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ANTIMICROBIAL EVALUATION OF NOVEL TRIPLE ANTIBIOTIC PASTE AND THE DIODE LASER BEAM EACH ALONE AND IN COMBINATION AGAINST ENTEROCOCCUS FAECALIS IN COMPARISON TO THE CONVENTIONAL TRIPLE ANTIBIOTIC PASTE

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ABSTRACT

Introduction: Local antibiotic administration has been investigated in addition to employing laser disinfection in light of the progress of non-instrumentation endodontic treatment, lesion sterilization, and tissue healing.

Methods: 96 single-rooted teeth were collected. At the CEJ point, their crowns were severed. The root canals were prepared to work up to file F3 ProTaper in length. The smear layer was removed using EDTA 17% and sodium hypochlorite 2.5% for 5 minutes, and composite was then utilized to close the apical foramen. The teeth were sterilised for 15 minutes at 121°C in an autoclave. The adverse control was then represented by 16 samples. The remaining samples spent 21 days submerged in an E. faecalis-containing solution. The samples were devided into 6 groups: group A received an 810 nm Diode laser beam, group B conventional TAP treatment, group C conventional TAP treatment + a diode laser, group D novel TAP treatment, group E novel TAP treatment + a diode laser beam, and group F control. Each group was divided two to test the impact of the intervention after one day and seven days.

Results: The negative control had the largest amount of bacteria (8687.501352.47), followed by laser (670.62221.22), old paste + laser (503.28186.12), old paste + laser (322.89150.85), and new paste + laser (195.6289.51), while new paste (64.0656.90) had the lowest amount.

Conclusion: When compared to utilizing Laser alone, employing a new Triple antibiotic paste has a promising inhibitory impact on biofilm formation and the development of the E. faecalis bacteria.

KEYWORDS: Ciprofloxacin, Metronidazole, Levofloxacin, Tinidazole, Tigecycline

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INTRODUCTION

An infected tooth with a root canal could get re-infected if *Enterococcus faecalis* survived, grew inside dentinal tubules, and survived. The most frequently mentioned infectiousness factors related to *E. faecalis* bacteria that could be connected with endodontic infection are material for aggregation, attachment to surface, , LTA , extracellular byproducts, the digesting enzymes which capable of degradation gelatin and HA acid , and toxins lead to cell death .[1] *E. feacalis* patients are substantially more likely to be asymptomatic than symptomatic, proving that it is closely linked to chronic infections.[2] The growth of highly ordered biofilms is beneficial for root canal systems. The adhering bacteria cannot be entirely removed by biomechanical techniques.[3,4] For the apical healing of adult or immature teeth, it has been discovered that the eradication of bacteria is crucial. To lessen the amount of germs in each root canal , intracanal medications are advised [5,6]. The intracanal system was tested for contamination using a variety of intracanal antibacterial pastes. Many pastes containing antibiotic combinations were employed, such as double antibiotic paste of metronidazole and ciprofloxacin, even though sodium hypochlorite is still working as an irrigating solution for disinfecting root canal systems[7]. Metronidazole, ciprofloxacin, and minocycline are three antibiotics known as the "triple antibiotic paste," or TAP, and they are hazardous to root dentin.[8]. As a way to eliminate germs that frequently exhibit resistance to the majority of irrigating solutions and the tested medications, it has been discovered that using laser technology at specified wave lengths is efficient, safe, and able to penetrate deep layers of the tooth tubules. Diode lasers with wavelengths between 800 nm and 1064 nm are suitable to dental applications.[9]. Due to the heat impact of the administered dose, the diode laser is renowned for its great strength and capacity to disinfect root canal systems and access to specific anatomical areas that other medications or treatments cannot reach.[10]. Dentin melting and sealing up exposed tubule orifices reduce dentin permeability and sensitivity, according to electron microscopic imaging of human dentinal tubules subjected to a the Nd:YAG laser.[11]. Using of Diode laser 980 nm wavelength with 1 and 1.5 watt on *E. feacalis* show high reduction in bacterial biofilm [12]. Diode laser and triple antibiotic paste compared The data indicated that Diode cleaned the root canals at a rate of 98.8%, followed by antibiotic paste at 99.9%.[13]. Diode laser proved useful for cleaning root canals infected by *E. faecalis*, especially when combined with NaOCl[14]. When a diode was used with conventional chemical and mechanical treatments, E. faecalis was considerably reduced in the apical third of root dentin.[15]. Because gram-negative bacteria are better at absorbing photonic energy than gram-positive bacteria, administering a diode laser alone inside the root canal has a minor antibacterial effect on the *E. faecalis* biofilm. However, it enhances root canal disinfection when used with 5.25% sodium hypochlorite because it boosts the bactericidal activity[16]. The intracanal microorganism, especially *E. faecalis*, was shown to be reduced more effectively by the 810 diode laser than the 980 diode laser.[17]. This study target to compare antibacterial effect between traditional TAP, new generation of antibiotics in Novel TAP and diode laser beam on canals infected with *E. faecalis bacteria*.

