® RGB for irradiation, since its design requires the use of specimens with biofilms on the bottom of a 24-well microtiter plate while the LEDs are positioned on the lid of the same plate.

Vitality of intact biofilms

Vitality essays were performed as described by Zaura-Arite et al. [15]. Glass slabs were collected and stained with 10 μl of 0.25 g L^{-1} of ethidium bromide for 30 min and 2.5 g L^{-1} of fluorescein diacetate for 7 min (Sigma Chemicals Co., St. Louis, USA). Then, samples were washed in CPW medium to remove excess reagents.

In each sample, three representative areas were chosen for visualization in the CLSM (model TCS-SPE, Leica, Wetzlar, Germany), using 488-nm excitation, double detection BP520 for fluorescein, and D650 for ethidium bromide, 40×, NA 0.65. To compare the images, the power and aperture of the laser were kept constant. At each site, the entire depth extension was analyzed and recorded, based on the biofilm thickness. The images were corrected and synthesized using Multicolor Analysis software (Leica, Wetzlar, Germany). The vitality of intact biofilms was determined by the percentage of green and red signals calculated by the Leica QWIN Image Analysis software (Leica, Wetzlar, Germany).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 21.0 (IBM® SPSS® Statistics, NY, USA). The hypothesis of a normal distribution of data was rejected by the Shapiro-Wilk test. Therefore, the differences between groups were detected by the non-parametric tests for independent samples, Kruskal-Wallis, and post hoc Dunn's tests. P values < 0.05 were considered significant.

Results

The vitality of intact biofilms is shown in Figs. 1 and 2. Regarding whole biofilms, curcumin + 75 J cm^{-2} LED, curcumin-EDTA + 37.5 J cm^{-2} LED, and curcumin-EDTA + 75 J cm^{-2} LED reduced significantly the vitality of biofilms in comparison with all other groups (Table 1).

Observing specifically inner and outer layers of biofilms, only treatments with curcumin-EDTA-mediated aPDT were effective in reducing the vitality of intact biofilms in relation to no treatment group.

Discussion

These results were based on the treatment of dentin caries microcosms, characterized by greater biofilm formation and virulence [26–29]. Therefore, these microbial communities are more resistant to antimicrobial agents than planktonic cells [30], and mono- and duo-species biofilms [3, 31–34], which explains the lower reductions observed in this study.

It has been reported that if bacteria could be eliminated from dentin caries, it could have positive consequences for dental health, such as remineralization of lesions [3]. With this aim, we seek the addition of EDTA to improve the effects of aPDT. It was demonstrated that the combination of curcumin and EDTA promoted the reduction of the vitality of dentin caries intact biofilms in half time of activation of a blue LED in comparison with the only curcumin usage. In addition, this combination decreased the vitality of all three layers equally, showing that EDTA favored the in-depth action of aPDT.

Similar to our results, EDTA did not show antibacterial activity on mono-species biofilms of *Enterococcus faecalis* ATCC 29212 and on polymicrobial biofilms grown in situ [35, 36]. However, the addition of 1% EDTA to curcumin promoted the decrease of the vitality of intact biofilms approximately 80%, probably due to the increased penetrability of cellular membranes produced by EDTA [37, 38], which could promote a better absorption of curcumin by Gram-positive and Gram-negative microorganisms, as noted for methylene blue, Rose Bengal, and indocyanine green [22].

Few studies aimed to investigate the use of EDTA on the improvement of aPDT. A recent study showed that the combination of curcumin and EDTA is capable of inactivating *Burkholderia cepacia* [39]. It was also shown that the addition of 20% EDTA improved the

