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Effect of Diode Laser Irradiation and Application of Nanoparticle Herbal Endodontic Irrigation Solutions on Candida Albicans and Enterococcus Faecalis Bacteria

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In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract

The present study investigate whether diode laser activation of two nanoparticle herbal irrigants affect Enterococcus faecalis (E.faecalis) biofilm and Candida albicans (C. albicans) compared with photodynamic therapy (PDT) utilizing methelyene blue as photosensitizer (PS). Material and methods: sixty single rooted teeth had undergo mechanical preparation. Teeth were divided into 3 groups (20 teeth each), G1 : 20 teeth inoculated with Enterococcus faecalis half (n= 10) irrigated with nanocurcumin and the other half irrigated with nanoneem both activated by diode laser, G2: 20 teeth inoculated with Candida albicans irrigated similar to group 1 and G3: 10 teeth inoculated with E.faecalis and 10 with Candida albicans undergo PDT using methylene blue as PS. Results: For E.faecalis no statistically significant difference neither between nanoneem and nanocurcumin ($p>0.05$) nor between nanocurcumin and PDT ($p>0.05$). While there was a statistically significant difference between nanoneem and PDT ($p<0.001$). While for C. albicans no statistically significant difference between nanocurcumin and PDT ($p>0.05$). While the differences were statistically highly significant between nanoneem and PDT ($p=0.002$) and between nanoneem and nanocurcumin ($p<0.001$). Conclusion: nanoneem and nanocurcumin irrigants activated by laser have an effect on root canal microorganisms and could be used as adjunctive endodontic irrigant.

Keywords: Root canal irrigants; antibacterial and antifungal effect; nanocurcumin; nanoneem, photodynamic therapy

1. Introduction

Many studies have shown that microbial organisms like Enterococcus faecalis (E. faecalis) and Candida albicans (C. albicans) are found in endodontic infections that lead to failure of root canal treatment (RCT) [1, 2]. Moreover, bacteria may reach the outer surface of the root by penetrating the dentinal tubules of the root, causing failure of root canal treatment [3]. Adequate penetration of antimicrobial irrigant solutions into anatomical complexities is essential for good debridement and disinfection [4]. Strategies of disinfection that use lasers or light can be divided into three groups: direct laser irradiation, laser-activated irrigation, and photodynamic therapy [5].

Laser wavelength, its energy, pulse frequency, and the design of the fiber optic tip, together with its position within the root canal, could influence the effect of LAI. Water, present in the endodontic irrigant solutions, blunts the thermal effect of the laser beam on the root dentin, but at the same time, when it becomes a target chromophore by mid-infrared laser activation, it can work synergistically to clean the canal [6]. Diode laser (DL) activation of irrigants has shown more favorable results, where 810-nm diode laser (DL) decreased the number of E. faecalis bacteria through invading dentinal tubules [7]. While different diode laser wavelengths (940- and 980-nm) are more highlighted because these are closely inconsistent with water absorption and are much more absorbed [8], For endodontic applications, activation of the irrigants could be done using a diode laser with parameters of 940-nm wavelength, a 50–60 Hz frequency system, and a maximum output power of 7 W using 200-μm plain-ended fiber, which consequently removes debris and the smear layer [9]. Photodynamic therapy (PDT) is based on utilizing light of an appropriate wavelength to activate photosensitizing molecules adhered to the bacterial or fungal membrane.

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It results in exciting its electronic layers to a so-called triplet state [10]. As photosensitizers, methylene blue (MB) and toluidine blue O (TBO) are amphibic; they can be used against both gram-positive and gram-negative bacteria present in root canal infections [11, 12]. PDT could be applied by a diode laser, in which the optical fiber is placed within the root canal near its full length, thus allowing for even light distribution both vertically and horizontally, resulting in better disinfection within the root canal system.

Herbal medicine has been widely used as a therapeutic agent because of its biocompatibility, safety, and cost-effectiveness, which raises the need to explore its application in the dental field [13]. Neem is one of the few herbal extracts that has antimicrobial properties along with other anti-cariogenic and anti-inflammatory activities [14]. It can be used as an effective substitute for sodium hypochlorite due to its high biocompatibility [15]. However, the major disadvantage of neem is its pungent taste, which reduces the patient's acceptance and can be neutralized by adding sweeteners [16]. Although neem has an efficient antimicrobial effect on many microbes, different studies have mentioned that neem has limited action against the main microbes in root canal infections like E. faecalis and candida albicans [17, 18].