MATERIALS AND METHODS

Preparing Samples

The Minia University's Faculty of Dentistry's ethics committee evaluated and approved the study procedure. (427/2020).

96 single-canal human anterior teeth which was arranged for extraction due to periodontal disease, prosthodontic or orthodontic purpose, etc. The crowns were separated with a diamond wheel stone till get length 15 mm. Then get straight access into the canals, and length was detected by introducing #10 K-file in each canal till it get out from the apex and apical patency was confirmed; and detect

(WL) 1mm before that length. Instrumentaion was continued with manual file #20 then 25 K files to cleaning roots, coronal part prepared by SX file of universal protaper system with speed 300rpm as manufacture's instruction. Apical part prepared by S1,S2,F1till F4 as MAF with using NaOCl 2.5% between rotary files.

Using 1 ml. 15% (EDTA) till 2 min as irrigation solution then use by 2.5% NaOCI and 1 ml saline solution. Last but not least, dry canals were created insertion sterile F4 paper points. To stop the flow of irrigant and the seeping of germs through the apical foramen, every one was closed with composite resin, and the root surface was coated with two layers of nail polish. Specimens were inserted in an Eppendorf tube" in order to simplify the manipulation during contamination and irrigation procedures. Samples were sealed in envelopes and autoclaved at 121 C for 30 minutes to disinfect them.

Classification of samples:

All samples (n=96) were divided according to the disinfection technique into 6 groups:

Group A: consisted of 16 samples and were subjected to 810nm diode laser beam.

Group B: consisted of 16 samples and were injected with triple antibiotic paste.

Group C: consisted of 16 samples and were injected with the triple antibiotic paste after subjected to the 810 nm laser beam.

Group D: consisted of 16 samples and were injected with novel triple antibiotic paste.

Group E: consisted of 16 samples and were injected with the novel triple antibiotic paste after subjected to the 810 nm laser beam.

Group F: A negative control group consisted of 16 samples and was not subjected to any disinfection protocol.

In accordance with the period of disinfection, every group was separated into two further subgroups:

Subgroup1: consisted of 8 samples that were tested on the day of the treatment.

Subgroup2: consisted of 8 samples that were tested 7 days after treatment.

Canals Biofilm Formation and Bacterial Contamination

Each sample was put into a clean microtube which filled with 2 ml. from suspension of *E. faecalis* (ATCC 29212) obtained from MIRCIN center, Faculty of agriculture, Ain Shams University, Egypt. suspension were made by culture pure bacterial which developd at 37°C in with 10% CO_2, for 24 hours. In blood culture PED the new bacteria were cultured, by spectrophotometer bacterial density was amended at 108 cells/ml at an optical density (OD) of 1 at 600 nm according to 0.5 MacFarland standard.

E.feacalis (ATCC 29212) cultured on different media

The specimen was then centrifuged at 1000 rpm with dration 5 minutes to force bacterial suspension inside the root canals. Then aspiration of the media surrounding the samples was performed and replaced by fresh bacterial soluion. All specimen were incubated at 37°C to 7 days at 100 % humidity. The broth culture medium was altered every other day to ensure the continuity of the culture media and the removal of extra bacterial cells in order to preserve the survival of E. feacalis. At the end of the incubation period the value of bacterial count was found to be ($1.49x10^5 \pm 0.17x10^5$).

