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Antimicrobial action of photoactivated C-Phycocyanin against *Enterococcus faecalis* biofilms: Attenuation of quorum-sensing system

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ABSTRACT

Background: The limitations of conventional chemo-mechanic therapy in endodontic have given rise to introduce and develop photoactivated disinfection (PAD) as an adjunctive treatment. The objective of this *ex vivo* study was to evaluate the effects of PAD using C-Phycocyanin, as a natural photosensitizer, on biological activities and expression of quorum-sensing system gene *fsrB* of *Enterococcus faecalis*.

Materials and methods: A total of 52 extracted single-rooted human premolar teeth were divided into five groups (n = 10) and were incubated with *E. faecalis* for biofilm formation, and they were treated according to following groups; A. C-PC only; B. Diode laser only (at wavelengths of 635 nm); C. PAD (C-PC plus diode laser); and D. Sodium hypochlorite (NaOCl). E. Control group (not treatment). Two remaining teeth were used to confirm *E. faecalis* biofilm formation in the root canal system by scanning electron microscopic. The effects of each treatment were measured on viability and expression level of *fsrB* gene of *E. faecalis* using microbial cell viability and quantitative real-time PCR assays, respectively. Additionally, intracellular reactive oxygen species (ROS) production was measured using 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) fluorescent probe.

Results: The C-PC alone and C-PC-PAD reduced the culture viability of *E. faecalis* by 38.1% and 89.45%, respectively (both; $P < 0.05$). According to the Bonferroni post hoc test, a significant difference in the reduction of *E. faecalis* count was observed between C-PC-PAD and other groups ($P < 0.05$), except the NaOCl group ($P > 0.05$). As well as, photoexcited C-PC in PAD could increase (3.8- fold) the intracellular ROS production in E. faecalis compared to the control group ($P < 0.05$). C-PC-PAD at sub-significant inhibitory concentration and NaOCl at sub-lethal dose could significantly decrease the expression levels of *fsrB* by 10.8- and 11.4-fold, respectively.

Conclusions: Our data support that C-PC alone and C-PC-PAD can serve as a potent irrigation solution and a disinfection method, respectively for microbial reduction and promising adjunct therapy in endodontic infection treatment against *E. faecalis* with clinical applications for infection control in endodontics.

1. Introduction

Effective root canal disinfection to eradicate microorganisms and prevent recontamination is the main objective and fundamental component of successful root canal treatment [1]. *Enterococcus faecalis* is one of the invasive pathogens in the oral cavity that leading causes of primary and secondary endodontic infections [2]. Among the virulence factors of *E. faecalis*, quorum sensing systems are especially important because of their biological effects, which lead to regulating the expression of a protein involved in biofilm formation [3]. Because of the biofilm formation capacity of *E. faecalis* in root canal space which empowers bacteria enhanced resistance to antimicrobial agents, confronting this microorganism in the successful treatment of endodontic infection is urgently needed [4].

In current canal system disinfection, chem-mechanical techniques, using intracanal irrigants such as sodium hypochlorite (NaOCl), cannot completely eliminate the microorganisms and may increase the risk of cytotoxicity and neurotoxicity of periapical tissues as well as residual microorganisms are readily detectable in infected root canal [5,6]. Regardless of the chemo-mechanical technique, photoactivated disinfection (PAD), also known as phototherapy, photoradiation therapy, photochemotherapy, antimicrobial photodynamic therapy (aPDT), or photoactivated chemotherapy (PACT) has been introduced as a non-invasive novel therapy to overcome the microorganisms involved in

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infected root canals [7–10].

PAD involves the use of a photosensitizer which is excited by a light source at a specific wavelength; transfers the energy to oxygen thus forming reactive oxygen species (ROS) that are highly reactive and harm microbial cellular components [11]. Recently, *in vitro*, *ex vivo*, and *in vivo* disinfectant effects of PAD with different synthetic photosensitizers have been widely reported and documented [7–10].

