Journal of Positive School Psychology http://journalppw.com
2022, Vol. 6, No. 8, 7877-7920

A Comparative Evaluation Of The Efficacy Of Three Irrigant Activation Systems: Fanta Af Max File, Xp Endo Finisher File And Irri-Safe Ultrasonic Tip On Enterococcus Faecalis Eradication In Long Oval Canals Using Confocal Laser Scanning Microscope. "A Randomized In-Vitro Study"

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Abstract

Purpose: To compare the efficacy of irrigant activation systems: Fanta AF Max, XP Endo finisher and Irri-safe ultrasonic tip on Enterococcus faecalis eradication and the maximum depth of the irrigant penetration into the dentinal tubules of long oval canals in single rooted teeth. **Methods:** Fifty-four extracted human single rooted teeth with oval canals were included. Teeth were prepared with Hyflex rotary system, the irrigation was done using 2.5% Sodium hypochlorite and 17% Ethylenediaminetetraacetic acid. After sterilization, the teeth were inoculated with Enterococcus faecalis for 21 days. The teeth were randomly allocated into three groups (n=14) according to the activation system used. The canals were irrigated using 5.25% Sodium hypochlorite then activated for 3 minutes. The teeth were splitted longitudinally, then treated by live /dead stains and examined using Confocal laser scanning microscope. **Results:** The XP Endo finisher file showed the highest percentage of bacterial death compared to the other groups where the difference was statistically significant regardless the root depths and levels (P-value <0.001). The results revealed that the coronal the middle parts showed the highest bacterial death percentage compared to the apical third with statistical significance (P-value<0.001) Regarding the irrigant penetration depth, the XP Endo finisher group showed that highest irrigant penetration depth values into the dentinal tubules when compared to the other groups. **Conclusion:** XP Endo Finisher had the highest ability to eradicate Enterococcus faecalis in addition, it showed the deepest irrigant penetration depths into the dentinal tubules as well.

Keywords Irrigant activation, Enterococcus faecalis, Sodium hypochlorite, Dentinal tubules, Confocal laser scanning microscope.

Introduction

Pulpal necrosis and apical periodontitis are considered to be sequelae of pulp inflammation and infection due to the presence of a variety of virulent bacterial species and their noxious by-products in the root canal system. The outcome of the treatment can be affected by the resistance & persistence of the bacteria during and after obturation which can end up with treatment failure.

Various bacterial species may cause endodontic infection; however, the most resistant bacterial species related to treatment failure and retarding the healing of the apical lesion is mainly the Enterococcus faecalis. It is a facultative

anaerobe that has the ability to survive in difficult conditions, due to its aggressive virulence factors enabling their intimate adherence to the dentine and finally the invasion of the dentinal tubules (Neuhaus et al. 2016). In order to combat these bacteria, efficient debridement of root canal system is very challengeable due to root canal irregularities and complexities. It has been shown that parts of the dentinal canal wall are left untouched after chemo-mechanical instrumentation as isthmus, lateral canals, ramifications, etc.., harboring debris and bacteria that survive, invade and colonize the dentinal tubules (Joy et al. 2015).

Therefore, the development of new disinfection protocols with specialized tools was a focus of attention to improve the canal cleanliness and enhance irrigant penetrability towards some areas difficult to reach enhancing its penetration deeper into the dentinal tubules

One of well-known method for the activation of irrigants is the passive ultrasonic irrigation using Irri-safe tip, as it accelerates the chemical reaction and creates cavitional effects and was proved to achieve superior cleansing action (Zehnder et al. 2006, Al-Jadaa et al. 2009).

The XP-endo Finisherwas recently introduced as a final irrigation system to activate the irrigant solution and maximizes its effectiveness (Zehnder et al. 2006). The XP-endo finisher is a nickel–titanium rotary finishing file with a small core size ISO 25 and zero taper. It is manufactured with MaxWire (Martensite-Austenite Electropolish-FleX) with an improved flexibility and an ability to remove a significant amount of bacteria from the main canal without compromising dentine.

Another approach for irrigant activation is the Fanta AF Max file. It is made of flexible NiTi wire with size ISO #25, 30 taper 1% and squared cross section. It has a unique sickle shape where the manufacturer clamied that it has superflexiability and ability to touch all the canal walls without shaping but only for the activation of the irrigant and the disruption of the bacterial biofilm inside the canal. In addition, it has a cheap price making it afforadable to all dentists.

The purpose of this study was to assess the effectiveness of both recent systems Fanta AF Max and XP Endo finisher compared to ultrasonic activation with Irri-safe ultrasonic tip on the E. faecalis eradication and the maximum depth of irrigant penetration inside the dentinal tubules.

LIST OF ABBREVIATIONS

Abbreviations	Full Term
CLSM	Confocal Laser Scanning Microscope
E. faecalis	Enterococcus faecalis
EDTA	Ethylenediaminetetraacetic Acid
NaOCl	Sodium Hypochlorite
NiTi	Nickel Titanium
PUA/I	Passive ultrasonic agitation/ irrigation
XPF	Xp finisher

Materials and methods

Methods:

1. Description of research question (PICO)

P: Population:

Single rooted teeth with long oval canal.

I: Intervention I: Fanta AF Max file:

Activation of sodium hypochlorite solution [5.25%] with Fanta AF Max® file.

I: Intervention II: XP Endo Finisher file:

Activation of sodium hypochlorite solution [5.25%] with XP-endo Finisher® file.

C: Comparator: Irri-safe ultrasonic tip:

Passive irrigation with sodium hypochlorite solution [5.25%].

- Six roots which were contaminated by E. faecalis and no irrigation was performed were considered as a positive control group (n=6).
- Six roots which were instrumented and sterilized and no bacterial contamination was done were considered as a negative control group (n=6).

O: Outcomes:

Table (2): Outcomes of the study.

Prioritization of Outcome	Outcome	Method of Measurement	Unit of Measurement
Primary outcome	Bacterial eradication in the dentinal tubules. Locations (coronal, middle and apical) Depths (50,100,150,300,400,1000µm).	Measuring the ratio of viable bacteria (red fluorescence) in relation to the viable and dead bacteria (red and green fluorescence)	Numerical
Secondary outcome	Depth of penetration of the endodontic irrigant: The maximum depth of penetration that the irrigant can reach in the form of maximum depth of dead bacteria.	[CLSM] Measuring the Red/Green fluorescence intensities Using the depth measuring tools (**Azim et al. 2016**).	Depth of irrigant penetration in µm.

2. Trial design:

Prospective, parallel, double blinded, randomized in-vitro trial with allocation ratio 1:1:1.

3. Samples:

3.1. Sample collection:

Fifty-four freshly extracted human single rooted, single oval canalled teeth were collected from the dental clinic of the national diabetes and endocrinology institute, Cairo- Egypt, and from the oral surgery department of the faculty of dentistry, Cairo University. The purpose of extraction was a periodontal problem.

3.2. Eligibility criteria:

Inclusion criteria:

- Single rooted, single long oval canalled teeth.

- Intact teeth [free of cracks, fractures, decay, previous root canal treatment and restoration]
- Mature root apices.
- The teeth are at 18- 24 mm average of length.

Exclusion criteria:

- Severe root curvature
- Complex root and root canal anatomy.
- Calcified root canals.
- Internal resorption.

3.3. Study setting:

All the endodontic procedures were done by a single operator, A PhD. degree student at the Endodontic Department, Faculty of Dentistry, Cairo University.

3.4. Sample size calculation:

Based on a pervious study done by Bedier et al. (2018) sample size was calculated as 14 sample in each group using (G power software). One-way analysis of variance power calculation for more than two groups was used to detect the proper sample size.

Means and standard deviations were determined according to the same study based on viability of bacteria. The results showed that at a power of 80% and a two-sided significance level of 5% was adequate to reject the null hypothesis that the group means are equal; i.e. there's no difference between groups regarding viability of bacteria.

As regarding the primary outcome (Detect the percentage of the viable Enterococcus faecalis colonizing the dentinal tubules), it was found that 14 teeth per group was appropriate sample size for the study with total sample size 42 teeth (3 groups).

F tests - ANOVA: Fixed effects, omnibus, one-way

Analysis:	A priori: Compute required sample size		
Input:	Effect size f	=	0.5
	α err prob	=	0.05
	Power (1-β err prob)	=	0.8
	Number of groups	**=**	**3**
Output:	Noncentrality parameter λ	=	10.500000
	Critical F	=	3.238096
	Numerator df	=	2
	Denominator df	=	39
	Total sample size	**=**	**42**
	Actual power	=	0.803414

The magnitude of the effect to be detected was estimated as the mean and standard deviation of the variable of interest and obtained from the scientific

literature.

3.5. Randomization steps:

3.5.1. Sequence generation:

Random allocation and sequence generation was performed using a computer random sequence generator program which was formulated in to three columns.

Computer generated random sequence table could be obtained through https//:www.random.org.

3.5.2. Allocation concealment:

To prevent the selection bias in the interventions, the allocated sequence was protected and concealed until assignment using sequentially numbered opaque sealed envelopes in which the teeth would be placed.

3.5.3. Implementation:

Random allocation, sequence generation and the allocation concealment was performed by the Co-supervisor and the technical procedures were carried out by the investigator.

4. Blinding:

To prevent detection bias, the images of the CLSM were taken by one trained blind observer, who was blinded to the sequence and prepared the reports of outcomes. The outcome assessor and the statistician were blinded.

5. Ethical consideration:

Research ethics approval

The protocol of the study was approved by the ethics committee of faculty of dentistry, Cairo university.

After receiving the results and finishing the experiment, all the instruments and teeth samples used in the research sterilized and discarded in a special incinerator under the supervision of Microbiology Department-Cairo University.

6. Intervention:

6.1. Sample preparation:

- The external surfaces of the roots were inspected and cleaned from blood, tissue debris, disinfected with 3% sodium hypochlorite for 5 minutes and scraped by a curette to remove the attached periodontal ligament fibers from the roots surfaces, then they were stored in a plastic container containing normal saline until their use.

- The teeth were inspected with a microscope for cracks and radiographed to verify the presence of a single canal, checking that the canal has buccolingual dimension greater than the mesiodistal dimension to ensure the long oval shape and absence of any resorption or endodontic treatment.

- The teeth were decoronated at the level of cemento-enamel junction by a low speed water cooled double sided diamond disc to obtain a standardized length of 15 mm. (figure 1,2).

- The canals were negotiated with K-file size 10 to establish apical patency and then the working length was adjusted at 1mm shorter after the first inspection of the file at the apex.

- The root canals were prepared by Hyflex rotary system in the following sequence (#20/.04), (#25/.04), (#20/.06), (30/.04) up to reach an apical preparation of (#40/.04) and were irrigated with 3% sodium hypochlorite (NaOCl) between each

instrumentation cycle using a plastic syringe and a side-vented 30-gauge needle that was positioned at 1 mm from the adjusted working length. The final rinse was performed with 2 ml of 17% EDTA for 2 minutes then flushed with 5.25% NaOCl to remove the smear layer and dentine mud ensuring patency of the dentinal tubules for bacterial growth and colonization. All the teeth were then irrigated with 5 ml of normal saline.

- A closed-end system was created in the teeth using flowable composite resin which was placed on the apex of each root (figure 3). All the root surfaces were double coated with nail varnish to ensure that the contamination occurs through the main root canal, and the teeth were placed in pouches and autoclaved at 121ºC for 20 minutes (Dai et al. 2018).

Fig. (1): Decoronation of a tooth sample into a standardized length

Fig. (2): Tooth sample after decoronation and measuring the root length 15mm

Fig. (3): Closure of apex with flowable composite

6.2. Contamination protocol:

Bacterial preparation phase:

A suspension of E. faecalis American type ATCC 29212 was prepared using blood agar media. Eight hundred Micro litter of the E. faecalis bacterial suspension was prepared in a Brain Heart Infusion broth (BHI) and maintained aerobically at 37°C for 24 hours. The bacterial culture was then adjusted to McFarland standard No.0.5 (1×10^8 CFU/mL) (figure 4).

Bacterial Inoculation phase:

This phase was achieved over a 21 days' period.

The first day:

- Each root was placed in an eppendorf in which 800 µl of sterile BHI broth was added and also injected into the canals. The eppendorfs were centrifuged at 1500g for 5 minutes prior to the bacterial contamination step.

- The sterile BHI broth of the previous step was removed using micropipette and 800 µl of the E. faecalis suspension was inserted with a 1-ml insulin syringe into each root canal.

- The roots in the eppendrofs were then centrifuged in two cycles, the first was for 5 minutes and the second was for 7 minutes (starting with a force of 1000g for 2 minutes and procceding with force of 1500g the rest of the cycles). A 30 µl fresh bacterial suspension was added inside the root canals between every centrifugation cycle where the solution already used was discarded with a 1-ml insulin syringe.

- A 30 µl of E. faecalis suspension was added to the full length of the canal with a 1-ml insulin syringe and a 30-gauge long needle, then incubated aerobically for 24 hours at 37°C and 100% humidity, this was achevied using wet cotton, all these measures were done to facilitate the bacterial recovery.