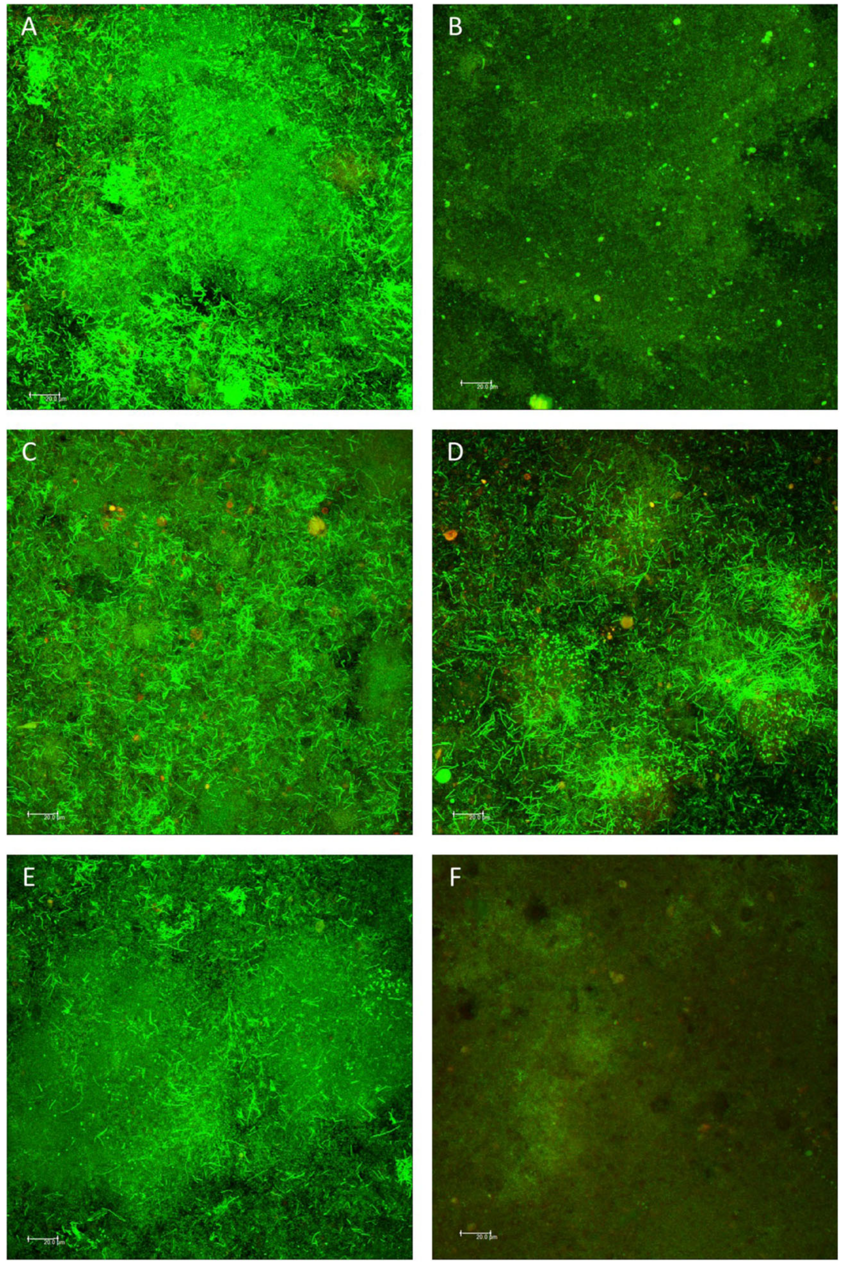

Fig. 1 CLSM images of intact microcosm biofilms. **a** L-C−, control group. **b** L-C+, 600 μmol L^{-1} curcumin. **c** EDTA, EDTA alone. **d** L-C + EDTA, 600 μmol L^{-1} + curcumin + 1% EDTA. **e** L37.5 + C−, 37.5 J cm^{-2} LED. **f** L75 + C−, 75 J cm^{-2} LED. **g** L37.5 + C+, 600 μmol L^{-1} curcumin + 37.5 J cm^{-2} LED. **h** L75 + C+, 600 μmol L^{-1} curcumin + 75 J.cm^{-2} LED. **i** L37.5 + C + EDTA, 600 μmol L^{-1} curcumin + 1% EDTA + 37.5 J cm^{-2} LED. **j** L75 + C + EDTA, 600 μmol L^{-1} curcumin + 1% EDTA + 75 J cm^{-2} LED

effects of chlorin- and poly-L-lysine-mediated aPDT against microorganisms from subgingival plaque [40].