So the use of nanotechnology to modify the particle size of neem extract may be beneficial to improve its antimicrobial action against various microorganisms present in endodontic infections. Curcumin, the main component of turmeric powder, has been used as a photosensitizer (PS) for PDT recently in dentistry [19]. Curcuma longa is a yellowish-orange powder that is a natural component of the rhizome, and it has a characteristic antimicrobial effect on bacteria, viruses, and fungi [20]. Gomes-Filho et al. (2016) [21] reported that PDT utilizing curcumin as PS had a benign effect on pulpal cells in addition to not interfering with fibroblast viability. Standard PDT performed with a diode laser and methylene blue performed similar bacterial inactivation as a combination of curcumin with the blue LED light [20]. Some studies showed that the antifungal properties of nanocurcumin were more potent than those of native curcumin [22].

Nanoparticles gained several properties that may improve the treatment of endodontic infections: synergistically increased antibacterial activity, increased reactivity, and the ability to interact with other reactive compounds. This leads to the innovation of various experimental nanoparticle-incorporated obturation materials and sealers that perform a range of favorable physicochemical properties, including enhanced antibacterial performance and bioactivity. Therefore, this study will investigate the effect of herbal irrigants, especially neem and curcumin, in nanoform after being activated by a diode laser in comparison with standard PDT using methylene blue.

2. Materials and methods

2.1. Preparation of nanoneem and nanocurcumin irrigants

The ultrasonic-solvent emulsification technique was used to prepare solid lipid nanoparticles (SLN), also called nanostructured lipid carriers, according to Adel [23]. Two phases were prepared: the oil phase and the water phase.

After volatilizing most of the solvents, the water phase was added to the oil phase. Finally, the impurity materials were filtered from the oil-loaded SLNs suspension and then stored at 4 °C for further bioassays.

Transmission electron microscopy (TEM) analysis was done, confirming their size below 100 nanometers. The effective electric charge on the nanoparticle's surface is measured by a test called Zeta, which quantifies the charges. This measure was done for both liquids showing negative Zeta potential.

2.2. Microorganism and Culture Conditions

Qualitative evaluations were carried out in nutrient broth according to Elboraey et al., 2021 [24] and Sultan et al., 2022 [25]. The pathogenic microorganisms used in this study were Gram-positive bacteria [Enterococcus faecalis **ATCC 29212]** and pathogenic yeast [Candida albicans **ATCC 10231**], prepared from fresh overnight broth cultures using nutrient broth medium.

20.0 µL of the above-prepared inoculum of each test strain was separately inoculated into each 2.0 mL-volume cryovial containing one tooth with one central hole filled with 1.0 ml of the sterile nutrient broth medium (NB). The inoculated cryovials with teeth were incubated at 37°C for 21 days for more test strain attachment. After the proper incubation period, the first inoculum was taken from each cryovial separately as a pre-inoculum or control sample.

2.3. Preparation of teeth

The extracted single-rooted teeth (n = 60) with one canal and well-developed apices were selected after checking their periapical radiograph. Teeth with caries, fracture, resorption, or malformation are excluded. De-coronation of all teeth was done using a diamond disc (Mani, Inc., Tochigi, Japan). Light cure composite (3M ESPE Z250, USA) was used to seal all the apical foramina of all teeth, which were

coated with two layers of varnish to prevent bacterial leakage.

Endodontic access was done with a 4-round carbide bur (DENTSPLY Maillefer, OK, USA). The working length was confirmed using a radiograph and was kept half a millimeter short of the radiographic apex. Preparation was done using the crown-down technique, where coronal preparation was done using Gates Glidden drills size 1:4. Mechanical preparation was done up to 30 stainless steel hand files, and rotary instrumentation was done using Pro-Taper (DENTSPLY Maillefer, OK, USA) up to F3. All the teeth were autoclaved twice at 121 °C for the elimination of all the microbes from the tooth and to prevent contamination of the study with other microbes.