Based on the literature, the use of photosensitizer with a natural and edible nature has attracted enormous interest [12]. Cyanobacteria (blue-green algae), red algae and the cryptomonads have emerged as an important source of phycobiliproteins (PBPs), a family of water-soluble light-harvesting protein-pigment complexes, counting important biotechnologically and bioactive chemicals. Based on their spectrum properties, PBPs are classified into three types: phycocyanin (PC) (dark cobalt blue: λmax 610–625 nm), phycoerythrin (PE) (bright pink: λmax 490–570 nm), and allophycocyanin (APC) (brighter aqua blue: λmax 650–660 nm). PC, PE, and APC have very similar three-dimensional structures which their coordinates of the model have been deposited in the Brookhaven Protein Data Bank (entry number 1CPC, 1LTA, and 1ALL, respectively). The PBPs assemble into phycobilisomes on the outer surface of the thylakoid or photosynthetic membrane. The PBPs absorb energy in portions of 450–650 nm that poorly utilized by chlorophyll and transfer electrons to the photosystems to drive photosynthetic electron transport [13,14]. C-PC as a non-toxic natural biological molecule found in microalga *Spirulina platensis*, is a novel class of fluorescent dye which can propagate singlet oxygen during PAD [15]. Moreover, C-PC is known to possess numerous bioactivities and the anti-inflammatory, anti-cancer, and antimicrobial functions that have been widely used in the food and pharmaceutical industries [16].

Although C-PC has been reviewed in the literature as a photosensitizer in PAD against cancer, there was no data about the effects of C-PC in PAD against *E. faecalis*. Therefore, the purpose of this study was to evaluate the effect of C-PC as irrigation solution, PAD efficiency on microbial cell viability, expression patterns of biofilm-associated genes, *fsrB*, of *E. faecalis* biofilms in the root canals of extracted teeth, and the intracellular ROS production by photoexcited C-PC.

2. Materials and methods

2.1. Sample preparation

After ethics committee approval by the Ethics Commission of Tehran University of Medical Sciences, Tehran, Iran under protocol IR.TUMS.DENTISTRY.REC.1397.126, 52 single-rooted human premolar teeth extracted for periodontal reasons were collected. All the teeth had completed root development without any visible cracks, fracture, caries, calcification, and no restoration history. After disinfecting the teeth with 5.25% sodium hypochlorite (NaOCl; (Merck, Darmstadt, Germany) for 30 min, the teeth were prepared with decoration the teeth with the use of a diamond disc (SS White, NJ, USA).

A crown-down technique was used to prepare the root canal. The patency of root canal was confirmed using a stainless steel #10 K file (Dentsply, Maillefer, Tulsa, OK) and the working length was recorded by subtracting 0.5 mm from the length in which the tip of the K file. The coronal one-third (4 mm) was prepared using size 1–3 Gates-Glidden burs based on the step-down procedure. The shaping of the root canals was then accomplished using X-Smart Endodontic Rotary Motor (Dentsply, Maillefer, Tulsa, OK) with 16:1 contra angle and 800 rpm speed and Gates-Glidden 0.8, 1.2, and 2.5 Ncm, respectively following the manufacturer's instructions. The coronal two-thirds was prepared using sizes 60–35 ProFile .04 instruments and apical part was cleaned and shaped using sizes 30 ± 15 ProFile .04 instruments. The apical stop was prepared using sizes 15–40 ProFile .04 instruments, each to working length. Eventually, the canal was flared by stepping-back with ProFile .04 instruments, sizes 45–60. After using each instrument during root canal preparation, 2 mL of 3% NaOCl were used as an irrigating solution and smear layer was removed by irrigating the canal with 2 mL of 17% ethylenediamine tetraacetic acid solution (Merck, Darmstadt, Germany) for 5 min. After that, the canals were washed with 2 mL of 5% sodium thiosulfate (Merck, Darmstadt, Germany) for 60 s to deactivate the residual NaOCl and with 10 mL of sterile saline solution for 5 min. All root canals were irrigated with a 30-gauge needle (Sigma-Aldrich, Steinheim, Germany). The apical foramen and the root surface of all samples were then sealed with a light-curable glass-ionomer (GC Gold Label, Kyoto, Japan) and two layers of nail-varnish, respectively. All samples were then sterilized by exposure to 40 kGray Gamma irradiation (GAMMACELL 220 irradiation unit. Ottawa, Canada).

2.2. Bacterial culture and inoculation

E. faecalis ATCC 29212 (Iranian Biological Resource Center, Tehran, Iran) was used in this study. The bacterium was inoculated into brain heart infusion (BHI) broth (Merck, Darmstadt, Germany), grown overnight at 37 °C, giving a final cell concentration of 1.5×10^8 colony forming units (CFUs)/mL. *E. faecalis* suspension was inoculated into each prepared root canal and the teeth were incubated for 2 weeks, changing the media twice weekly. Two teeth were processed for scanning electron microscopic (SEM) analysis to confirm *E. faecalis* biofilm formation in the root canal system. SEM microphotographs confirmed *E. faecalis* bacterial biofilm formation in the root canal system after inoculation (Fig. 1).