Preparing the Medicament:

Old Triple antibiotic paste preparation:

TAP was prepared by combining Ciprofloxacin (Ciprofloxacin 500mg European Egyptian pharm) Metronidazole (Flagyl 500 mg **SANOFI**) and tetracycline (Tetracid 250 mg **CID)** in equal amounts by volume in the ratio 1:1:1. The resultant powder was added to 0.9 sterile saline solution in the ratio 3:2 wt. /vol. The mix was homogenized to obtain a uniform powder then mix with saline to obtain a paste like consistency.

Novel Triple antibiotic paste preparation:

TAP was prepared by combining Levofloxacin (levofloxacin 500 mg **MEMPHIS**), Tinidazole (Fasigyn 500 mg PFIZER) and Tigecycline (Standiga 50 mg AMOUN) in equal amounts by volume in the ratio 1:1:1. The resultant powder was homogenized with 0.9 sterile saline solutions wih ratio 3:2 wt. /vol. The mix was homogenized to obtain a uniform powder then mix with saline to obtain a paste like consistency.

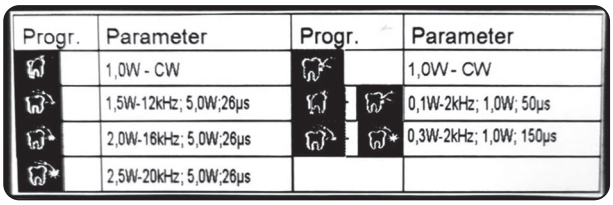
Moving the Medicine into the Canal:

Medications were placed in sterile container ready to be transferred into the canal .Then specimen were washed by 5 mL sterile saline to eliminate the remaining culture. After that, sterile gauze used for drying samples, after that, the surfaces of the teeth were covered with nail polish to expunge the bacteria and create a sterile layer such as cementum. Some of the substance was taken from the container containing it using a sterile spatula to insert in plastic syringe with disposable plastic irrigation tips at the end. Then the medication packed by Gutta-Percha.

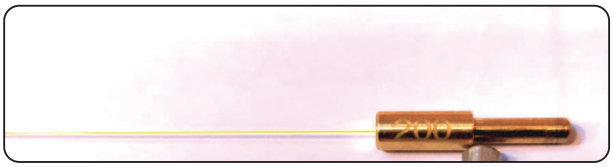
Treating the Samples Using laser:

Elexxion device emitting 810nm diode laser beam was set at power 2.5 watt will be checked by power meter and frequency 20 KHz. A fiber optic of 200 μm diameter was emitted in the canal, in the way that the laser fiber were stopped 1 mm before (WL) and was pulled out with the speed 2 mm per second, for 5 seconds. This was repeated for four times with a10-second between the cycles for all of group (A) samples.

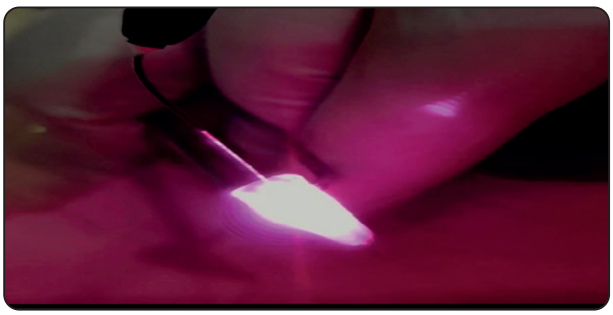
Elexxion Diode laser device



Elexxion Diode laser device programs and parameter



A fiber optic of 200 μm diameter



Application of laser into canals

Statistical analysis

In the form of mean and standard deviation (SD) values, numerical data were given. They were examined for normality using the Shapiro-Wilk test and the data distribution. Data had a parametric distribution, thus one-way ANOVA was used to examine it before the Tukey's post hoc test. At p 0.05, the significance level was established. R statistical analysis software for Windows, version 4.1.3, was used to conduct the statistical analysis.

RESULTS

1- Effect of disinfection technique

There was a significant difference between different groups ($p<0.001$). Table (1) showed that new paste has the highest effect on the inhibition of microbial growth in comparison with other medicaments, followed by the effect of new paste in Combination with Laser, old paste and old paste with laser while the least effect was shown by the application of laser alone.