Sixty tooth samples are divided into three groups and undergo different interventions, as follows:

G1: 20 teeth were inoculated with Enterococcus faecalis; 10 teeth were irrigated with nanocurcumin; and another 10 teeth were irrigated with nanoneem. All irrigated teeth undergo activation by a diode laser system (Sirona, Bensheim, Germany) at 980 nm, power 0.5 W, with pulse settings ranging from 10 to 10,000 Hz in eight increments. In this intervention, a 200 µm laser tip was placed into the root canal at a preset position 1 mm from the closed end, and the fiber tip moved slowly in an up-and-down motion within the canal for 20 seconds, repeated three times at 10-second intervals. The panel settings were 2.5 W/15 Hz with an average power of 1.2 W for the diode laser.

G2: 20 teeth inoculated with C. albicans. Irrigated similarly to group 1 and undergo laser activation with the same parameters for an equal period of exposure.

G3: 10 teeth inoculated with E. faecalis and 10 with candida albicans. For both groups, 1 mL of methylene blue was injected in the root canal using a 25-gauge needle and irradiated with a 200 µm fiber tip using a diode laser of wave length 650 nm (Pioon, Wahan, China) applied with settings of 200 mW and an energy of 60 joules. The photosensitizer is activated for 60 seconds in a circular motion with slight up-and-down movements.

In this step, a serial dilution from each [pre-inoculum or control group] and from each sample treated has been done (10^{-1}, 10^{-4}) separately.

The microbial inhibition was determined using the total viable count technique by counting the colony-forming units (CFU) using the Scan 100 colony counter system (France).

A diode laser is an electrically pumped semiconductor laser in which the active laser medium is formed by a p-n junction of a semiconductor diode. A 980 nm diode laser was used for its high transmission due to its higher absorption in water. This allows for microbial reduction at a depth of 1.000µm. Siro-Laser Advance Plus is a diode laser device with both an infrared and a red diode. The established infrared 980 nm wavelength covers all indications in the fields of soft-tissue surgery, periodontology, and endodontics. This enables it to cover more than 20 indications.

3. Results

Table (1) shows a comparison between nanoneem (median190.0, IQR 135.3-207.0) and nanocurcumin (median 69.0, IQR 18.5-137.3) activated by laser and PDT using methylene blue (median 4.5, IQR 0-39.5) on Enterococcus faecalis bacteria. G1 showed efficient antimicrobial activity against E. faecalis, with significant results compared to pretreatment samples. The results revealed that there was a statistically highly significant difference among groups ($p < 0.01$), while comparisons between each group and another showed no statistically significant difference, neither between nanoneem and nanocurcumin ($p > 0.05$) nor between nanocurcumin and PDT ($p > 0.05$). The difference was statistically highly significant between nanoneem and PDT ($p < 0.001$).

Fig. (1) Shows a comparison between nanoneem, nanocurcumin activated by laser, and methylene blue CFU*10^{-4} of bacterial Enterococcus faecalis . There is a statistically highly significant difference between nanoneem and methylene blue ($p<0.001$). While the differences between nanoneem and nanocurcumin and between nanocurcumin and methylene blue are statistically insignificant ($p > 0.05$).

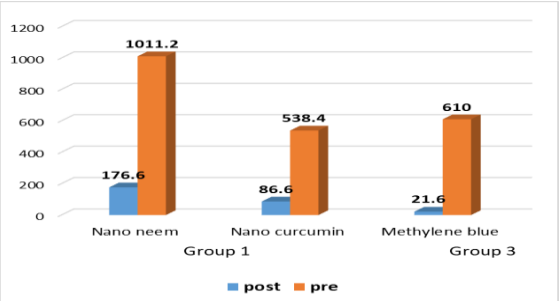

Fig. (1): Comparison between nanoneem and nanocurcumin group1 and MB group 3 samples on E. faecalis CFU*10-4

Table (2) shows a comparison between nanoneem (median 1.0, IQR 0-1.0), nanocurcumin (median 307.5, IQR 219.3-508.5) activated by laser, and PDT (median 257.0, IQR 242.3-276.5) on Candida albicans. Both nanoherbal irrigants activated by diode laser showed significant results against Candida albicans compared to pre-inoculum samples. The results revealed that there was a

statistically highly significant difference among groups (p<0.01), while comparisons between each group and another showed no statistically significant difference between nanocurcumin and methylene blue (p>0.05). The differences were statistically highly significant between nanoneem and methylene blue (p=0.002) and between nanoneem and nanocurcumin (p<0.001).

Fig (2) shows a comparison between the second and third group samples of Candida albicans CFU*10-4. There is a statistically highly significant difference between nanoneem and methylene blue (p=0.002) and between nanoneem and nanocurcumin (p<0.001) with the lowest growth in nanoneem by laser group. While the difference between nanocurcumin and methylene blue is statistically insignificant (p>0.05).