2.3. Determination of minimum inhibitory concentration (MIC) of C-PC

The MIC of C-PC against *E. faecalis* was determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [17]. Briefly, 100 µL of 2X BHI broth was added to the well of a round-bottom 96-well microtiterplate (TPP, Trasadingen, Switzerland) and 100 µL of C-PC (2 mg/mL) was then added to the first well in column 1 and the C-PC concentration was diluted to 1:2. Afterward, 100 µL of an overnight culture of *E. faecalis* that was re-suspended in BHI broth to an OD_{600} of 0.08-0.12 (1.0×10^6 CFU/mL) was added to a 96-well microtiterplate and incubated at 37 °C for 24 h. According to the CLSI, MIC was defined as the lowest concentration of the agents that inhibits the visible growth of microorganisms [17].

2.4. Determination of sub-lethal dose of diode laser irradiation time

The sub-lethal dose of diode laser irradiation time against *E. faecalis* was determined according to a previous study [18]. Briefly, 200 µL of *E.*

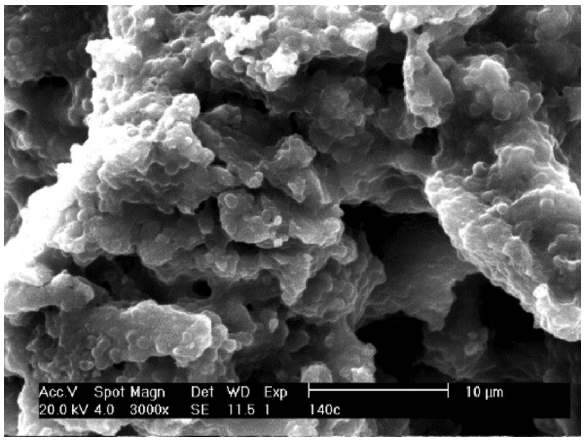

Fig. 1. SEM image of *Enterococcus faecalis* biofilm formation inside the root canals of extracted tooth at 3000x magnification (scale bar represents 10 µm).

faecalis suspension (1.0×10^6 CFU/mL) was transferred to the wells of a round-bottom 96-well microtiterplate and exposed to 1, 2, 3, 4, and 5 min diode laser irradiation times, with energy density of 34.37, 68.75, 103.12, 137.5, and171.87 J/cm^2, respectively, at wavelength 635 nm. The distance between the optical fiber and microplate surface was < 1 mm, and the sheet of black paper was placed under the microplate during diode laser irradiation to prevent the beam reflecting of the table-top. The sub-lethal dose of diode laser irradiation time was determined according to the methods mentioned above (Section 2.3).

2.5. Quantification of E. faecalis cells viability in biofilm structures in root canals

After the formation of biofilms in root canal systems, the specimens were assigned randomly to five experimental groups with 10 teeth in each group by Random Allocation Software (https://random-allocation-software.software.informer.com/2.0/) as followed:

A **C-PC:** 10 µL of C-PC (Sigma-Aldrich, Steinheim, Germany) at MIC dose was inoculated into root canals and specimens were incubated in the dark condition for 5 min.
B **Diode lasers:** Root canals with *E. faecalis* biofilm were exposed with a sub-lethal dose of diode laser irradiation time at wavelengths of 635 nm (DX62, Konftec, Taiwan) with the output power of 220 mW delivered. The laser beam was directed into the root canal by the fiber optic cone (Konftec, Taiwan). It was placed at a distance of one mm from the working length (Fig. 2).
C **PAD:** 10 µL of C-PC at MIC dose was inoculated into root canals and specimens were incubated in the dark condition for 5 min and were then exposed with a diode laser as in group B.
D **NaOCl:** 10 µL of 2.5% NaOCl was inoculated into root canals and specimens were incubated at room temperature.
E **Control:** 10 µL of sterile normal saline was inoculated into root canals without exposure to C-PC and diode laser.