TABLE (1) Mean, Standard deviation (SD) values of bacterial count (CFU/ml) for different disinfection techniques

Bacterial count (CFU/ml) (mean±SD)						p-value
Laser	New paste	New paste + Laser	Old paste	Old paste + Laser	Negative control	
670.62±221.22^{B}	64.06±56.90^{C}	195.62±89.51^{BC}	322.89±150.85^{BC}	503.28±186.12^{BC}	8687.50±1352.47^{A}	<0.001*

Different superscript letters indicate a statistically significant difference within the same horizontal row *; significant (p≤0.05) ns; non-significant (p>0.05)

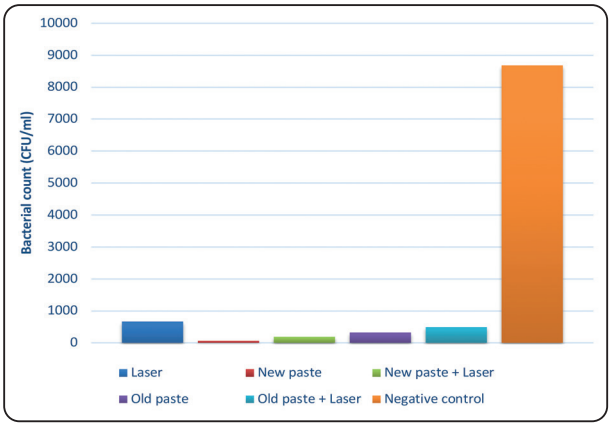


Fig. (1) Bar chart showing average bacterial count (CFU/ml) for different disinfection techniques.

2- Effect of time:

Mean, Standard deviation (SD) values of bacterial count (CFU/ml) for different times were presented in table (2) and figure (2)

Value measured at 24 hours (1881.61±3213.83) was higher than value measured at 7 days (1599.71±3169.43) yet the difference was not statistically significant (p=0.666).

TABLE (2) Mean, Standard deviation (SD) values of bacterial count (CFU/ml) for different times

Bacterial count (CFU/ml) (mean±SD)		p-value
24 hours	7 days	
1881.61±3213.83	1599.71±3169.43	**0.666ns**

; significant (p ≤ 0.05) ns; non-significant (p>0.05)

3- Effect of time within each disinfection technique

Mean, Standard deviation (SD) values of bacterial count (CFU/ml) for different times within each disinfection technique were presented in table (3) and figure (2). By testing the effect of each disinfection technique according to time, it was that new paste has the highest antimicrobial activity after 24 hours and 7 days in comparison with tested techniques. Also, all tested medicaments showed much better activity after 7 days in comparison with 24 hours results (P<0.001). In addition, our results showed that the tested pastes new or old paste have higher activity when tested alone but showed lower activity when combined with laser technique (Table 3).

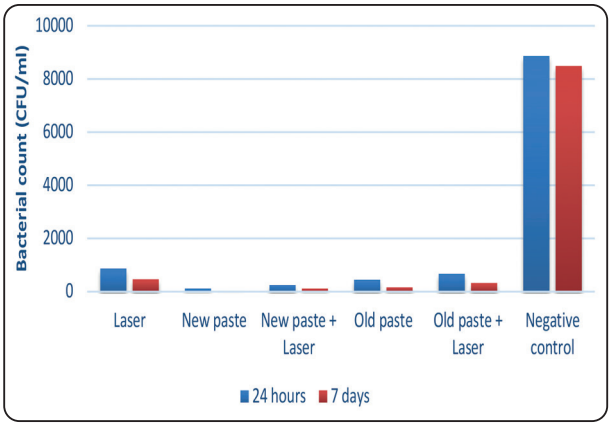


Fig. (2) Bar chart showing average bacterial count (CFU/ml) for different times within each disinfection technique

TABLE (3): Mean, Standard deviation (SD) values of bacterial count (CFU/ml) for different times within each disinfection technique

Disinfection technique	Bacterial count (CFU/ml) (mean±SD)		p-value
	24 hours	7 days	
Laser	876.25±84.17	465.00±33.81	**<0.001***
New paste	118.12±14.13	10.00±7.56	**<0.001***
New paste + Laser	271.25±62.44	120.00±14.14	**<0.001***
Old paste	467.50±25.50	178.28±17.79	**<0.001***
Old paste + Laser	681.56±17.88	325.00±35.46	**<0.001***
Negative control	8875.00±1356.20	8500.00±1414.21	**0.597ns**

; significant (p ≤ 0.05) ns; non-significant (p>0.05)