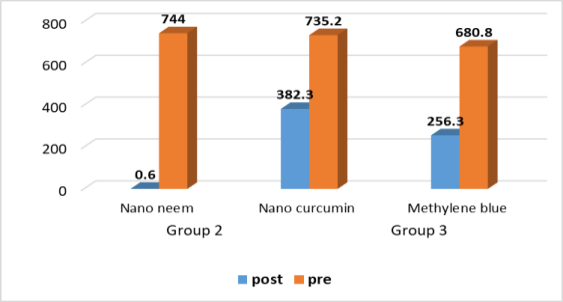

Fig. (2): Comparison between nanoneem and nanocurcumin of group 2 and MB of group 3 samples on Candida albicans CFU*10-4 (n=10 samples in each group)

Table (1): Statistical comparison between CFU*10^{-4} pre and post treatment of laser activated nanoneem and nanocurcumin irrigants against PDT on E. feacalis.

		Group 1 activated by laser		Group 3 PDT using Methylene blue
		nanoNeem	***nanoCurcumin***	
Post treatment	Mean±SD	176.6±40.8	86.6±82.5	21.6±33.5
	SE	12.9	26.1	10.6
	Median IQR	190 135.3-207.0	69 18.5-137.3	4.5 0-39.5
	Range (min-max)	106.0-224.0	13.0-265.0	0-95.0
pre	Mean±SD	1011.2±358.8	538.4±91.2	610.0±91.9
	SE	160.5	40.8	41.1
	Median IQR	1024 692.0-1324.0	556 454.0-614.0	618.0 522.0-694.0
	Range (min-max)	484.0-1444.0	420.0-664.0	404.0-788.0
	P value	0.006*	<0.001*	<0.001*
			P value	Adjusted significance
Group 1 nanoneem vs. Group 3			0.001	0.017*
Group 1 nanocurcumin vs. Group 3			0.06	0.9

Table (2): statistical comparison between CFU*10-4 pre and post treatment of activated nanoneem and nanocurcumin irrigants against PDT on C. albicans.

		Group 2		Group 3 PDT using Methylene blue
		NanoNeem	***NanoCurcumin***	
Post treatment	Mean±SD	0.6±0.5	382.3±222.2	256.3±34.8
	SE	0.2	70.3	11.0
	Median (IQR)	1 0-1.0	307.5 219.3-508.5	257.0 242.3-276.5
	Range (min-max)	0-1.0	177.0-870.0	180.0-305.0
pre	Mean±SD	744.0±208.2	735.2±150.7	680.8±158.1
	SE	93.1	67.4	70.7
	Median (IQR)	724.0 558.0-940.0	664.0 608.0-898.0	720.0 560.0-782.0
	Range (min-max)	556.0-1056.0	596.0-904.0	404.0-788.0
	P value	0.001*	0.007*	0.003*
			P value	Adjusted significance
Group 2 nanoneem vs. Group 3			0.012	0.185
Group 2 nanocurcumin vs. Group 3			0.635	1.0

4. Discussion

The photodynamic therapy (PDT) principle is effective against bacteria, viruses, fungi, and protozoa [26].

Mature (3 weeks old) bacterial biofilms resist antimicrobial treatments more than young biofilms, so the culture for the microorganisms used in the present study was 21 days old, as Shen et al. (2011 [27].

In modern clinical trials, toluidine blue O (TBO) and methylene blue (MB) were used as photosensitizers (PSs) with absorption wavelengths ranging from 600–660 nm [28, 29, 30, 31]. Phenothiaziniums, such as MB, attack the plasma membranes of yeast and bacteria and produce bacterial DNA damage [32]. In the present study, MB was used and activated with a diode laser with a wavelength of 650 nm. Methylene blue alone, used at concentrations of 100 µg/mL, could destroy 83% of E. faecalis bacteria inside a root canal [33].

A laser was used to activate the MB, in accordance with earlier research by Bago et al. and Rios et al. The irradiation times were 30 and 60 seconds, respectively. There is no agreement in the literature concerning the most reliable PDT parameters for E. faecalis destruction inside root canals [34, 35].