After treatment based on the experimental groups, the viable bacteria quantitated by colony enumeration of bacteria harvests from the root canals and reported as CFU/mL as previously described [19]. Briefly, before sampling, the root canal was washed with sterile normal saline solution and was dried by a sterile paper point size 30 (Meta Biomed, Chungcheongbuk-do, Korea). 0.01 g of dentine chips were collected from the root canal using sterile long shank round #4 burs. Dentine chips were transferred to tubes containing 1 mL of sterile normal saline solution, vortexed for 60 s, and diluted serially ten-fold stepwise before plating on culture plates (BHI agar). Eventually, CFUs/mL was counted using Miles and Misra method [20], and a log transformation was calculated. The microbial cell reduction was calculated according to the following equation:

$$\text{Microbial cell reduction (\%)} = \frac{\log \text{in the treated group} \times 100}{\log \text{in the untreated group}} - 100$$

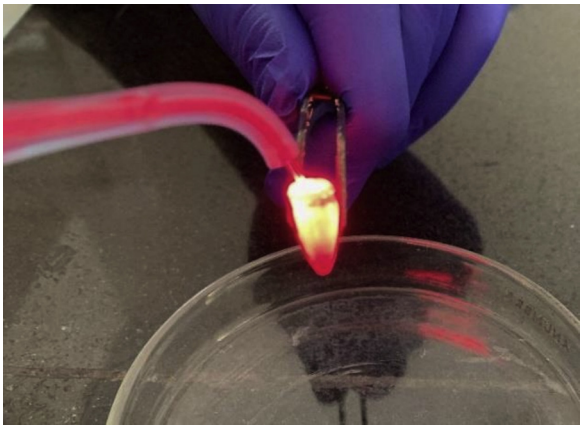

Fig. 2. Irradiation of diode laser at wavelengths of 635 nm to the root canal with *Enterococcus faecalis* biofilm.

2.6. Measurement of intracellular ROS

ROS level was measured in C-PC-treated cells compared to control cells with 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) fluorescent probe as previously reported [21]. In brief, 1.0×10^8 CFU/mL of *E. faecalis* was washed three times with phosphate-buffered saline (PBS) at pH 7.4 and resuspended in PBS by adjusting the cell density to 10^7 CFU/mL. DCFH-DA was added to the culture at a ratio of 1 : 2000 and the mixture was shaken for 10 min at 37 °C. The cells were then centrifuged at 10,000 rpm for 15 min and the pellets were washed twice with PBS to remove the DCFH outside the cell. Afterward, the cells were treated with C-PC for 5 min and irradiated with a diode laser at wavelengths of 635 nm for 60 s, while the control was left untreated. The fluorescence intensity of 2′,7′-dichlorofluoroscein (DCF), which is the oxidized product of hydrolyzed DCFH-DA by intracellular esterases was measured by fluorescence spectrophotometer at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

2.7. Gene expression assay

2.7.1. Determination of MIC of NaOCl

The MIC of NaOCl against *E. faecalis* was determined according to the method described for the C-PC (Section 2.3.), except that the test concentrations of NaOCl were 2.5-0.078%.

2.7.2. Determination of sub-significant inhibitory concentration (SSIC) of C-PC-PAD

To define SSIC of C-PC-PAD, C-PC at 2 × MIC (100 µL) was serially diluted two-fold in microtiter plates to 1/64 MIC in 2X BHI broth according to the method mentioned above in Section 2.3. The wells were then inoculated with fresh BHI bacterial cultures (100 µL/well; final bacterial cell concentration: 1.0×10^5 CFU/mL). The C-PC concentrations were in the range of 1/2–1/64 MIC. The microplates were incubated at room temperature for 5 min in the dark. The treated bacterial cells in the wells were exposed to a sub-lethal dose of the diode laser. The SSIC of C-PC-PAD was determined according to the methods mentioned above. SSIC of C-PC-PAD was defined as the lowest concentration of C-PC with the shortest irradiation time of diode laser in the last well-showing growth.