The second day:

- All the roots in the eppendrofs were agitated in the vortex for 10 seconds and the fluidinside the canal was discarded with 1-ml insulin syringe. Then 1 ml of sterilized BHI broth was inserted into the root canal with the same syringe, which

again was agitated in the vortex for 10 seconds and then incubated aerobically at 37°C for 24 hours and 100% humidity.

On the third and fourth days:

- On the third day, the same steps were repeated as the first day, while the steps followed on the second day were repeated on the fourth day.

On the fifth day till the twenty-first day:

- During the incubation period, a fresh sterile culture medium (30µm) was injected inside the root canals every 3 days to keep the bacteria alive.

On the twenty-first day:

- The roots were removed from the eppendorf and prepared for the next step which is the activation of the irrigant.

Fig. (4): E. faecalis suspension

6.3. The Activation Protocol for the Final Rinse of the Endodontic Irrigant:

- The activation protocol was done as follows: 3ml of 5.25% NaOCl solution was delivered into the canals within 30 seconds using a disposable plastic syringe with a side-vented 30G needle that was positioned at 1mm from the adjusted working length until the canal was filled.

- The teeth were randomly divided into three groups of 14 samples each according to the final irrigant activation method.

Group (A): Intervention group I: Fanta AF Max file:

The irrigant was activated using Fanta AF Max file mounted on X smart motor device with 800-1000 Rpm speed ,1 N/cm torque according to the manufacturer's instructions (Fanta Shanghai Dental Materials Co., Ltd. China). The file was positioned at 1 mm from the adjusted working length using slow and gentle vertical in and out movements. The activation cycle was repeated three times each lasted for 1 minute (figure 5, 6).

Fig. (5): Fanta AF Max file

Fig. (6): X-Smart rotary motor

Group (B): intervention group II: XP endo finisher file:

The irrigant was activated using XP endo finisher file mounted on X smart motor device with 800-1000 Rpm speed ,1 N/cm torque according to manufacturer's instructions. The file was positioned at 1 mm from the adjusted working length using slow and gentle vertical in and out movements of 7–8 mm inside the canal. The activation cycle was repeated three times each lasted for 1 minute (figure 7).

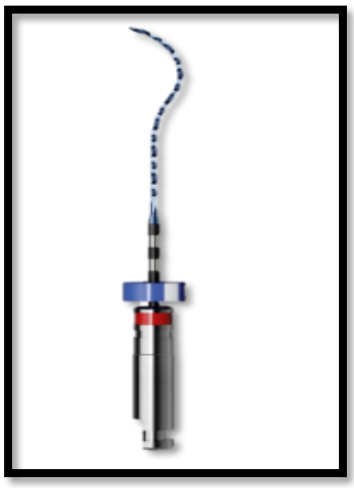

Fig. (7): XP endo Finisher file

Group (C): Control group: Irrisafe ultrasonic tip:

The irrigant was activated using Irrisafe ultrasonic tip mounted on an ultrasonic unit adjusted to a power setting of 5. The tip was positioned at 1 mm from the adjusted working length using in and out movements. The activation cycle was repeated three times each lasted for 1 minute (figure 8, 9).

Fig. (8): Irrisafe Ultrasonic Tip

Fig. (9): Ultrasonic unit device

All the procedures were done under laminar flow with safety cabinet to prevent and protect the investigator from bacterial infection, and the instruments were sterilized each time the procedure was performed.

6.4. Specimens Scanning Using Confocal Laser Scanning Microscope (CLSM): (Live/Dead) Essay:

- All the disinfected root samples were attached to acrylic blocks and were sectioned longitudinally into two halves in the buccolingual direction using IsoMetTM precision sectioning saw (figure 10, 11,12).

a) Each specimen (one half of each root) was placed in an eppendorf tube and was immersed in 1 ml of distilled water.

b) the specimens were stained by Live / Dead Stains: Acridine Orange (AO) for living bacteria and Propidium Iodide (PI) for dead bacteria (figure 13). Ten microliter of Acridine Orange (AO) (100µg/ml) and ten microliter of Propidium Iodide (PI) (100µg/ml) were added respectively to the eppendorf using a micropipette.

Fig. (10): IsoMetTM precision sectioning saw

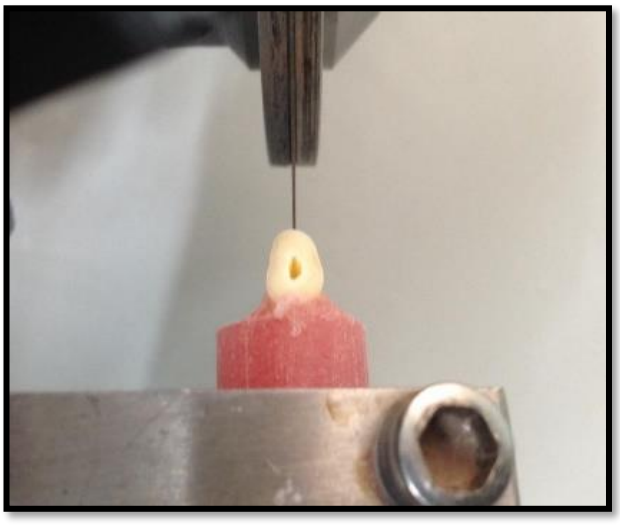

Fig. (11): IsoMet cutting through a tooth sample

Fig.(12): Longitudinal cut section of a tooth sample

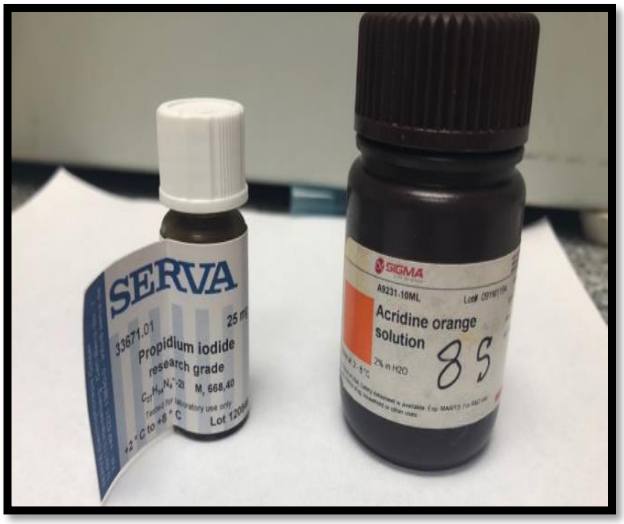

Fig. (13): Acridine Orange (AO) and Propidum Iodide (PI) stains

c) The dying solutions in the eppendorf were mixed using a Thermo scientific LP Vortex Mixer for 30 seconds (figure 14).

d) Each specimen was then incubated in the darkness for 15 minutes at room temperature.

e) After 15 minutes the solution of each specimen was discarded from the eppendorf and the specimen was gently washed with 1ml of deionized water for three times (figure 15).

f) The specimen was placed on a microscope cover slip of 170 µm thickness and was scanned using Confocal Laser Scanning Microscopy (CLSM) (figure 16) with EC Plan Neofluar 20x/ 0.50 M27 lens. An excitation/emission wavelength of 458 nm was used for the excitation of Propidium Iodide (PI) stain, and 514 nm for Acridine Orange (AO) stain. PI would thus emit red fluorescence light representing the dead cells, while AO would emit green fluorescence representing the living cells.

g) Sequential dual-channel imaging was used to display the green fluorescence (live cells) and red fluorescence (dead cells).

h) The border of the root canal and the outer surface of the root was first located with the microscope and then full length of dentinal tubules were scanned in each section.

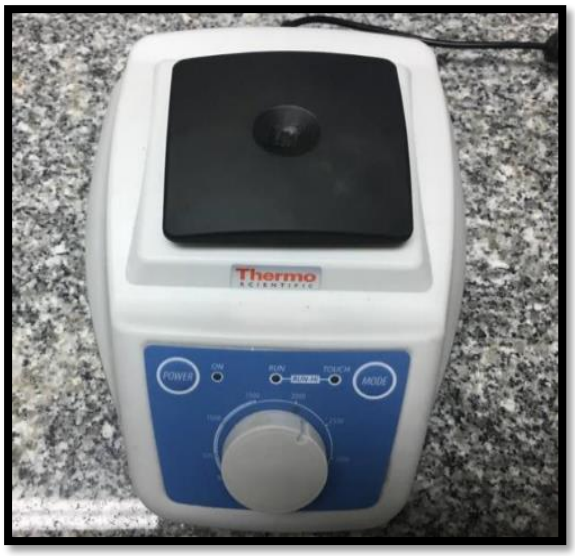

Fig. (14): Thermo scientific LP Vortex Mixer.

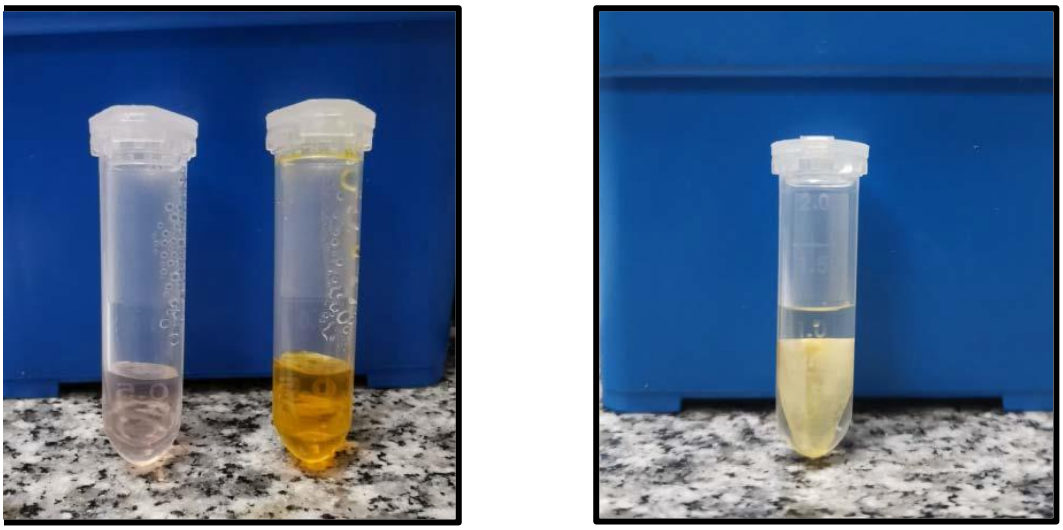

Fig. (15): Staining the root halves with fluorescent LIVE/DEAD Bacterial Viability stain

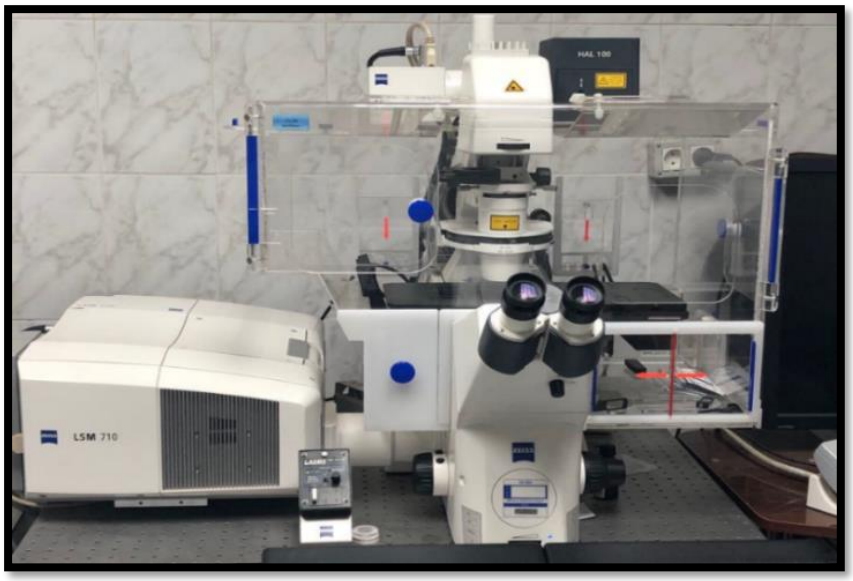

Fig. (16): Confocal Laser Scanning Microscopy

Primary outcome (Bacterial Eradication in The Dentinal Tubules):

Quantification and analysis of CLSM images and calculation of the percentage of dead bacterial cells was done at coronal, middle and apical thirds at different dentinal tubules depths 50, 100, 150, 300, 400 and 1000 μm from the canal surface outwards. This was done using ZEN 3.0 software lite edition (Blue and black Edition) in each of the scanned images (Azim et al. 2016) (figure 17).

$$\text{percentage of Dead bact. cells} = \frac{\text{intensity of Dead bact.cells}}{\text{intensity of of live bact.cells+intensity of Dead bact.cells}} \times 100$$

Secondary Outcome (Depth of Irrigant Penetration in the form of the Maximum depth of the Dead Bacterial Cells):

The maximum depth of penetration of the endodontic irrigant was measured in the dentinal tubules of (coronal, middle, apical thirds) is clued by the percentage of the dead bacteria in specific points along the entire length of the dentinal tubules using the depth measuring tools in the software of the microscope (Image browser version 4.2.0.121) (figure 18)

The Null Hypothesis:

The null hypothesis of this study was adopted the idea that there was no difference in the effect of the three irrigant activation systems: Fanta AF Max file, XP Endo finisher file ultrasonic activation with Irri-safe ultrasonic tip on Enterococcus faecalis eradication and the maximum depth of the irrigant penetration into the dentinal tubules of long oval canals in single rooted teeth.