Despite these findings, our team previously showed a significant reduction of the vitality of intact biofilms by employing aPDT with methylene blue [41], toluidine blue O [42], and curcumin [43] activated by both 37.5 and 75 J cm^{-2} blue LED, with a trend of dose-dependence effect. Nevertheless, our results are not completely comparable because of the differences found in the atmospheres of biofilm growth. In theory, microaerophily favors the formation of a more virulent biofilm with the predominance of Gram-positive and facultative anaerobes, such as *Streptococcus mutans* and *Lactobacillus*, in detriment to anaerobic conditions [44]. Also, these biofilms were grown on non-fluorescent glass slabs with similar surface roughness of dental tissues [45], which allows biofilm scanning by the avoidance of dental surface staining that might directly interfere on the analyses of CLSM images. Due to this design, active biofilm models were not proper for the adequate fixation of specimens [46–48].

Pilot studies were developed to determine the best concentration of EDTA (data not shown), characterized by the minimum concentration needed to evidence the improvement of the antimicrobial effect of curcumin.

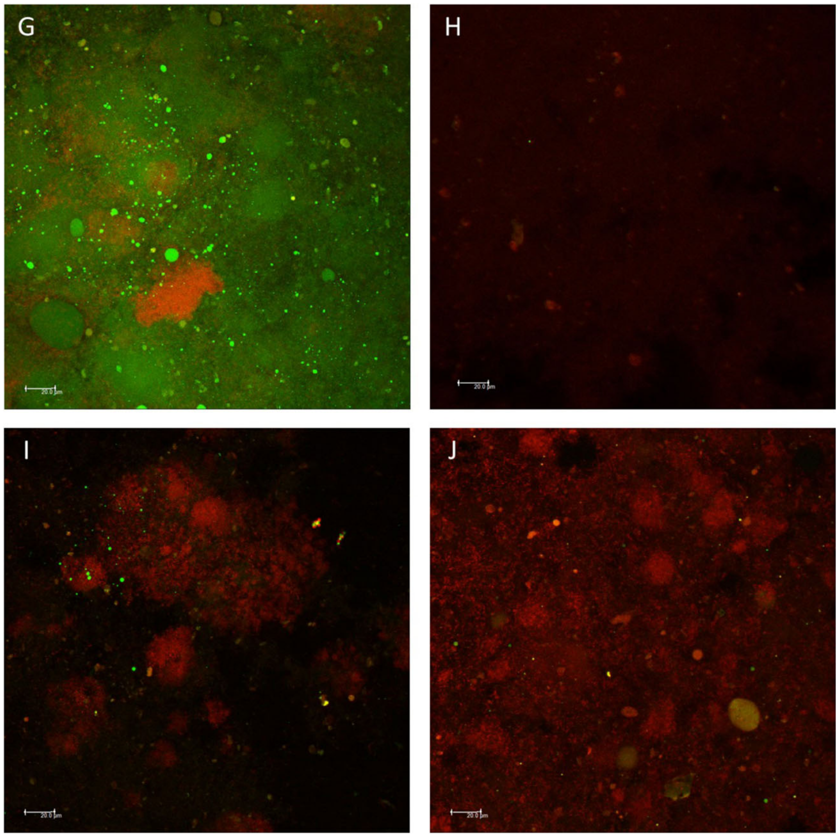

Fig. 1 continued.

The combination of curcumin and EDTA did not change the original color of the dye. The pre-irradiation time of 2 min was applied similarly to previous studies that considered the effects of photosensitizing agents [41, 42] and chelants [49] on microbial cells. Additionally, the densities of energy were chosen with basis on previous studies [42, 43]. Due to low input and output powers of the light source, non-feasible clinical times were applied