DISCUSSION

This study compared the bactericidal results of three methods conventional triple antibiotic paste, novel triple antibiotic paste and diode laser beam on the *E. faecalis* growth and their ability to develop biofilm after one day and 7 days from treatment. The study referring to the count of CFU/mL of TAP, NTAP and Diode laser sample was smaller than that of the untreated group; the novel TAP cleansed the canal till 99.26%, combination between novel paste and laser reduced the count of CFU/mL till 97.74%, conventional TAP cleansed the canal till 96.17%, combination between conventional TAP and laser minimize the count of CFU/mL up to 94.21%, finally Diode laser beam cleansed the canal till 92.28%, the best results were shown by the replacement of the old ingredient of conventional TAP (ciprofloxacin, metronidazole and tetracycline) by the new generations (Levofloxacin, Tinidazole and Tigycycline). The study pointed out that the count of CFU/mL of TAP, NTAP and Diode laser sample was smaller than that of the control group; the novel antibiotic paste cleansed the canal till 99.26%, combination between novel paste and laser reduced the amount of CFU/mL till 97.74%, conventional TAP cleansed the canal till 96.17%, combination between conventional TAP and laser reduced the count of CFU/mL till 94.21% and finally Diode laser beam cleansed the canal till 92.28%, the best results were shown by the replacement of the old ingredient of conventional TAP (ciprofloxacin, metronidazole and tetracycline) by the new generations (Levofloxacin, Tinidazole and Tigycycline). The lowest effect was related to Diode laser beam in comparison with conventional and novel TAP. Combination between laser and pastes improved antibacterial effect of laser and decreased the effect of pure TAPs. Results after 7 days were better than that after 1 day.

The Actinobacteria species Streptomyces produces the broad-spectrum polyketide antibiotic tetracycline. Preventing incoming aminoacyl tRNA by adhesion in revers way to the bacterial 30S ribosomal subunit from contact with the ribosome acceptor site, it has a bacteriostatic impact on bacteria. Additionally, it partially attaches to the bacterial 50S ribosomal subunit and has the potential to change the cytoplasmic membrane, allowing for the leakage of intracellular components from bacterial cells. Tetracycline resistance was created through many ways, which reduced its effectiveness. The glycylcyclines were created to get tetracycline analogues that got around these resistance mechanisms. The minocycline 9-tert-butyl-glycylamido derivative, also called tigecycline (GAR-936), is the most developed glycylcycline. The antibacterial properties of the glycylcyclines are similar to those of previous tetracyclines, although they are more effective against tetracycline-resistant pathogens. As Tigecycline beat on the two key tetracycline resistance mechanisms (efflux pumps and ribosomal protection) and hasn't any other bacterial mechanisms of resistance, such as extended-spectrum β-lactamases[18]. Methicillin-resistant staphylococci, penicillin-resistant Streptococcus pneumoniae, and vancomycin-resistant enterococci are only a few examples of the resistant microorganisms that the glycylcyclines are effective against. Contrary to other tetracyclines currently on the market, tigecycline is only accessible to clinical usage in an injectable formulation. Comparing its volume of distribution to that of the other tetracyclines (which ranges from 0.14 to 1.6 L/kg), tigecycline is noticeably bigger (> 10 L/kg). 68% or so of proteins are bound. Although experiments in rats employing radiolabeled tigecycline showed excellent tissue penetration, there are currently no data available about the tissue penetration of tigecycline in humans. [19].

Metronidazole is form of nitroimidazole that shows a broad spectrum of activity versus protozoa and anaerobic bacteria. Has strong antibacterial activity against anaerobic cocci as well as Gram-negative and Gram-positive bacilli, it could use both systemically and topically in the treatment of periodontal disease. Bacterial cell membranes

are easily penetrated by metronidazole, which subsequently binds to DNA to destroy its helical helix, causing fast cell death. [20, 8]. The antibacterial activity of metronidazole was examined in vitro by ***Roche and Yoshimori*** [21] versus clinical isolates from odontogenic abscesses. They demonstrated that metronidazole had no effect on aerobes but was very effective against anaerobes.