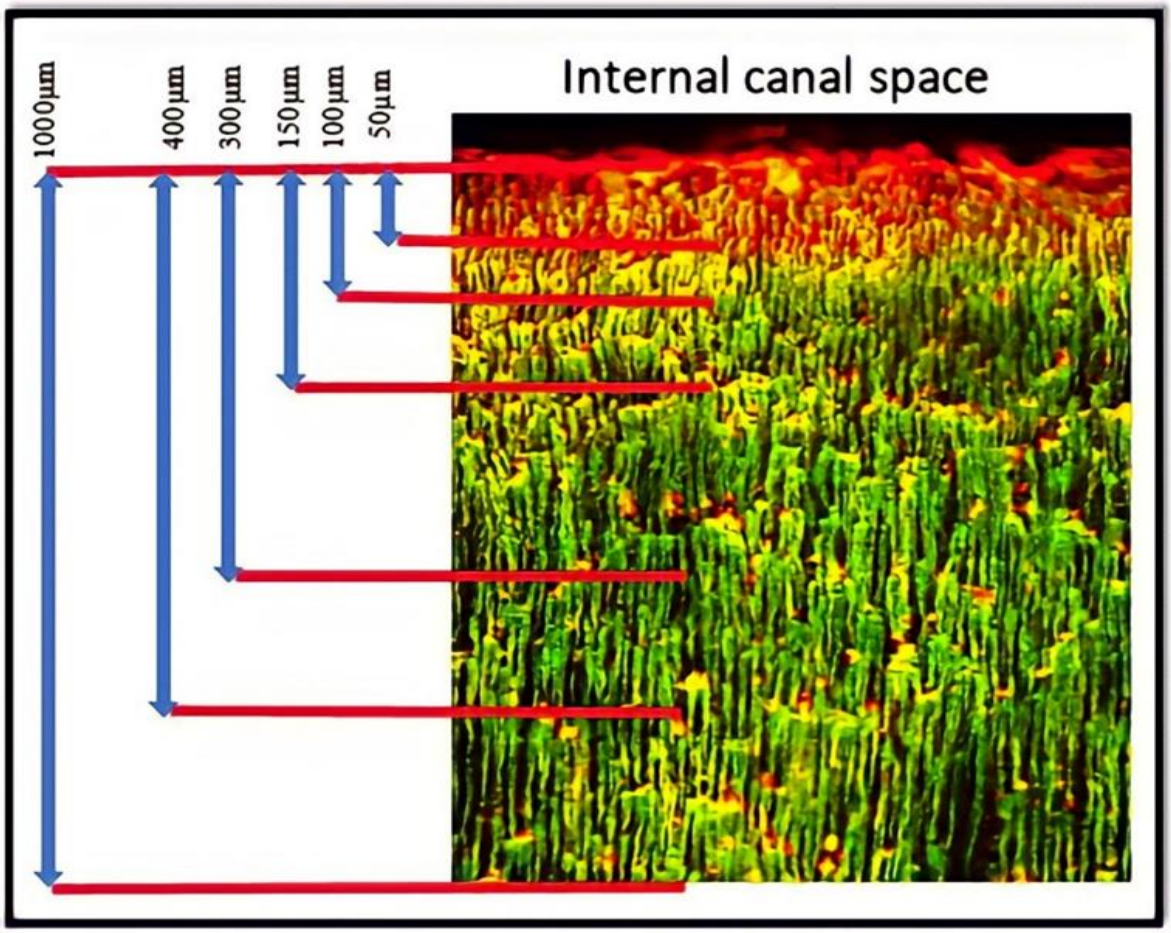

Fig. (17): A CLSM image showing the intended measuring depths.

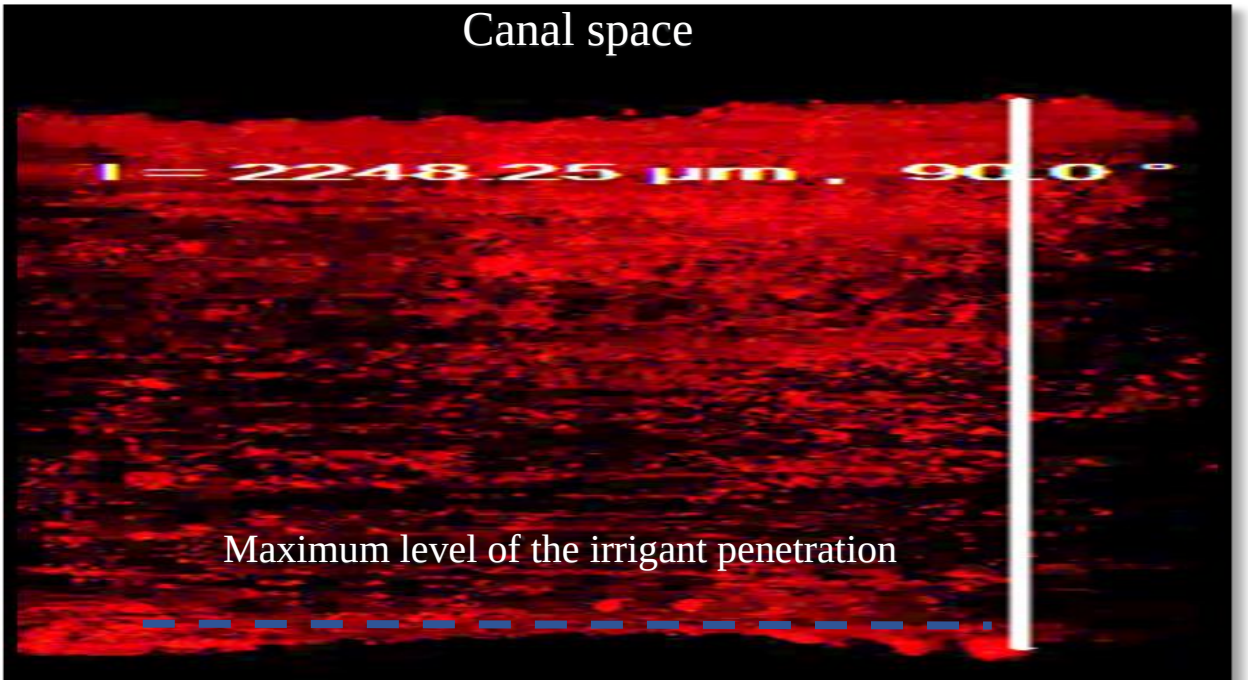

Fig. (18): A CLSM image showing the measurements with the intended measuring tools in the software of the microscope.

7. Statistical Analysis:

Numerical data were explored for normality by Kolmogorov-Smirnov and Shapiro-Wilk tests. All data showed normal (parametric) distribution. Parametric data were presented as mean and standard deviation (SD). Repeated measures Analysis of Variance (ANOVA) was used to compare between the systems, root levels as well as the depths. Bonferroni's post-hoc test was used for pair-wise comparisons when ANOVA test is significant. The significance level was set at ($P \leq 0.05$). Statistical analysis was performed with IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.

RESULTS

Primary outcome:

Bacterial eradication in the dentinal tubules

a. Comparsion of the overall intratubular percentages of dead bacterial cells among the three tested irrigation activation groups irrespective to the intracanal location or the intratubular depth (Table 3, Figure 19):

XP endo finisher group recorded the highest overall values of intratubular bacterial reduction with a mean value and SD of 56.8±2.5, followed by the (Irrisafe ultrasonic tip) group with a mean value and SD of 50.8±1. (Fanta AF Max) group recorded the least values with a mean value and SD of 49.9±2.1.

Pair wise comparsion revealed no statistical significant difference between Fanta AF Max and Irrisafe ultrasonic tip groups, both showed significant lower mean percentage of dead bacteria than XP endo finisher.

Table (3): The mean, standard deviation (SD) values and the results of the repeated measures ANOVA test for the comparison between the percentages of dead bacteria (%) following using the three different irrigant activation systems regardless of the root level and depth.

Fanta AF Max		XP endo finisher		Irri-safe ultrasonic		P-value
Mean	SD	Mean	SD	Mean	SD	<0.001*
49.9 ^{B}	±2.1	56.8 ^{A}	±2.5	50.8 ^{B}	±1	

***: Significant at $P \leq 0.05$, Different superscripts are statistically significantly different**

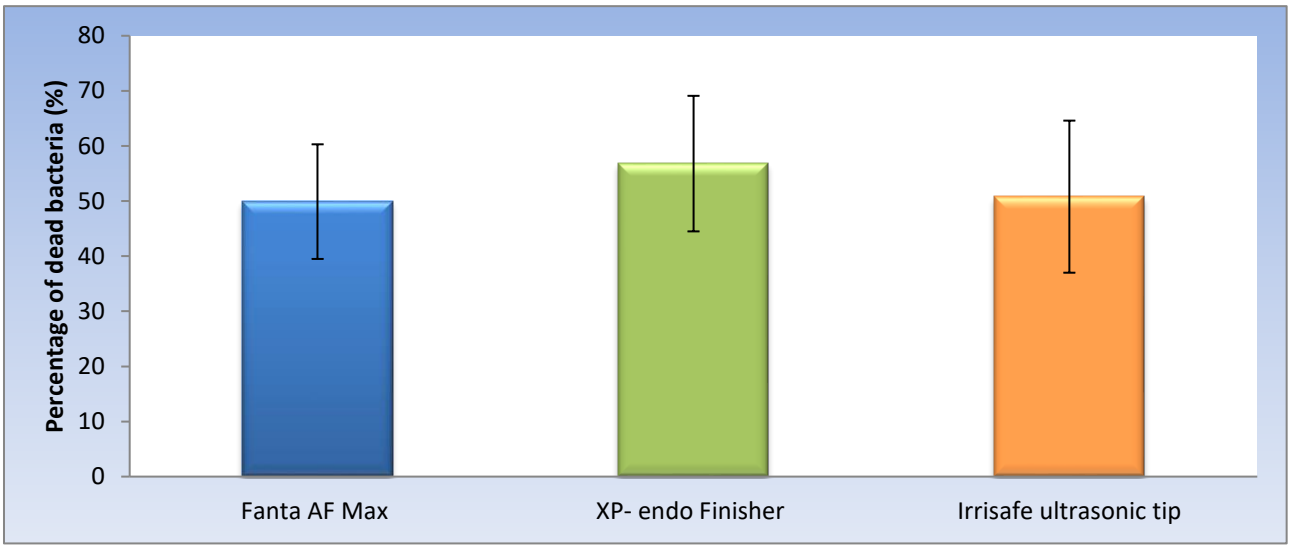

Fig. (19): Bar chart representing the mean and standard deviation values for the percentages of dead bacteria after using the three different irrigant activation systems regardless of the root level and depth.

b. Comparison the percentage of dead bacterial cells between the different root levels for each irrigant activation system (Table 4, Figure 20):

1. Fanta AF Max

The results of the percentage of dead bacteria of Fanta AF Max group at different root level revealed the highest value in the coronal third with a mean value and SD of 51.75±10.55 followed by the middle third with a mean value and SD of 50.47±10.85, while the least value was recorded in the apical third with a mean value and SD of 47.48±9.35.

2. XP-endo Finisher

The results of the percentage of dead bacteria of XP endo finisher group at different root levels revealed the highest value found in the coronal third with a mean value and SD of 59.8±14.35 followed by the middle third with a mean value and SD of 55.15±14.36 while the least value was n the apical third with a mean value and SD of 53.6±11.96.

3. Irri-safe ultrasonic tip

The results of the percentage of dead bacteria of Irrisafe ultrasonic tip group at different root levels revealed the highest value in the middle third with a mean value and SD of 50.7±9.71 followed by the coronal third with a mean value and SD of 51.53±8.05, while the least value was recorded in the apical third with a mean value and SD of 50.3±17.20.

All three groups showed significant difference between the coronal, middle and the apical thirds ($p<0.05$). Pair wise comparison revealed no statistical significant difference between the coronal and middle thirds both showed a signoificant higher values of dead bacteria than the apical third.

c. Comparsion of percentage of dead bacterial cells among different root levels in each irrigant activation systems (Table 4, Figure 21)

There was a statistical significant difference in the percentage of dead bacterial cells among the three root levels in all the irrigant activation systems where P-value for Fanta AF Max group is 0.014, XP endo finisher is 0.032 and Irrisafe ultrasonic tip is 0.05.

Pair wise comparsion revealed no statistical

significant difference between Fanta AF Max and Irrisafe ultrasonic tip groups, both showed significant lower mean percentage of dead bacteria than XP endo finisher.