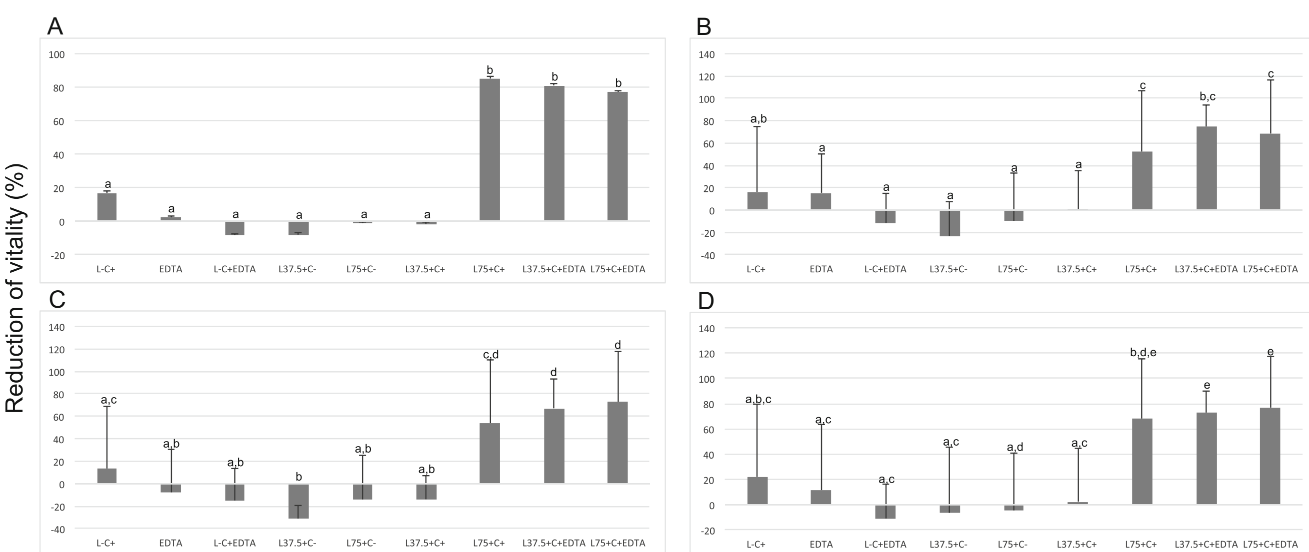

Fig. 2 Percentage of reduction of the vitality of whole biofilms (**a**), outer layer biofilms (**b**), medium layer biofilms (**c**), and inner layer biofilms (**d**) after distinct treatments. These values are presented in function of no treatment group (L-C−). Distinct letters represent significant statistical differences between treatments

Table 1 Vitality of intact microcosm biofilms, represented by the ratio of live cells/live + dead cells. Distinct superscript letters represent significant statistical differences between groups