2.7.3. Gene expression analysis following treatment groups by quantitative real-time polymerase chain reaction (qRT-PCR)

To compare the relative quantities of *fsrB*-specific mRNA, the *E. faecalis* cells were treated based on the experimental groups using sub-MIC of C-PC and NaOCl, the sub-lethal dose of diode laser and SSIC of C-PC-PAD. In all comparative analyses, 16S ribosomal RNA gene used to normalize mRNA quantities among the specimens. Total bacterial RNA of specimens were extracted using the GeneAll Hybrid-R™ RNA purification kit (GeneAll Biotechnology Co., Korea), as recommended by the manufacturer. Extracted RNA concentrations were determined at a wavelength of 260 nm and 280 nm using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, US). The residual contaminating genomic DNA was removed by RNase-free DNase I treatment (Thermo Scientific GmbH, Germany) to avoid false-positive amplification in qRT-PCR. For single-stranded cDNA synthesis, a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, US) with random primers was used according to the manufacturer´s recommendations.

qRT-PCR was performed using the Light Cycler® 96 System (Roche, Basel, Switzerland). The target qRT-PCR primer sets that were designed

using Primer3 software version 4.0 (http://bioinfo.ut.ee/primer3/) were *fsrB*-F, 5´-TCTTCTGTGAGCTTACCGTTT-3´; *fsrB* -R, 5´-GACCGTA GAGTATTACTGAAGCA-3´; *16S rRNA*-F, 5´-AGAGTTTGATCCTGGCT CAG-3´; *16S rRNA*-R, 5´-AAGGAGGTGATCCAGCCGCA-3´. Thermal cycling conditions were: an initial cycle of denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 57 °C for 15 s and 72 °C for 15 s; a final extension step was performed at 72 °C for 5 min. Positive and negative control reactions were included at all times. The expression levels of target genes were analyzed using the Livak and Schmittgen method [22].

2.8. Statistical analysis

Data were entered in statistical package for social sciences (SPSS) software version 25 and analyzed using One-way Analysis of Variance (ANOVA). The Bonferroni post hoc test was used for the comparison between the experimental groups. P values < 0.05 were considered significantly different.

3. Results

3.1. C-PC reduced the E. faecalis growth

As shown in Fig. 3, C-PC at 125–2000 µg/mL concentrations statistically reduced *E. faecalis* growth (38.1% to 73.3%) when compared with untreated bacteria (P < 0.05), but there was no significant effect on the numbers killed when C-PC was decreased from 62.5 to 3.9 µg/mL (21.0% to 3.41%). Therefore, the MIC of C-PC was 125 µg/mL.

3.2. Diode laser irradiation did not completely inhibit the E. faecalis growth

The treatment of *E. faecalis* cells with diode laser exposure up to 5 min could not completely inhibit *E. faecalis* growth, but significant decreases in bacterial cell survival were observed, with energy densities of 68.75–171.87 J/cm^2 (P < 0.05; Fig. 4). There was no significant difference in the count of *E. faecalis* cells in an energy density of 34.37 J/cm^2 (P > 0.05). Therefore, the sub-lethal dose of diode laser irradiation time against *E. faecalis* was 1 min with an output energy density of 34.37 J/cm^2.

3.3. Bacterial growth was considerably affected by C-PC- PAD

The mean and standard deviation of $\log_{10}$ CFU concentrations of *E. faecalis* following the different treatment are shown in Table 1. Analysis of *E. faecalis* count showed logarithmic bacterial reduction was considerably affected by C-PC-PAD in the combination of 1 mg/mL C-PC plus 5 min irradiation time of diode laser (89.45%) and 2.5% NaOCl (85.72%) groups compared to control (non-treated bacteria) group (P < 0.05). Most notably, C-PC- PAD showed a significantly higher inhibitory activity than the other treatment groups (P < 0.05). Also,

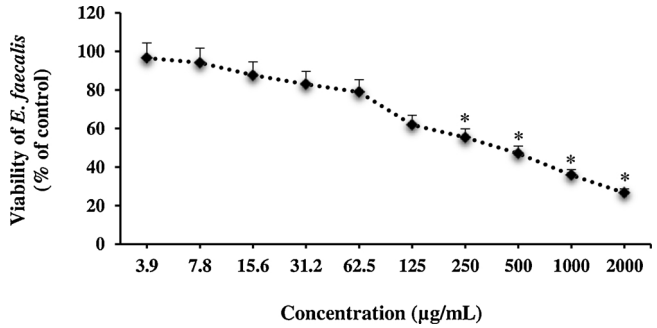

Fig. 3. Effect of different concentration of C-PC on viability of *Enterococcus faecalis* cell. *Significantly different from the control (no treatment), P < 0.05.