Table (4): The mean, standard deviation (SD) values and the results of repeated measures ANOVA test for the comparison between the percentages of dead bacteria (%) at different root levels within each irrigant activation system

Irrigant activation system	Coronal		Middle		Apical		P-value
	Mean	SD	Mean	SD	Mean	SD	
Fanta AF Max	51.7	± 10.5	50.47	±10.89	47.48	±9.35	0.014*
XP-endo Finisher	59.8	±14.3	55.15	±14.36	53.6	±11.96	0.030*
Irri-safe ultrasonic tip	50.7	±9.71	51.53	±8.05	50.13	±17.20	0.040*
P-value	0.014*		0.05*		0.032*		

*: Significant at $P \leq 0.05$

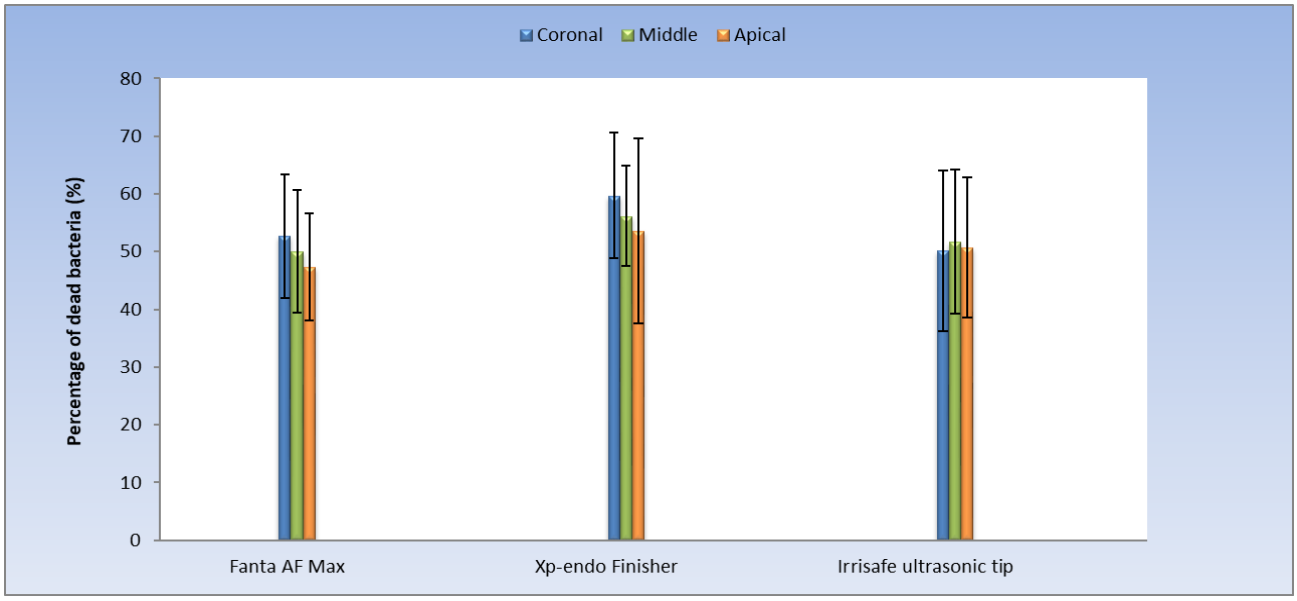

Fig. (20): Bar chart representing the mean and standard deviation values for the percentages of dead bacteria at the different root levels within each irrigant activation system group.

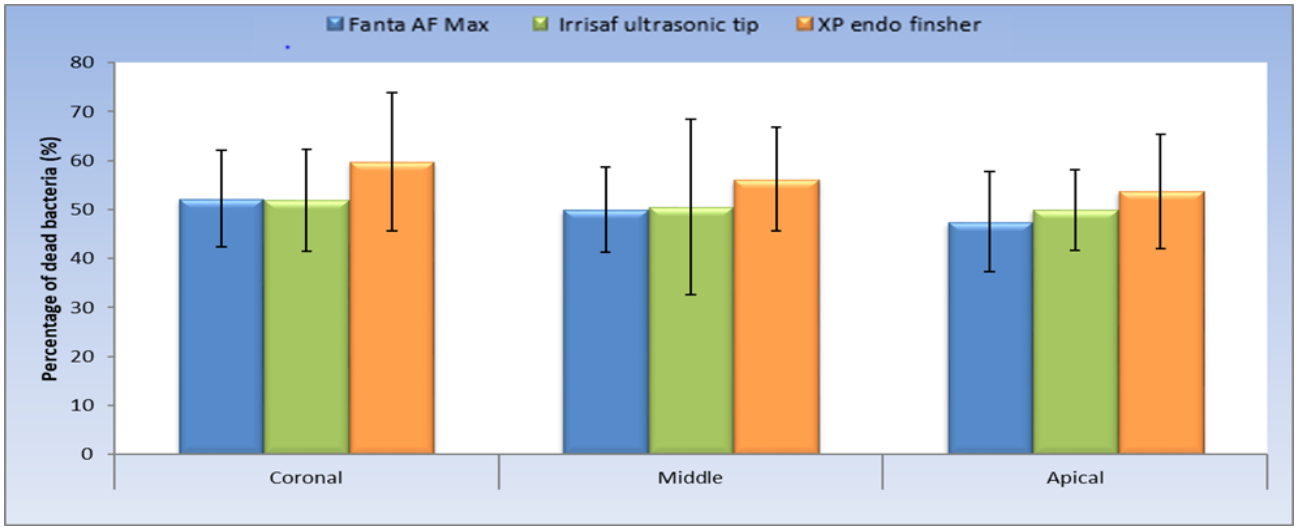

Fig. (21): Bar chart representing the mean and standard deviation values for the percentages of dead bacteria at the different irrigant activation systems within each root level.

d. Overall effect of the depth on the percentages of dead bacterial cells inside the dentinal tubules irrespective of the irrigant activation method and the root level (Table 5, Figure 22):

There was a statistically significant difference between the mean percentages of dead bacteria at all scanned depths (P-value = 0.0135) where the highest value was found at 50µ depth with a mean and SD of 59.8 ±4, 100 µ depth with a mean and SD of 52 ±4.1, 150µ depth with a mean and SD of 47.2 ±3.8, 300µ depth with a mean and SD of 42.3 ±3.6, 400µ depth with a mean and SD of 38.4 ±3.9 while the least was found at 1000µ depth with a mean and SD of 32.2 ±3.3.

Table (5): The mean, standard deviation (SD) values and the results of the repeated measures ANOVA test for the comparison between the percentages of dead bacteria (%) at different depths regardless of the irrigant activation method and the root level

Depth	Mean	SD	P-value
50 µ	59.8^{A}	±4	0.0135*
100 µ	52^{B}	±4.1	
150 µ	47.2^{C}	±3.8	
300 µ	42.3^{D}	±3.6	
400 µ	38.4^{E}	±3.9	
1000 µ	32.2^{F}	±3.3	

*: Significant at $P \leq 0.05$ Different superscripts are statistically significantly different

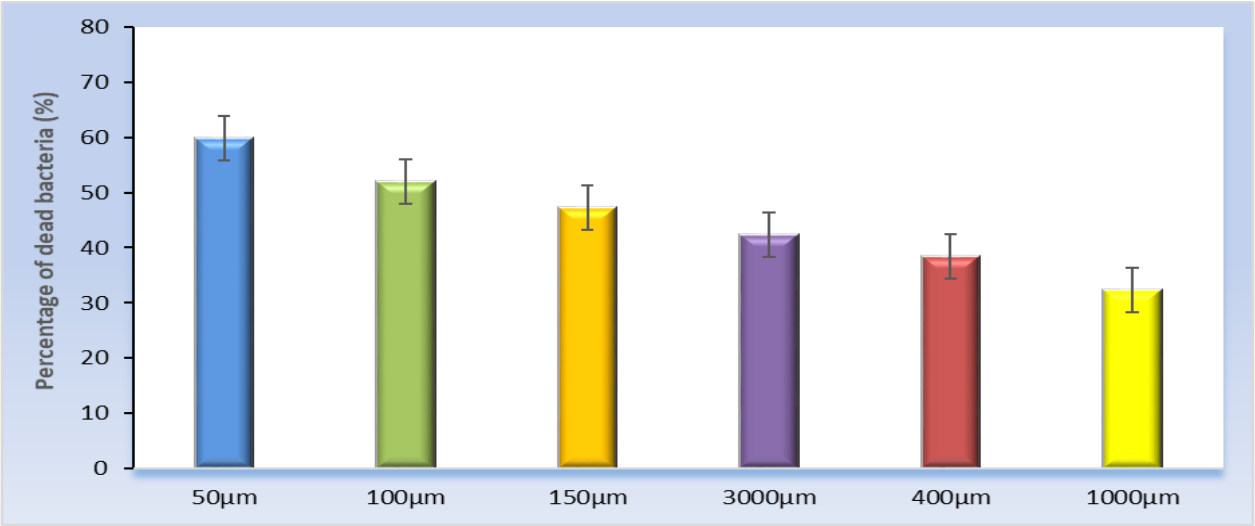

Fig. (22): Bar chart representing the mean and standard deviation values for the percentages of dead bacteria at different depths

e. Comparison of the percentages of dead bacteria amoung the three irrigant activation systems within different root levels at each tested intratubular depth (Table 6, Figure 22):

1. At coronal third

There was a statistical significant difference between the mean percentage of dead bacteria among the three irrigant activation systems at all the investigated intratubular depths where the highest value was recorded with the XP endo finisher group followed by the Irrisafe ultrasonic tip, while the least value was recorded with Fanta AF Max group.

2. At the middle third

There was a statistical significant difference between the mean percentage of dead bacteria among the three irrigant activation systems at all the investigated intratubular depths where the highest value was recorded with the XP endo finisher group followed by the Irrisafe ultrasonic tip, while the least value was recorded with Fanta AF Max group.

3. At the apical third:

There was a statistical significant difference between the mean percentage of dead bacteria among the three irrigant activation systems at all the investigated intratubular depths where the highest value was recorded with the XP endo finisher group followed by the Irrisafe ultrasonic tip, while the least value was recorded with Fanta AF Max group.

Table (6): The mean, standard deviation (SD) values and the results of the repeated measures ANOVA test for the comparison between the percentages of dead bacteria (%) after using different irrigant activation systems at each root level and each depth

Root level	Depth	Fanta AF Max		XP-endo Finisher		Irri-safe ultrasonic		P-value
		Mean	SD	Mean	SD	Mean	SD	

Coronal	50µ	52.7 ^{A}	±9.9	65.2 ^{B}	±14.1	54.2 ^{C}	±10.4	0.049*
	100µ	49.1 ^{A}	±12.6	63.4 ^{B}	±13.3	51.4 ^{C}	±8.3	0.040*
	150µ	47.3 ^{A}	±10.7	60.8 ^{B}	±13.9	48.5 ^{C}	±8.7	0.015*
	300µ	43.6 ^{A}	±10.8	57.6 ^{B}	±15.8	44.4 ^{C}	±8.2	0.010*
	400µ	39 ^{A}	±11.3	54.2 ^{B}	±15.8	40.5 ^{C}	±7.8	0.033*
	1000µ	36.2 ^{A}	±11.8	48.4 ^{B}	±15.6	37.5 ^{C}	±20	0.025*
Middle	50µ	50.6 ^{A}	±9.8	63.2 ^{B}	±10.9	56.8 ^{C}	±8	0.038*
	100µ	48.3 ^{A}	±11.8	61.3^{B}	±11.6	54.6 ^{C}	±10.9	0.022*
	150µ	45.1 ^{A}	±10.6	58.6 ^{B}	±12.5	51.3 ^{C}	±10.9	0.022*
	300µ	42 ^{A}	±11.2	55.1 ^{B}	±12	46.2 ^{C}	±9	0.011*
	400µ	38.3 ^{A}	±12.1	52.4 ^{B}	±12.2	41.3 ^{C}	±9.9	0.041*
	1000µ	34.3 ^{A}	±12.6	46.3 ^{B}	±16.5	38 ^{C}	±8.6	0.021*
Apical	50µ	48.2 ^{A}	±8.7	55.7 ^{B}	±10.6	52.7 ^{C}	±17.9	0.039*
	100µ	45.6 ^{A}	±8.7	52.5 ^{B}	±11.6	49.1 ^{C}	±15.9	0.013*
	150µ	42.2 ^{A}	±9.3	50.3 ^{B}	±12.2	46.2 ^{C}	±16	0.042*
	300µ	39.1 ^{A}	±9.5	48.6 ^{B}	±13.6	42.1 ^{C}	±17.5	0.042*
	400µ	35.4 ^{A}	±10.2	45.2 ^{B}	±11.5	38.2 ^{C}	±18.6	0.012*
	1000µ	30.5 ^{A}	±11.1	40.8 ^{B}	±12.8	32.3 ^{C}	±8.6	0.05*

*: Significant at $P \leq 0.05$, Different superscripts are statistically significantly different