	Groups	Unprocessed data (Mean ± SD)	Mean ± SD	Median	CI (95%)	
					Lower	Upper
Whole biofilm	L-C-^{a}	0.87 ± 0.14	100.00 ± 35.61	108.14	77.37	122.63
	L-C +^{a}	0.66 ± 0.35	83.10 ± 43.70	97.46	55.33	110.86
	EDTA^{a}	0.77 ± 0.24	97.60 ± 30.11	99.93	78.47	116.73
	L-C + EDTA^{a}	0.86 ± 0.13	108.32 ± 16.31	109.81	97.96	118.69
	L37.5 + C-^{a}	0.86 ± 0.23	108.00 ± 29.26	124.55	89.41	126.59
	L75 + C-^{a}	0.80 ± 0.27	101.43 ± 33.73	113.13	80.00	122.86
	L37.5 + C +^{a}	0.81 ± 0.21	101.71 ± 26.74	109.80	84.72	118.69
	L75 + C +^{b}	0.12 ± 0.24	14.83 ± 30.84	3.03	− 4.76	34.43
	L37.5 + C + EDTA^{b}	0.15 ± 0.14	18.92 ± 18.24	11.89	7.34	30.51
	L75 + C + EDTA^{b}	0.18 ± 0.32	22.96 ± 40.44	2.16	− 2.74	48.65
Outer layer	L-C-^{a}	0.63 ± 0.37	88.20 ± 51.52	108.34	53.59	122.81
	L-C +^{ab}	0.55 ± 0.41	77.95 ± 57.62	87.56	41.34	114.57
	EDTA^{a}	0.63 ± 0.37	88.36 ± 51.94	104.69	55.36	121.36
	L-C + EDTA^{a}	0.79 ± 0.19	110.82 ± 26.72	116.32	93.84	127.79
	L37.5 + C-^{a}	0.76 ± 0.37	106.76 ± 52.41	136.97	73.46	140.06
	L75 + C-^{a}	0.68 ± 0.38	104.70 ± 45.82	114.95	73.92	135.49
	L37.5 + C +^{a}	0.69 ± 0.30	97.49 ± 42.22	108.11	70.66	124.32
	L75 + C +^{c}	0.23 ± 0.34	31.91 ± 47.71	1.65	1.60	62.23
	L37.5 + C + EDTA^{bc}	0.19 ± 0.12	27.02 ± 17.12	29.12	16.14	37.89
	L75 + C + EDTA^{c}	0.16 ± 0.29	23.09 ± 40.19	4.77	− 2.44	48.62
Middle layer	L-C-^{ab}	0.69 ± 0.33	97.67 ± 47.09	111.69	63.99	131.36
	L-C +^{ac}	0.61 ± 0.39	86.45 ± 55.51	110.56	51.19	121.72
	EDTA^{ab}	0.76 ± 0.27	107.59 ± 38.12	119.75	83.37	131.81
	L-C + EDTA^{ab}	0.82 ± 0.20	114.85 ± 28.08	123.57	97.01	132.69
	L37.5 + C-^{b}	0.93 ± 0.09	131.27 ± 12.34	138.40	123.43	139.12
	L75 + C-^{ab}	0.74 ± 0.36	114.00 ± 39.15	134.35	85.99	142.01
	L37.5 + C +^{ab}	0.81 ± 0.15	113.79 ± 20.67	115.71	100.65	126.93
	L75 + C +^{cd}	0.33 ± 0.40	46.47 ± 56.35	16.47	10.67	82.27
	L37.5 + C + EDTA^{d}	0.23 ± 0.19	32.80 ± 26.33	23.68	16.07	49.52
	L75 + C + EDTA^{d}	0.19 ± 0.32	27.23 ± 44.83	9.78	− 1.25	55.72
Inner layer	L-C-^{ac}	0.73 ± 0.30	101.97 ± 41.99	115.48	71.94	132.01
	L-C +^{abc}	0.60 ± 0.42	83.67 ± 58.36	99.65	46.59	120.76
	EDTA^{ac}	0.84 ± 0.25	117.50 ± 34.72	132.70	95.44	139.56
	L-C + EDTA^{ac}	0.80 ± 0.19	111.55 ± 26.74	118.54	94.56	128.54
	L37.5 + C-^{ac}	0.88 ± 0.22	123.15 ± 31.12	138.86	103.38	142.92
	L75 + C-^{ad}	0.72 ± 0.37	109.77 ± 42.51	134.53	81.21	138.33
	L37.5 + C +^{ac}	0.71 ± 0.25	99.04 ± 34.42	112.53	77.17	120.91
	L75 + C +^{bde}	0.34 ± 0.39	47.99 ± 54.61	23.11	13.30	82.69
	L37.5 + C + EDTA^{e}	0.18 ± 0.14	25.27 ± 19.45	24.14	12.91	37.63
	L75 + C + EDTA^{e}	0.22 ± 0.35	31.46 ± 48.36	5.44	0.73	62.19

for irradiation in this study; however, the application of high-power LED found in dental offices could overcome that limitation for treating patients, e.g., 75 J cm^{-2} could be achieved in ≈180 s with a 400-mW LED device [41].

In conclusion, this study demonstrated that the combination of curcumin and EDTA improves the effect of aPDT against dentin caries microcosms, especially regarding the application of lower light densities and deeper layers of biofilms. These findings indicate the rejection of both hypotheses *H0* and *H0'*.

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Compliance with ethical standards

Ethical approval This research protocol was approved by the Committee for Ethics in Human Research of the Bauru School of Dentistry (CAAE: 34559314.6.0000.5417), following ethical standards of the Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflict of interest.

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