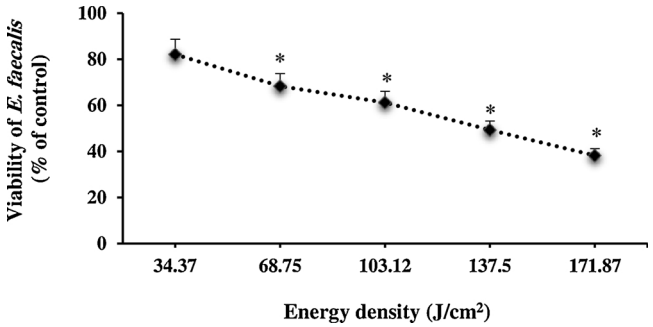

Fig. 4. Effect of different energy density of diode laser at wavelength 635 nm on viability of *Enterococcus faecalis*. *Significantly different from the control (no treatment), P < 0.05.

Table 1
Mean and standard deviation values (in $\log_{10}$ CFU/mL) of *Enterococcus faecalis* in the different treatment groups compared with control group.

Groups	Mean ± SD[a] of $\log_{10}$ CFU[b]	% of microbial cell reduction	*P* value
C-PC[c]	6.71 ± 0.04	18.31	> 0.05
Diode laser	7.22 ± 0.06	12.15	> 0.05
PAD[d]	0.86 ± 0.06	89.45	< 0.05
NaOCl[e]	1.17 ± 0.06	85.72	< 0.05
Control	8.22 ± 0.06	–	–

*Abbreviation: a. Standard deviation; b. Colony forming unit; c. C-Phycocyanin; d. Photoactivated disinfection; e. Sodium hypochlorite.

there was no significant difference between the $\log_{10}$ CFU concentrations in the biofilm treated with PAD (1.17 ± 0.06) versus the $\log_{10}$ CFU concentrations (1.45 ± 0.09) in the biofilm that were treated with NaOCl.

3.4. ROS production was raised by C-PC-PAD

The results of the current study showed a significant increase to 3.8-fold in the DCF fluorescence in C-PC-PAD group compared to the control group (P < 0.05), indicating generation of ROS.

3.5. fsrB gene expression was considerably decreased by SSIC of C-PC-PAD

After treatment of *E. faecalis* with different experimental groups, the profile of gene expression was determined. As the results reveal, the *fsrB* gene expression profiling downregulated in *E. faecalis* cells almost 1.3- and 1.6- fold following diode laser and C-PC, respectively. The expression level of *fsrB* reduced 10.8-fold in C-PC-PAD. As well as, the 11.3-fold reduction was reported following NaOCl. Based on the results, the level of gene expression in C-PC-PAD compared with C-PC and diode laser was remarkably different (P < 0.05), while no statistically significant differences were observed in the expression of *fsrB* following PAD and NaOCl groups (P > 0.05).

4. Discussions

The ability of *E. faecalis* to cause root canal infections has been linked to possessing a wide range of virulence factors [2]. Biofilm formation ability is one of the dominant characteristics of *E. faecalis* extending into resistance towards conventional intracanal irrigants [23]. With the advent of proteomic studies, it is revealed that biofilm formation in *E. faecalis* can manage by a cell to cell communication mechanism via signaling molecules, called quorum sensing phenomena

[3]. The role of FsrB quorum-sensing system as a regulator of *E. faecalis* pathogenicity has been documented in several studies [24–26]. On the other hand, it has been assessed in several experimental studies that FsrB is involved in virulence, the degradation of host tissues, and the formation of biofilms in *E. faecalis* [23–27]. Many features of bacterial biofilms contribute to their enhanced antimicrobial resistance relative to planktonic cells, including decreased antibiotic penetration, antibiotic sequestration, and the presence of persister cells. Furthermore, the genetic elements involved in biofilm-associated antimicrobial resistance have been determined for *E. faecalis* [28–30]. These genetic elements include genes of the operon encoding two glycosyltransferases (GTFs), enterococcal polysaccharide antigen (*epa*), *epaOX* and *epaI*, *gelE* which encodes gelatinase, and the fsr quorum-sensing system. Dale et al. [27] also demonstrated that the GTFs play additional roles in *E. faecalis* including determination of cell shape, maintenance of cell envelope integrity, polysaccharide composition. Additionally, an *E. faecalis epaOX* deletion results in the most noticeable phenotypic differences in biochemical composition and biofilm architecture. Dale et al. [28] demonstrated that the wild type biofilms exhibit a similar architectural arrangement upon exposure to the daptomycin, a cell membrane-active antibiotic. It suggesting a link between biofilm architecture, cell envelope stress, and the *epa* operon. In this study, basic information obtained from FsrB via bioinformatics analysis showed that it was a valid and stable protein with acceptable quality which can be considered as a protein encoded by target gene for PAD [10].