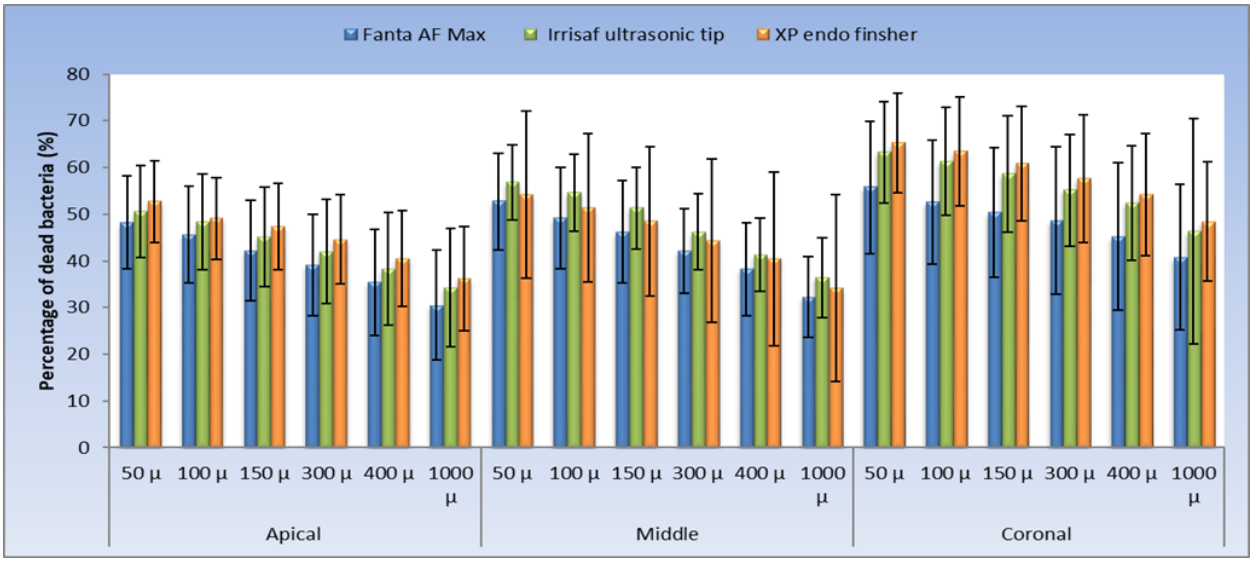

Fig. (23): Bar chart representing the mean and standard deviation values for the percentages of dead bacteria after using the three different irrigant activation systems at each root level and each dept.

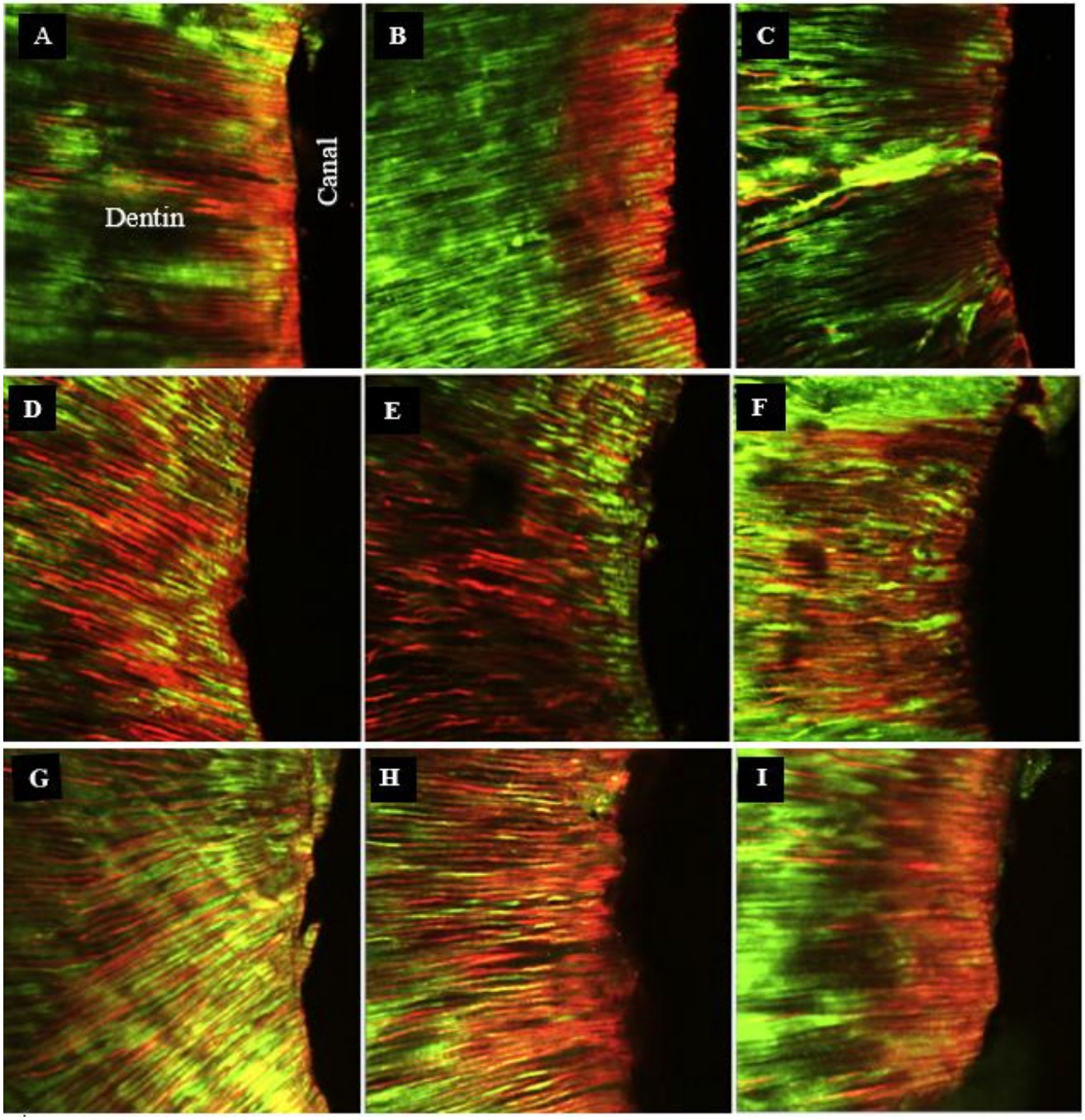

Fig. (24): CLSM images showing the dead and live bacteria of at the tested root levels (Coronal, Middle, Apical) in Fanta AF Max group (A, B, C), in Irrisafe ultrasonic group (D, E, F) and in XP endo finisher group (G, H, I) respectively.

Secondary outcome:

Depth of penetration(µm) of endodontic irrigant:

The depth of penetration of NaOCl into the dentinal tubules valued by maximum depth of dead bacterial cells observed:

a. Comparison of the overall depth of penetration of NaOCl among the three

tested irrigation activation systems groups (Table 7, Figure 24)

XP endo finisher group recorded the highest overall value of irrigant penetration with a mean and SD of 2133.9±600.8, followed by Irrisafe ultrasonic tip group with a mean value and SD of 1964.3±692 and Fanta AF Max recorded the least value with a mean and SD of 17.22.9±601.8.

There was a statistically significant difference between the mean depths of penetration of NaOCl in the three irrigant activation system groups (P-value = 0.017). Pair-wise comparisons between the three systems revealed that there was no statistically significant difference between the XP-endo Finisher and the Irri-safe ultrasonic tip; both showed a statistically significant higher mean depth of penetration than the Fanta AF Max.

Table (7): The mean, standard deviation (SD) values and the results of repeated measures ANOVA test for the comparison between the depths of penetration (µ) of NaOCl the three irrigant activation system groups regardless of the root level

Fanta AF Max		XP-endo Finisher		Irri-safe ultrasonic tip		P-value
Mean	SD	Mean	SD	Mean	SD	
1722.9 ^{B}	±601.8	2133.9 ^{A}	±600.8	1964.3 ^{A}	±692	0.017*

*: Significant at P ≤ 0.05, Different superscripts are statistically significantly different

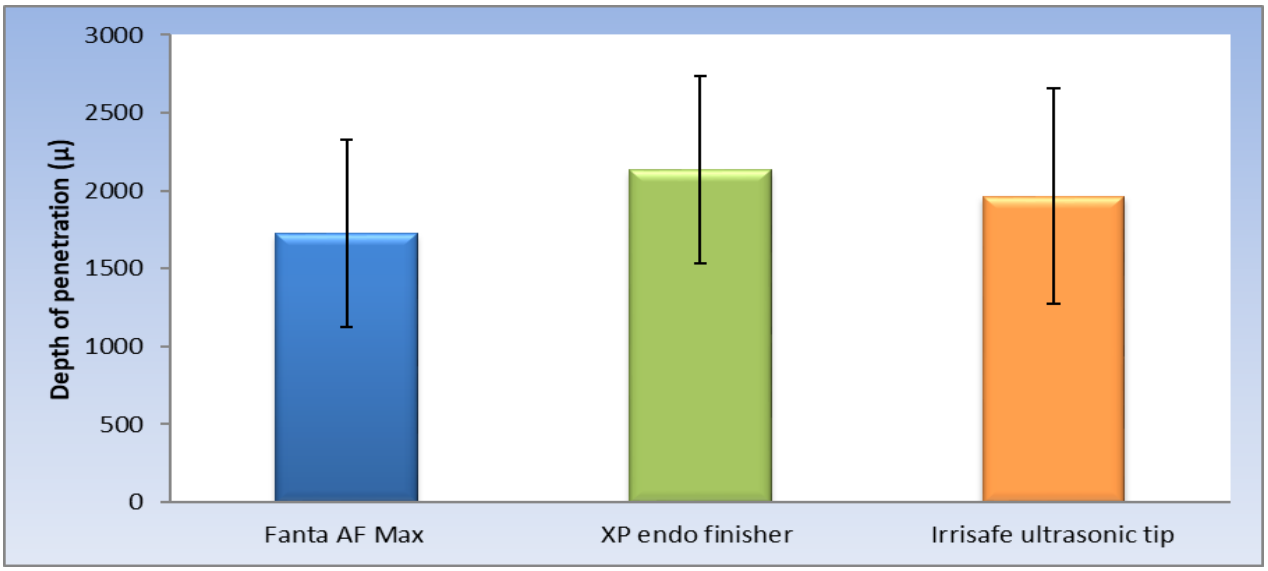

Fig. (25): Bar chart representing the mean and standard deviation values for the depth of penetration of the three irrigant activation system groups regardless of root level

b. Effect of root level on the depth of penteration of NaOCl into the dentinal tubules irrespective to the irrigant activation system used (Table 8, Figure 26):

The highest value was found in the coronal

third with a mean value and SD of 2211±554.2, followed by the middle third with a mean and SD of 2050.8±622.2 and the least value was found in the apical third with a mean and SD of 1559.4±599.

There was a statistically significant difference between the mean depths of penetration at the three different root levels (P-value <0.001). Pair-wise comparisons between the levels revealed that there was no statistically significant difference between the middle and coronal root levels; both showed statistically significantly higher mean depth of penetration of NaOCl than the apical root level.

Table (8): The mean, standard deviation (SD) values and the results of repeated measures ANOVA test for the comparison between the depths of penetration (µ) of NaOCl at the different root levels irrespective to the tested irrigant activation system

Apical		Middle		Coronal		P-value
Mean	SD	Mean	SD	Mean	SD	
1559.4 ^{B}	±599	2050.8 ^{A}	±622.2	2211 ^{A}	±554.2	<0.001*

*: Significant at P ≤ 0.05, Different superscripts are statistically significantly different

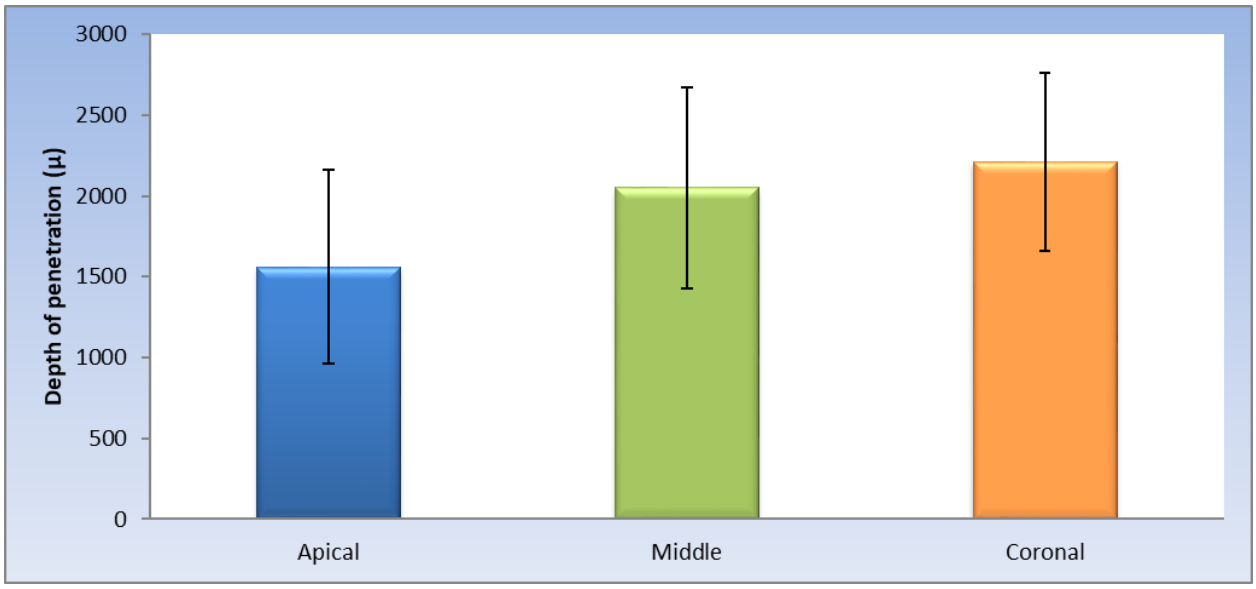

Fig. (26): Bar chart representing the mean and standard deviation values for the depth of penetration of NaOCl at different root levels irrespective to the tested irrigant activation system used

c. Comparison of the depths of penetration (µ) of NaOCl among the three tested irrigant activation system groups at each root level (Table 9, Figure 27)

At the apical root level; XP endo finisher group recorded the highest value of irrigant penetration with a mean and SD of 1949.3±635.1, followed by Fanta AF Max group with a mean value and SD of 1435.4±546.7 and the least value was recorded in

Irrisafe ultrasonic tip group with a mean and SD of 1293.4±415.9.