A previous study reported that methylene blue in PDT along with the diode laser could result in a 96.7% reduction of E. coli (910 nm, 1W at pulsed mode) [36]. The laser parameters used in the present study were similar to those of Pourhajibagher & Bahador et al. (2018 [37], whose results revealed that the microbial count significantly decreased ($P < 0.05$) when using an output power of 220 mW at 635 nm for 60 s.

The average pre-irradiation time, according to the literature, is 5 to 15 minutes [38]. In this study, PS was left for 5 minutes before irradiation.

Oda et al.'s 2019 comparison of 30 µL of curcumin as PS activated by LED light 430 for 5 min and standard PDT (1% methylene blue activated with diode laser 660nm for 4 min) resulted in no significant difference between both groups. We reported the same results, although nanocurcumin was used as an irrigant, not PS, and with a different laser parameter, 980 nm, for 60 sec at 20 Hz [39].

Nardini et al. reported that MB, when activated at 660 nm, presented similar reactive oxygen species (ROS) production to curcumin photosensitizer [40]. Moreover, a significant reduction in planktonic cultures and biofilms of E. faecalis could be achieved by activating curcumin with a red light LED (660 nm). Likewise, nanocurcumin and nanoneem, when activated by laser, produce significant antimicrobial effects compared with pretreatment groups.

In a study investigating irrigation with 2.5% NaOCI in comparison to NaOCl followed by either MB-PDT or curcumin-PDT using a LED lamp emitting a red spectrum with a power peak of 630 nm, there were no significant differences among these groups against E. faecalis [41]. Diode laser activation for nanocurcumin showed comparable results to MB-PDT, but for nanoneem, it showed a significant difference to MB as the latter was better.

Hendi. et al. (2021, compared the activated silver nanoparticle irrigant by diode laser 940 nm for 15 sec at 3 times interval together with 5% NaOCI on E. faecalis bacteria and found that NaOCI was significantly more effective [42]. They concluded that laser irradiation could not enhance the effect of silver nanoparticles. On the contrary, the present study showed that diode lasers with the same parameters enhanced the effects of both nanoherbal irrigants compared to pretreatment samples.

Some studies reported that conventional cleaning and irrigation protocols produce a similar reduction in *E. faecalis* counts in infected root canals compared to PDT [43]. Likewise, others use methylene blue (50 mg/mL) as photosensitizers and red light (665 nm), with an irradiation time of five minutes and an output power of one watt, to obtain the same results. [44]. Some studies reported that after mixing methylene blue with 0.5% hydrogen peroxide or poly (lactic-co-glycolic acid) nanoparticles, there was a decrease in the number of colony-forming units compared with conventional PDT [7, 10].

In accordance with the present study's results, on activation of 0.05 mg/mL MB using a 670 nm diode laser for 30 sec on E. faecalis biofilm cultures, the results showed a significant decrease in bacterial account in PDT-treated groups [45]. While Soukos et al. applied PDT with longer treatment times (5 minutes), they eliminated nearly 95% of the bacterial biofilm [46].

Diode laser activation of nanocurcumin showed an insignificant difference compared to PDT, similarly to the study that evaluated the efficacy of antimicrobial activity for NaOCI activated by laser for 20 seconds, repeated three times, and PDT applied for 60 seconds using indocyanine green (12 mg/ml) as a photosensitizer with an 810 diode laser and 1.5 output power, showed no difference in both groups, although they used a lower wavelength [47]. Bago et al. activated 2.5% NaOCl by endoactivator, comparing its effect with PAD using diode laser (100 mW, 60 seconds) on the 7-day biofilm of Enterococcus faecalis. They concluded that PAD was significantly more effective than activated NaOCl irrigation in reducing CFUs ($P < 0.05$) [34].

All nanoparticle herbal irrigants, together with PDT, were significantly effective compared to pretreatment samples. Literature found that a combination of photodynamic therapy and 2.5% NaOCl was effective in reducing E. faecalis biofilms [48, 49]. The combination of PDT with NaOCl offers many advantages, like faster permeation of the drug into the root canal for eliminating bacteria in a short time and the absence of thermal side effects in the nearby tissues [50].

In this study, PDT was performed at 650 nm and 200 mW for 60 sec, which was significantly better than nanoneem against E. faecalis. The same parameters were used by Asnaashari M., who reported an 80% decrease in the bacteria inside the canal compared to NaOCI [51].