Similar to metronidazole, tinidazole is a 5-nitroimidazole that minimize ability of anaerobic bacteria to active intermediates that interact with nucleic acids to cause breaking and instability (mediated by flavoprotein and ferredoxin systems and a low oxidation-reduction potential). Tinidazole shows double half-life (12–14 h) that of metronidazole, tinidazole is bactericidal against anaerobic bacteria, shows high concentration in saliva ,blood and crevicular fluid concentrations throughout the dosing interval, and has appropriate pharmacodynamic and pharmacokinetic activity superior to metronidazole against anaerobic periodontal pathogens. Tinidazole kills bacteria quickly, its effectiveness is unaffected by the size of the inoculum, and it seldom causes resistance to develop during therapy. [22,23]. When used against periodontal bacteria that were both -lactamase-positive and -negative, tinidazole exhibited strong anti-anaerobic action. [24].

Levofloxacin has more bactericidal efficacy than ciprofloxacin, according to ***Montanari et al***. (25) levofloxacin-containing paste shown greater efficacy against Gram-positive *Enterococcus faecalis* bacteria. The levofloxacin ((S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido(1,2,3-de)-1,4-benzoxazine-6-carboxylic acid), found as the L-isomer of the racemate Ofloxacin [26], Additionally, a third-generation FLQs has broad-spectrum action against both Gram-positive and Gram-negative bacteria. Furthermore, the levofloxacin also demonstrates higher activity versus Gram-positive bacteria than the ciprofloxacin. Also, levofloxacin has longer post-antibiotic effect (PAE) which means count of microorganisms after removing the antibiotic or paste containing antibiotic[26,27]. Before and after antibiotic withdrawal, the size and quantity of bacterial colonies were counted. The best second-generation fluoroquinolones (FLQs) against Gram-negative bacteria is (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid), which quickly effect without interfering with penicillin, cephalosporins, or aminoglycosides. [28]. Instead of other FLQs, the ciprofloxacin inhibits the DNA gyrase (topoisomerase II) and lacks the DNA replication.

The results showed that the effect of triple antibiotic paste increased after 7 days than 1 day of applying the paste and that due to increase contact time between paste and bacteria leading to increase effect which is the same conclusion obtained by ***Lakhani et al.*** [29] Increase contact time more than 7 days affected microhardness of dentin[30] and the results were agreed with ***Ghabraei et al.*** [31] According to ***Ghabraei et al***., TAP must be used for at least seven days in order to completely remove *E. faecalis* from the root canal system. TAP use for longer than a week may cause coronal discolouration. (32). Using of Diode laser alone in group A and with both pastes in groups C and E shows less effect than using TAPs alone in groups B and D and it may due to sealing of dentine pores(33) Antonio et al 2004 that plays against TAPs to reach bacteria to get maximum effect from combination between laser and pastes. Comparison between laser and conventional TAP, Antibiotic paste minimize bacteria count in root canal up to 99.9%. Photodynamic therapy minimize the count of CFU/mg to 98.8%, and calcium hydroxide minimize the count of CFU/mg to 94.13[13]. By employing electron microscopic scanning to analyze the Nd:YAG laser's effect on dentinal tubules, it was discovered that the dentin was melting and the uncovered dentinal tubule orifices were closing. On dentinal tubules, the Nd:YAG laser had a closure depth of around 4 microns. [11]. Investigation the

effect of Diode laser 980 nm wavelength with 1 and 1.5 watt on E.feacalis and figured the 980 nm and show high reduction in bacterial biofilm ($1x10^3$-$13x10^5$) CFU/ml [12] Comparing the capacity of the Nd:YAG and Er:YAG lasers to eradicate bacteria in experimentally infected curved root canals, it was discovered that the Er:YAG laser exhibited superior bactericidal effects to the Nd:YAG laser by 6.4-10.8%. In contrast, the Er:YAG laser outperformed the Nd:YAG laser in the curved root canals by a margin of 1.5–3.1%. The Er:YAG laser has a substantially weaker bactericidal impact in curved root canals than it does in straight ones(14) Diode laser, triple antibiotic paste, and calcium hydroxide were compared, and the findings revealed that antibiotic paste cleaned the root canal to a level of 99.9%, followed by diode at 98.8%, and calcium hydroxide at 94.13. [13].

CONCLUSION:

Using a novel Triple antibiotic paste (1 mg/mL) has a promising inhibitory effect on biofilm formation and the growth of *E. faecalis* bacterium in comparison to using Laser alone.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, priori approval (No.427/2020) by the ethical committee of Faculty of Dentistry, Minia University.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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