There was a statistically significant difference in the mean depth of penetration of NaOCl among the three irrigant activation system groups (P-value = 0.007) Pair-wise comparisons between the systems revealed that there was no statistically significant difference between the Fanta AF Max and the Irri-safe ultrasonic tip systems; both showed statistically significant lower mean depth of penetration of NaOCl than the XP-endo finisher.

At the middle and the coronal root levels; the highest value was recorded in the Irrisafe ultrasonic tip group with a mean values and SD of 2261.6± 565.2 and 2338±536.2 respectively, followed by the XP endo finisher group with a mean values and SD of 2128.8±654.36 and 2323.5±480.6 respectively and the least value found in the Fanta AF Max goup with a mean value and SD of 1762.1±573.5, 1971.4±597.7 respectively with no significant difference between the three tested groups at both levels.

d. Comparison of the depth of penetration of the irrigant among different root levels in each irrigant activation system (Table 9, Figure 28):

There was a statisticaly significant difference in the depth of irrigant penteration among the different root levels (coronal, middle, apical) in both Fanta AF Max and Irrisafe ultrasonic tip groups with (P-value 0.05,0.0023) recepectivly.

For the XP endo finisher group there was no statistical significant difference among the different root levels (coronal, middle, apical) with (P- value 0.09).

Table (9): The mean, standard deviation (SD) values and the results of the repeated measures ANOVA test for the comparison between the depths of penetration (µ) of NaOCl in the three irrigant activation system groups at each root level

Root level	Fanta AF Max		XP-endo Finisher		Irri-safe ultrasonic tip		P-value
	Mean	SD	Mean	SD	Mean	SD	
Apical	1435.4	±546.7	1949.3	±635.1	1293.4	±415.9	0.007*
Middle	1762.1	±573.5	2128.8	±654.6	2261.6	±565.2	0.086
Coronal	1971.4	±597.7	2323.5	±480.6	2338	±536.2	0.140
P- value	0.05*		0.09		0.0023*		

*: Significant at $P \leq 0.05$

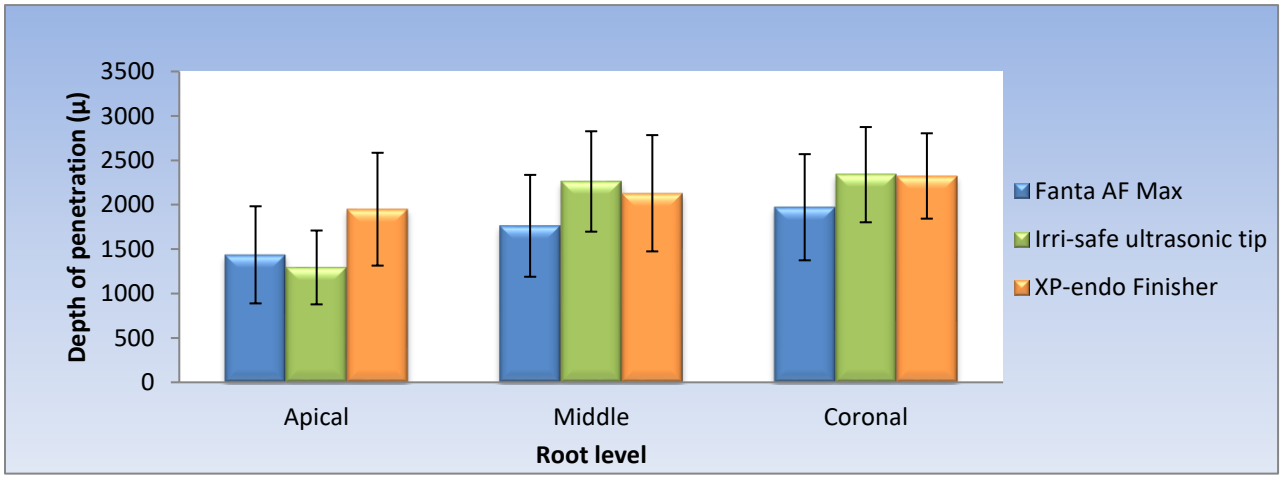

Fig. (27): Bar chart representing the mean and standard deviation values for the depths of penetration of NaOCl in the three tested irrigant activation system groups at each root level

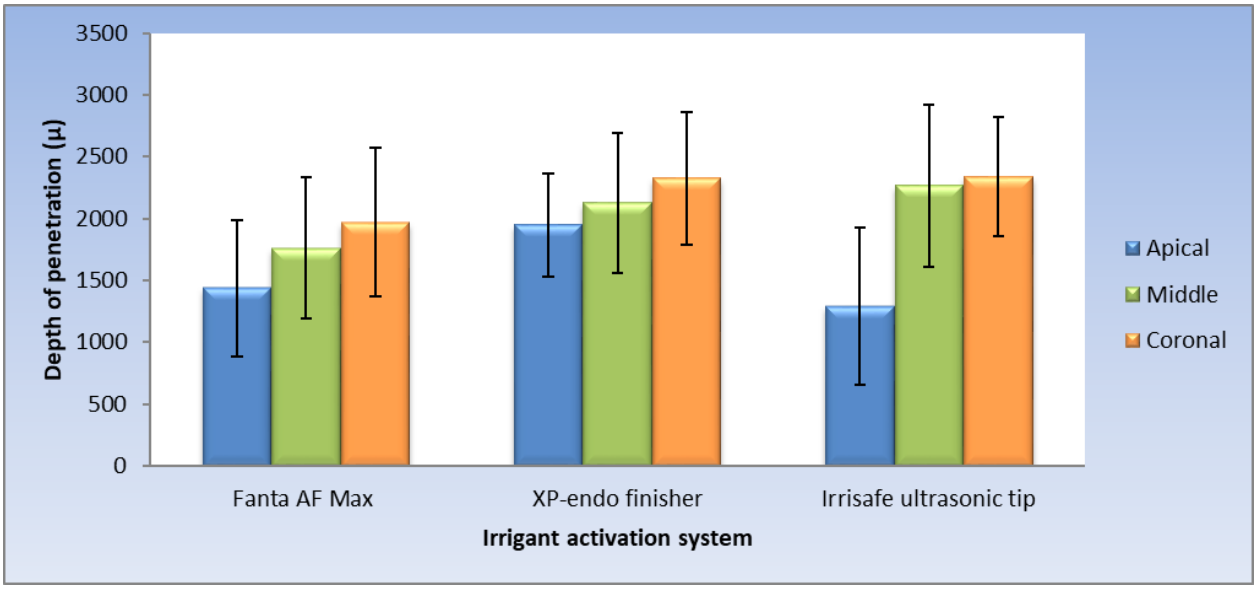

Fig. (28) Bar chart representing the mean and standard deviation values for the depths of penetration of NaOCl at the different root levels among each irrigant activation system.

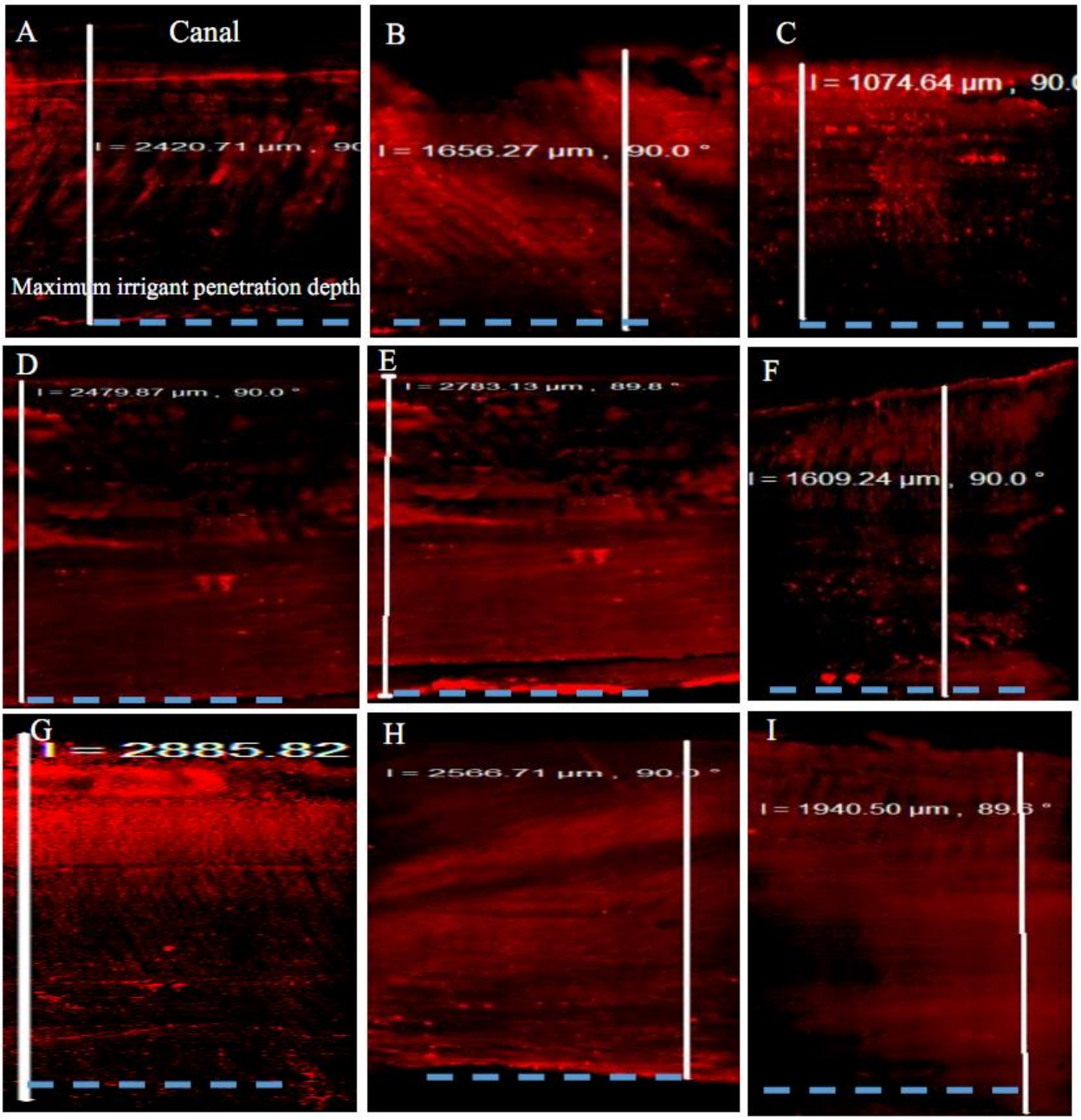

Fig. (29): CLSM images showing the depth of irrigant penetration of at the tested root levels: Coronal, Middle, Apical in Fanta AF Max group (A, B, C) , in Irrisafe ultrasonic tip group (D, E, F) and in XP endo finisher group (G, H, I) respectively. The dotted blue line representiong the maximum depth of the irrigant penetration (no dead bacteria after this line).

DISCUSSION

The main goal of endodontic treatment is to provide the ultimate disinfection of the root canal space. This principally depends on the efficient eradication of bacterial biofilm, and elimination of bacteria-loaded smear layer. E. faecalis was proved to be one of the most persistant types of bacteria causing endodontic treatment failure (Costerton et al. 2001, Socransky et al. 2002, Alves et al. 2016).

Chemomechanical preparation of the infected root canals using irrigating solutions having the ability to dissolve pulp tissues, wash away debris and eliminate microoragnisms played an important role in cleaning the canal space. Recently introduced irrigant activation systems proved to enhance irrigant penetration into dentinal tubules to a depth equivalent to that reached by E. faecalis thus eliminating it better than conventional irrigation methods (Alves et al. 2016).