Recently, natural products have been considered as photosensitizers *in vitro* PAD experiments. Bioactive potentials of several microalgae have been exploring which among them, *Spirulina* is one of the well-known reservoirs. C-PC, from the light-harvesting phycobiliprotein (PBP) family, is a natural compound produced by microalgae that have led to significant therapeutic results against several diseases including atherosclerosis, neurodegenerative diseases, cancer, diabetes mellitus, rheumatoid arthritis, and inflammatory diseases [31]. As well as, C-PC has a wide molar extinction coefficient spectrum at 625 nm and fluorescence with a high quantum yield (50%) that can be used in the immunological analysis as fluorescent reagent [32,33]. PBPs were introduced as a novel class of fluorescent dyes in 1982 [34] that can absorb a broad spectrum of visible light and transfer the energy unidirectional to the target sites [12,15,16]. PBPs are the brilliantly colored, highly fluorescent, and the water-soluble proteins with a similar three-dimensional structure, with each unit composed of two kinds of subunits, the α-subunit, and β-subunit. The subunits of α and β are heterologous polypeptide chains of approximately 160–180 amino acid residues with the molecular weights of 18–20 kDa and 19.5–21 kDa, respectively. Additionally, C-PC carries three phycocyanobilin (PCB) as a blue phycobilin that covalently attached to Cys-84 of α-subunit and Cys-82 and Cys-153 residues of β-subunit [35].

Numerous studies have reported C-PC showed beneficial efficacy against microbial pathogens. Fan et al. [36] reported that C-PC had certain antibacterial activity on *Vibrio parahemolyticus*, *Bacillus mucilaginosus*, *Sarcina lutea*, and *V. harveyi*. In another *in vitro* study, the activity of C-PC was assayed against *B. cereus*, *Staphylococcus aureus*, *Escherichia coli*, as well as *Klebsiella pneumonia* using agar well-diffusion method by Sitohy et al. [37]. The results of their study have shown that this new product has antibacterial action and maybe a good candidate to be a partial substitute for some antibiotic. As well as, Sarada et al. [38] demonstrated C-PC was a potent agent against *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, and *S. aureus*.

The results of this study were consistent with previous studies that show C-PC has a broad-spectrum antimicrobial activity [36–38]. According to the results of the current study, C-PC in MIC and lethal concentration was able to reduce the cell survival of *E. faecalis* in the root canal by 38.1% to 73.3%. Interestingly, the effect of C-PC in inhibiting microbial biofilm is very close to the effect of 2.5% NaOCl. So, it can be used as an intracanal irrigant in endodontic treatment. On the other hand, to increase the effectiveness of C-PC in this study, it was used as the photosensitizer and was exposed to a diode laser with the respective wavelength.

To the best of our knowledge, there is no report on the effects of C-PC in combination with a diode laser in the PAD method against microorganisms. In the present study, we evaluated the efficacy of MIC of C-PC- mediated PAD on biofilm formation ability and changes of gene expression in *E. faecalis*. As the results of this study proposed, the activity of C-PC in *E. faecalis* was significantly increased in combination with the laser light compared with other groups. However, none of the groups were able to completely decontaminate the infected root canal system. These results are in accordance with previous studies which have shown difficulty in total eradicating *E. faecalis* from the root canal space [7–10].

Also, based on the current results, C-PC- PAD could be potential therapeutic agents for attenuating the *fsrB* activity as a major virulence factor of *E. faecalis*. Although several studies have investigated the effect of PAD with different photosensitizers on the expression of other genes involved in the formation of *E. faecalis* biofilm [7–10], the superiority of this study is that this natural product possesses pharmacological activity with no toxic side effects and can be considered as an optimal substitute for chemical photosensitizer.

5. Conclusion

C-PC represented great performance against biofilm of *E. faecalis* inside the root canal system and down-regulation of *fsrB* as a major virulence factor associated with the biofilm formation in *E. faecalis* when it was exposed to diode laser light in PAD. Forasmuch as C-PC is a comestible photosensitizer which can be used for the treatment of endodontic infection *in vivo*.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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