Photoactivated therapy using 1.2 mg/L tolonium chloride with a 100 mW diode laser for 120 seconds against irrigation with 5 mL of 2.5% NaOCl showed that NaOCl was significantly better than PDT [52, 53, 54]. This may be due to the high susceptibility of the real-time PCR technique for detecting bacteria used in Janani's study, unlike the CFU used in the present study. In the same way, NaOCI was significantly better as an antibacterial agent (98% against E. faecalis) compared to PDT, which utilized a high output power of 2 W [55].

Nanoneem was activated for one minute to allow more penetration into dentinal tubules, unlike another study, which reported that 5 minutes of PDT and NaOCI were statistically significant compared with 1 minute of PDT. This may indicate that short irradiation time may be the cause of incomplete elimination of bacteria and consequently less amount of arisen ROS in dentinal tubules, where it is difficult for the photosensitizer agent to diffuse deeper [56]. So, the present results showed no statistically significant difference between nanocurcumin and methylene blue ($p > 0.05$). While the differences were statistically highly significant between (nanoneem, methylene blue) ($p = 0.002$) and between (nanoneem, nanocurcumin) ($p<0.001$)

Nanoneem almost succeeded in eliminating all C. albicans from pretreatment groups, unlike nanocurcumin and PDT-MB, which were significantly effective against C. albicans but failed to remove all microorganisms from pretreatment groups; similarly, Oliveira et al. (2015, Eldeniz et al. (2015 reached the same results when comparing the effect of PDT with classical endodontic irrigation against Candida albicans. They concluded that incomplete microbial removal could be associated with several factors related to PDT, such as the type of chromophore, its concentration, the light source wavelength, the maximum absorption of each chromophore, and the time of irradiation [57, 58]. This coincided with the results of the other studies [59, 60].

No significant difference was found between activated nanocurcumin and PDT; the same results were obtained when comparing 1 and 5 minutes of PDT using photosensitizer phenothiazine chloride at a concentration of 10 mg/ml with an output power of 100 mW/cm^2 at 66 nm with the gold standard of irrigation of 2.5% NaOCI on candida albicans [56].

PDT antifungal efficacy has been reported in the present study, as Wiench et al. evaluated this antifungal effect using a diode laser with a wavelength of 635 and toluidine blue (TB) as PS on selected candida species cultured on an acrylic surface. They used different parameters for laser (400 mW, 24 J/cm), (300 mW, 18 J/cm2), and (200 mW, 12 J/cm^2) all for 30 sec using 8 mm optical fiber; the reduction in CFUs was statistically significant; using MB on an acrylic plate with parameters 660 nm laser (CW, 137 J/cm2 and 480 sec.); obtaining the same results [61, 62]. Likewise, Ferreira et al. (2016 who used MB activated with a 660 nm diode laser with 690 mW and an energy of 60 J/cm2 [63], and Kato et al. (2013 used the same parameter with a 10-minute exposure time, which led to the complete elimination of fungi [64].

Few in vivo studies performed for PDT using Methylene blue at 660 nm and energy 40 J combined with potassium iodide showed almost complete eradication of C. albicans [65].

Even when using the same PS and activated light or laser source, the variety of irradiation procedures, including time and light power, in addition to the difference in PS concentration, make comparisons between the literatures difficult [66].

There is a lack of studies about the laser activation of herbal irrigants and their nanoforms in comparison with standard endodontic irrigants.

5. Conclusion

Activation of nanoparticle herbal irrigants are effective against E. faecalis specially nanocurcumin, while nanoneem is powerfully effective against C. albicans. They can be used as adjunctive in cleaning process of endodontic treatment.

6. List of abbreviations

- NaOCl----------Sodium hypochlorite
- *E. faecalis*------- *Enterococcus faecalis*
- *C. albicans*------ *Candida albicans*
- SLN---------- Solid lipid nanoparticles
- TEM----Transmission Electron Microscopy
- PCR---------- Polymerase chain reaction
- *C.longa*---------- *Curcuma longa*
- *A. indica*---------- *Azadirachta indica*
- CFU---------- Colony forming units
- FtsZ: Filamenting temperature-sensitive mutant Z

7. Ethics approval and consent to participate

In this study, ethical approval was obtained from the medical research ethics committee at the National Research Center in Egypt for the study titled Effect of photodynamic therapy on Enterococcus faecalis and Candida albicans compared to diode laser-activated nanoparticle herbal irrigants. Final approval number: (1483042021).

8. Competing interests

The authors declare no competing interests.

9. Funding

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