XP endo finisher file and Fanta AF Max file tested in this study are two of the innovative methods for activation of irrigants and disinfection of the root canal. Irrisafe Ultrasonic tip was chosen to be the control of the study; as it is one of popular passive ultrasonic activation methods for irrigation (PUI) that has been shown to be able to enhance the antibacterial activity of sodium hypochlorite. This was done through the formation of acoustic stream for the irrigant with high velocity creating micro bubbles (cavitaions) that hit the dentin walls of the roots canal, causing disagglomeration of the biofilm, changing it to a planktonic form which is more susceptible to antibacterial agents, also it weakens the bacterial cell membrane thus increasing the permeability to antibacterial agents. In addition, it allows better penetration of the irrigant into dentinal tubules (Xhevdet et al. 2014, Mohmmed et al. 2018, Singh et al. 2019).

Thus, the aim of the present study was to assess and compare the efficacy of three irrigant activation systems: Fanta AF Max file, XP Endo finisher file on Enterococcus faecalis eradication and the maximum depth of the irrigant penetration into the dentinal tubules of long oval canals in single rooted teeth. A comparative in vitro study design was selected for this study to ensure the control of variables and uniformity of results. The study was designed to be randomized and blinded. In order to avoid selection bias, allocation concealment was done, and to avoid the detection bias, both the outcome assessor and the statistician were blinded about both the interventions and the control groups of the study (Alves et al. 2016).

The choice of single rooted teeth with single oval canal was made because of it suitability for the study design. It is well known that oval shaped canals are unlikely to be fully cleaned using rounded cross-section endodontic files, leaving some areas of the main canal untouched after instrumentation. Researchers have shown that these untouched areas can reach up to 35% of the total area of the canal walls. The elimination of the microorganisms in these areas is fully dependent on the efficacy of the irrigating solution. Accordingly, the role of irrigation along with the irrigant activation is of utmost importance in the disinfection of these root canals. (Dutertre et al. 1990, Sjögren et al. 1997, Palareti et al. 2016). to a depth equivalent to that of the E. faecalis thus eliminating it better than conventional irrigation methods (Alves et al. 2016).

All the teeth were decoronated to a standardized length of 15mm to reduce the variability between the samples, even though this is considered as a technical limitation as there is

no coronal depot for the irrigation solution. (Neuhaus et al. 2016).

The teeth were instrumented and prepared to receive the bacterial biofilm using Hyflex rotary system, a well-shaped and uniformly tapered canal is necessary to act as an adequate reservoir of irrigant. Hyflex was used owing to its manufacturing technology (electrical discharge machining), the alloy of construction (CM wire) and its unique cross section, which allowed achieving a fully tapered canal with fewer files. This resulted in a predictable preparation shape with greater procedural efficiency (Kaya et al. 2019).

Sodium hypochlorite was chosen for irrigation in our study as it is the most commonly used irrigant and is considered to be the gold standard endodontic irrigant. Along with its antimicrobial action, as it possesses the capability to dissolve necrotic tissue, vital pulp tissue and the organic component of the smear layer (Iqbal et al. 2012, Del Carpio-Perochena et al. 2015). It is usually used in a concentration range of 0.5-6%. The concentration of 5.25% was used in this study as it was demonstrated that the antibacterial efficacy of NaOCl and its ability to penetrate the dentinal tubules up to a depth of 50% could be enhanced by increasing its concentration. (Zou et al. 2010, Vandrangi 2016, Verma et al. 2019). Others stated that activation of NaOCl with various methods was proven to penetrate through the dentinal tubules approximately 77-300 µm without any sort of agitation based on time, volume and temperature (Joy et al. 2015), while E. faecalis can penetrate about 150 µm to half the distance of the dentine depth and can reach approximately 1000 µm (Vieira et al. 2012).

A 30-gauge needle with a tip diameter of 0.30 mm was used in this study, along with an apical preparation up to size 40 taper 4% (Hyflex), whose tip corresponds to 0.40 mm and would shape a canal diameter of 0.44 and 0.48 at 1 and 2mm from the apex, respectively (Kucklick et al. 2006). This allowed for a better reaching and free movement of the needle tip without binding at 1-2mm from the apex which in turn would provide better and deeper action of the irrigant especially at the apical third (Zhu et al. 2013, Urban et al. 2017).

Following the instrumentation protocol, 17% EDTA was used to remove the inorganic part of the smear layer resulting from the cutting procedures, thus enhancing deeper penetration of E. faecalis inside the dentinal tubules, as presence of smear layer was found to inhibit the bacterial colonization into the dentinal tubules (Drake et al. 1994, Schoop et al. 2004, Bago et al. 2013, Neelakantan et al. 2015).

Closure of the apices of the teeth with flowable composite and painting the external root surface with double layers of nail varnish was done in the study. As clinically, the tooth roots are encased in the bony socket and the periapical tissues which cause an air entrapment in the root canal and periodontal ligament hindering the irrigation penetration in apical portion of the canal (Urban et al. 2017). For this reason, the sealed root apices allow for creating a room in the canals for the irrigant solution to act as a reservoir for the irrigation activation process without leakage periapically. The double layers of the nail varnish ensure that the bacterial contamination occurred internally inside the dentinal tubules, only through the main root canal (Andrade et al. 2015).

Sterilization of the samples was done using the autoclave after the chemo-mechanical preparation, to render the canal sterile before the bacterial inoculation (Estrela et al. 2003, Mathew et al. 2014 Dai et al. 2018).

The E. faecalis strain (ATCC 29212) was used to infect the root canals; since E. faecalis was found to be the most common and

occasionally the easiest to isolate bacteria from the root canals of teeth with persistent periapical inflammation (Peciuliene et al. 2001, Portenier et al. 2003, Baumgartner et al. 2004, Rôças et al. 2004[a], Rôças et al. 2004[b]). The inherent antimicrobial resistance and the acquired adaptation to changing environment help E. faecalis to survive in the difficult environmental conditions present in endodontically treated teeth (Hancock et al. 2001, Distel et al. 2002). Moreover, E. faecalis (ATCC 29212) strain has the ability to grow in a biofilm state, which is an additional survival strategy to overcome the strict environmental changes in the root canal system. This strain possesses enhanced adhering capacity, increased virulence factors and higher resistance to antimicrobial agents, that are all characteristics of the biofilm style of growth (Hazlett et al. 1999, Love 2001, Podbielski et al, 2003, Tronstad et al. 2003, Nagayoshi et al. 2004).

Teeth were incubated aerobically for 21 days at 37°C and 100% humidity to allow biofilm growth. The 3-week incubation protocol followed was similar to the protocol utilized in many previous studies, who stated that as the incubation time for E. faecalis is prolonged, the biofilm ages, gets biomineralized and calcified resulting in more resistance to antimicrobial agents (Al Shahrani et al. 2014, Frough-Reyhani et al. 2016, Azim et al. 2016).

The contamination protocol used in this study was based on a modification to the contamination method by (Ma et al. 2011). In this protocol, the eppendrofs containing the samples with the sterile BHI were centrifuged. This was to ensure better penetration of the broth inside the dentinal tubules providing the needed nutrients to the bacteria (Andrade et al. 2015).

On regard to the force of the centrifugation of eppendrofs containing samples with infected broth, the force was gradually increased. If the highest force was used at the beginning of the centrifugation, it could cause the bacterial cells to promptly accumulate and aggregate on the dentin surface blocking the openings of the dentinal tubules and hence less bacterial cells penetrating the dentin (Ma et al. 2011).

During the 21-day period of the contamination protocol, an incubation everyday was established every day to enable the E. faecalis to have a chance to recover from any damage could happened during the centrifugation of the first and the third day (Ma et al. 2011). On the second day after 24 hours, a new sterilized BHI broth media was added with agitation to supply the bacteria with additional nutrients while recovering for further bacterial proliferation (Ma et al. 2011, Andrade et al. 2015).

Activation of Sodium hypochlorite was carried out by the three tested systems in accordance to other studies (Mancini et al. 2015, Mohmmed et al. 2018) to improve the irrigant dispersal into the root canal system, and to increase the penetration of the irrigating solution into the canal recesses and the apical portion of the canal that can't be fully reached by conventional syringe irrigation (Gu et al. 2009). Moreover, it enhances the bactericidal effect of Sodium hypochlorite irrigating solution (Huque et al. 1998, Gonçalves et al. 2011, Pawar et al. 2012, Andrabi et al. 2014, Mancini et al. 2015, Mohmmed et al. 2018), and aids in the removal of smear layer that blocks the openings of the dentinal tubules up to 40 μm depth hindering the irrigant to access the infected dentinal tubules (Singh et al. 2019).

The irrigation protocol of alternative periods of irrigation and activation was chosen in accordance to a previous study by (Mohmmed et al. 2017). It was considered a suitable design that allowed for standardization of the irrigation time and volume during both passive and active irrigation, thus allowing for the irrigation

technique to be the only variable.

Sectioning the teeth after the activation of the irrigant longitudinally into two halves was done to evaluate the effect of irrigant activation on bacterial reduction and the penetration of irrigant where proper visualization and evaluation for the dentinal surface for the whole root canal length can be done (Andrade et al. 2015, Vatkar et al. 2016, Azim et al. 2016, Mahmoufz 2020, Li et al. 2020).

Acridine orange (AO) dye was used to stain bacterial cells in the current study as it can permeate live cells, staining all nucleating cells to generate a green fluorescence. Propidium iodide (PI) can only enter and stain dead cell with poor membrane integrity generating a red fluorescence (Bogachev et al. 2018).

Testing the biofilm eradication at the apical region was considered as a must, because one of the apparently dilemmatic phenomena associated with conventional syringe irrigation is the apical air entrapment known as "vapor lock", which might preclude this region from contact or disinfection by the various irrigants (Senia et al. 1971). In order for the irrigant to penetrate and flood the apical region, a time frame of hours to days is needed (Pesse et al. 2005), which definitely considered clinically impractical. It was found that a high percentage of microorganisms resides in the apical third of the root canal system after chemo mechanical preparation (Migoun et al. 1996). This conclusion was in accordance with the fact that the high incidence of canal ramifications, accessory canals and lateral branches occurs in the apical region, rendering it a microbial threat for the ideal root canal disinfection procedure (Chow 1983). was found to be effective on both planktonic and biofilm when combined with NaOCl

On the other hand, checking the bacterial reduction in the coronal and middle regions had a strong evidence on the success of the endodontic treatment through proper disinfection for the total canal length, where the number and the diameter of the dentinal tubules are larger than that in the apical region and this may lead to more harboring bacteria with deep penetration inside the dentinal tubules. So by removing the bacterial load in these areas, better disinfection will be obtained (Chu et al. 2010, Giuseppe et al. 2015).

The selected depths for evaluation of bacterial eradication after activation of irrigant were 50, 100, 150, 300, 400, 1000 µm as each depth had a significant value in studying the effect of sodium hypochlorite on bacteria and to be able to compare the results with pervious literature, where 50 µm, 100µm and 150µm showed the direct effect of irrigant on the dentin surface as in (Azim et al. 2016, Bedier et al. 2018). A depth 300µm depth was selected to measure the effect of irrigant activation on bacteria as stated by (Sequeria et al. 2002, Saleh et al. 2004, Gurgel-Filho et al. 2007) who found that penetration of E. faecalis into dentinal tubules was seen at this depth, and 400 µm depth was chosen in the current study in accordance with Orstavik and Haapasalo 1990 who found that Enterococcus faecalis penetrated deeply in dentinal tubules, up to this depth invitro. Choosing the depth 1000 µm to evaluate the dead and live bacteria was in accordance with Vieira et al. 2012, Eyal Rosen 2020 who clamied that the bacterial biofilm could penetrate deeply to this depth.

Evaluation of the biofilm was conducted using Confocal Laser Scanning Microscope (CLSM). Since CLSM has been widely used in the study of biofilms structure, composition and metabolism. It allows for a nondestructive in depth analysis of the biofilms ecosystem at the cellular scale (Cerca et al. 2012). CLSM can provide a better visualization for the presence of bacteria in the dentinal tubules than SEM. It has been shown that it can penetrate 50 µm from the

surface of the specimen which provides images of the specimen ranging in thickness up to 1000 μm. This feature enables visualizing and generating quantitative data for the dentinal tubules that are not open to the surface (Zapata et al. 2008, Nair et al. 2017). CLSM was chosen over the more conventional method of colony forming units (CFU) counting as the sensitivity of the CFU method was deemed insufficient for detecting possible viable bacteria in lower concentration, this coupled with its inability to provide in-situ evaluation of biofilms and bacterial reduction favored the use of CLSM. Thus it provided a direct, more accurate and reproducible method for in-situ detection of live and dead bacteria inside the dentinal tubules (Mathew et al. 2014). Furthermore, colony forming units (CFU) only provides information about the existence of the bacteria and their estimated number rather than the exact cell count Also, it cannot obtain any information about the distribution and viability of the bacteria present in the examined of the dentinal tubules. In addition, it can not reveal the dead bacteria that was killed by the irrigant which diffused through the dentinal tubules (Wong & Cheung 2014).

Regarding the primary outcome, the present study compared the ability of the three different irrigation activation methods to kill the bacterial cells using the CLSM by calculating the percentage of live/dead bacteria in the infected dentinal tubules at the coronal, middle and apical thirds at different intratubular depths, as employed by Azim et al. 2016, Alves et al. 2016. The study results showed that none of the three experimental groups could result in complete elimination of E. faecalis from the dentinal tubules and demonstrated a significant higher overall percentage of bacterial reduction in the dentinal tubules when compared to the positive control group. This was in accordance with Wong and Cheung. 2013, Bao et al. 2016, Azim et al. 2016, Qiang et al. 2020, Vasudev et al. 2020 who found that none of the irrigant activation systems could totally eliminate the bacterial biofilm inside the root canals at the different root levels.

Upon comparing the overall antibacterial action of the three tested irrigant activation systems regardless the root level and the measured depths, there was a statistical significant difference between the three groups, where the XP endo finisher group showed statistically the highest percentage of dead bacteria compared to the other two groups, this was in accordance with Azim et al. 2016, Teves et al. 2019 who showed a maximum antibacterial action when using the XP endo finisher file for irrigant activation when compared to the passive ultrasonic irrigant activation.

This could be due to XP endo finisher unique flexibility and its small core size with adjusted speed that enables it to move freely and touch more canal walls which may lead to increase the antibacterial efficacy of the irrigating solution with better removal of the smear layer and bacterial biofilm from the root canal wall (Carvalho et al. 2019).

The superiority of XP endo finisher group over the other two groups in the antibacterial efficacy where it showed a significantly higher overall antibacterial activity than the Irrisafe ultrasonic tip, this is due to its special manufacturing process from a proprietary alloy with a shape-memory based design, allowing this single file with a size 25 diameter and zero taper to change from the M-phase to the A-phase within the root canal during the rotation mode thus enables the file to adapt properly to the canal shape. Its unique spoon shape, with a length of 10 mm from the tip and a depth of 1.5 mm, allows the file to expand to 6 mm in diameter, or 100-fold compared to an equivalent-sized file, permitting it to easily clean canal irregularities, and to detach biofilm (Bedier et al. 2018, Giovanna et al. 2018), and when compared with

Fanta AF Max, the XP endo finisher is better owning to its unique spoon like shape rather than the wavy shape of Fanta AF Max file, where its unique shape together with its unique metallurgy aids in better scraping of the canal walls in addition to better disruption of the intracanal biofilm which enhances its antibacterial action (Garcia et al. 2018, Giovanna et al. 2018).

The results were in accordance with Sasanakul et al. 2019 who tested the efficacy of XP-Finisher as an adjunctive approach in improving the reduction of bacterial load in the root canal system and found that the XP finisher files could improve the cleaning efficacy and bacterial reduction in the root canals. The results were also in accordance with Leoni et al. 2017 who showed that XP-endo Finisher file succeeded in reducing the level of smear layer and bacterial biofilm inside the root canal.

Although there was no statistical significant difference between the Irrisafe ultrasonic tip group and Fanta AF Max in the antibacterial action , the Irrisafe ultrasonic tip group showed slightly higher overall antibacterial activity than the Fanta AF Max. thiscould be related to the great amplitude of the passive ultrasonic tip and the acoustic streaming that is created by its motion this causes greater forces which allows the dislodgment of the bacterial biofilm (Al-Obaida et al. 2018, Nagendrababu et al. 2018). Our results were in agreement with Qiang et al. 2020 who showed that passive ultrasonic irrigation activation had a better antibacterial action compared with M3 Max file which is an instrument that is considered to be similar to the Fanta AF Max in its wavy shape, its flexibility and the small core size. The manufacturer recommends its operation with vertical motions to “scrape” the root canal walls, thereby disturbing the smear layer or biofilm.

All the tested activation irrigation method groups showed a significantly higher intratubular bacterial reduction in the coronal and middle thirds when compared with the apical third. This might be attributed to the more peritubular and sclerosed dentin that is found in the apical third with decreased number of dentinal tubules in addition to the more available space in the coronal and middle thirds of the canal due to the increased canal taper which provide enough room for more volume of the irrigant to be activated and circulated within the entire canal also the wider surface area of the coronal and middle thirds allowing free movement of the irrigant activation system. (Brunson et al. 2010, Azim et al. 2016).

Our results were in accordance with Azim et al. 2016 who demonstrated a significantly higher bacterial reduction efficacy in the dentinal tubules of both coronal and middle thirds in comparison to the apical third using XP endo finisher to activate 2.5 % NaOCl. Also it was in agreement with El Naghy et al. 2017, Sanabria-Liviac et al. 2017 who demonstrated that the canal cleanliness and smear layer removal in the apical portion of the root canal system were minor compared with the middle and coronal portions, which could be related to the small canal diameter in the apical portion which can impact the effectiveness of debris removal, the volume and exchange of irrigant at the working length.

About comparing the root levels, the results of our study showed that the XP endo finisher had the maximum bacterial death at the coronal third followed by the middle third and the least value was found at the apical third where there was a statistical significant difference between the three root levels. This could be explained by the virtue that the coronal third has wider surface area where it could allow the XP endo finisher to touch more canal walls so this would provide better antibacterial action when compared with the passive ultrasonic tip as stated by (Azim et al. 2016, Bedier et al. 2018, Carvalho et al. 2019, Teves et al. 2019).

Irrisafe ultrasonic tip group, the results of our study showed non-significant higher percentage of dead bacteria at the middle third followed by the coronal third and the least was found at the apical one. This could be explained by the virtue that the ultrasonic irrigation has been claimed to be highly effective within 3 mm from the irrigant activation tip and that the acoustic cavitation decreases markedly with increasing distance from the tip (Ahmad et al. 1988, Malki et al. 2012).

Upon comparing percentage of dead bacterial cells among different root levels in each of the tested systems, the XP endo finisher showed higher percentage values than the other two groups. Our results were in agreement with Azim et al. 2016 who showed that the maximum bacterial death following irrigant activation with XP endo finisher files when compared with passive ultrasonic irrigant activation was in the coronal third followed by the middle then the apical third. Our results were in disagreement with Sousa et al. 2018, Vasudev et al. 2020, Villalta-Briones et al. 2021 who compared the irrigant activation with the XP endo finisher file and the Irrisafe ultrasonic tip in bacterial reduction ability and found that there was no significant difference among them in removing bacteria from all the canal levels.

The comparsion between the Irrisafe ultrasonic tip and Fanta AF Max file, there was no statistical significant difference between the two groups, where the Irrisafe ultrasonic tip had a slightly higher antibacterial activity than Fanta AF Max group in the coronal, middle and the apical third. This is in agreement with Nagendrababu et al. 2018, Qiang et al. 2020 who stated that the ultrasonic tip showed significaly better antibacterial action at all the root levels when compared with other irrigant activation files such as M3 Max file, XP clean.

Regarding the percentages of dead bacteria at all the tested depth (50, 100, 150, 300, 400, 1000µm) regardless the irrigant activation systems and the root levels, there was a statistical significant difference between all the measured depths where the highest value was found at 50µm and the least value was found at 1000µm. This could be because the maximum antibacterial effect of any activated irrigant which found to be at the nearest depth from the canal lumen where the irrigant is in close contact with the canal wall. This effect decreases with increasing the intratubular depth (Ghorbanzadeh et al. 2016). In addition, it was claimed it could be attributed to the buffering effect of the dentin that causes inhibition of NaOCl when diffused further into the tubules. Also it may be due to the presence of organic materials as a component in dentin structure which may interact with the irrigant resulting in weaking the action of the irrigant on the bacterial cells (Morgental et al. 2013).

The results were in agreement with Azim et al. 2016 who found that the XP finisher groups showed highest bacterial reduction at 50µm depth and also it was with agreement with Bedier et al. 2018 who stated that XP endo finisher showed the highest bacterial reduction at (50,100,150µm) from the canal. Our results were in accordance with Azzawi et al. 2017, Bao et al. 2017, El Naghy et al. 2017, Sanabria-liviac et al. 2017, Ulusoy et al. 2018 who demonstrated that XP-Finisher file has the highest bacterial reduction at (50 µm) then dropped with increasing the depth in the dentinal tubules.

Upon stratification of the dentinal tubules, there was a statistically significant difference among the three tested groups where XP endo finisher showed significantly higher antibacterial activity than the other two groups at all the tested depths. This may due to the shape of the XP endo finisher that can change according to the surrounding temperature as when it is exposed to the body temperature, it changes into a spoon shape which allows it to expand during rotation.

This unique property promoted the agitation of the irrigating solution inside the canal system which could allow for the disruption and removal of the smear layer debris and bacterial biofilm (Leoni et al. 2017).

Comparing the bacterial reduction ability of the Irrisafe ultrasonic tip group with the Fanta AF Max group at different depths of the root canal levels higher values were found in the Irrisafe group at middle and apical thirds at (50,100,150,300,400,1000µm) depth. These results were found to be in accordance with Qiang et al. 2020 who showed that passive ultrasonic is better than M3 Max file. This could be attributed to the acoustic streaming the irrigant generated around the vibrating tip, forming microscopic bubbles in the irrigant which are both associated with the improvement of cleaning efficiency.

For the secondary outcome, the depth of penetration of NaOCl was evaluated through the extension of its antibacterial efficacy by observing and tracing the maximum depth limit of the dead bacterial cells in the dentinal tubules with CLSM. The comparisons between the three irrigant activation systems revealed that there was no statistically significant difference between XP-endo Finisher and Irrisafe ultrasonic tip; both showed a statistically significant higher mean depth of penetration than Fanta AF Max.

It was claimed that the ability of the XP endo finisher to enhance the penetration capacity of the irrigating solution deeper into the dentinal tubules was through the removal of the smear layer and tissue debris from the root canal wall (Carvalho et al. 2019) and the ability of Irrisafe group to increase the depth of irrigant penetration in relation to the Fanta AF Max file, this could be through its vibratory motion which increases the velocity of the irrigant and its penetration depth inside the dentinal tubules

Our results were in accordance with Qiang et al. 2020, who stated that the passive ultrasonic irrigant activation is better than the Fanta AF Max file in the irrigant penetration depth but in disagreement with Lauritano et al. 2019, Abdelghany et al. 2020 who stated that the XP endo finisher showed signifcantly higher irrigant penetration inside the dentinal tubules in comparison to the passive ultrasonic irrigant activation.

In the present study the coronal and the middle thirds both showed higher depth of penetration for the irrigant than the apical third. These results could be attributed to the fact that the diameter of the dentinal tubules at the coronal and middle thirds are wider in diameter and are more patent with less ramification than the apical third thus deeper penetration of the irrigant as stated by Morgental et al. 2013.

These results were in agreement with Rajakumaran and Ganesh. 2019 who stated that in the coronal and middle thirds of root canal, dentinal tubules are larger and densely packed while they are narrower in the apical third of root canal contributing in better irrigant penetration in the coronal and middle thirds.

Unlike Fanta AF Max and Irrisafe ultrasonic tip, XP endo finisher was capable of pushing the irrigant into the dentinal tubules at all the root levels with no significant difference with a statistically deeper irrigant penetration through the apical third when compared to the other two systems, This could be explained by the unique alloy of the XP endo finisher which can expand inside the canals enabling it to hits the full length of the canal walls together with its motion making the irrigant easily penetrating inside the tubules at all the root levels. This may also explain the superior antibacterial effect of XP endo finisher system than the other studied systems.

The results were in accordance with Bedier et al. 2018, Carvalho et al. 2019, Teves et al. 2019

who stated that XP endo finisher files showed no significant difference in penetration depth of the irrigant inside the dentinal tubules at all the root levels.

Regarding the coronal and middle, there was no a statistical significant difference in the depth of irrigant penetration among the three tested groups. Our results were in accordance with Zou et al. 2010 who found that the extent and depth of irrigant penetration using passive ultrasonic activation were significantly less in the apical third of the canal than in coronal and middle parts of the root canal.

Our results were in disagreement with Ghorbanzadeh et al. 2016, Rajakumaran and Ganesh. 2019 who stated that passive ultrasonic irrigant activation was less effective than XP endo finisher in increasing the depth of irrigant penetration inside the dentinal tubules at the middle and the coronal thirds although not significant, Irrisafe ultrasonic tip showed slightly better irrigant penetration depth in the middle and coronal thirds than XP endo finisher and Fanta AF Max. this might be attributed to the fact that the maximum activation of irrigant is found of a few millimeters beyond the tip and it decreases with increasing the canal length Ghorbanzadeh et al. 2016, Qiang et al. 2020.

The difference between our results and these study results could be due to the difference in the irrigant activation methods such as the PUI, laser and conventional needle irrigation and manual agitation by master cone.

Comparing the results of the tested irrigation activation methods in this study; Fanta AF Max and XP-Endo Finisher as the two interventions and Irrisafe ultrasonic tip as a comparator, showed a strong evidence against the null hypothesis ($P < 0.001$). Thus, the null hypothesis was rejected.

CONCLUSION

Within the limitation of this study it could be concluded that:

- Activation of the irrigant is a must to disrupt the biofilm and penetrate dentinal tubules to the depth invaded by Enterococcus faecalis.
- All the three types of irrigant activation methods couldn't completely eliminate the intracanal bacterial biofilm.
- XP endo finisher efficiently reduced bacterial count and increased irrigant penetration depth at the apical third.
- The intratubular depth and the root level position inside the canal may affect the bacterial eradication ability of the different irrigant activation systems.

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