

## Evaluation of Antimicrobial Effects of Three Antimicrobial Agents Against Pathogenic Root Canal Microbe *Enterococcus Faecalis*: An in Vitro Study

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### Abstract

Triple antibiotic paste (TAP) consisting of a mixture of 3 antibiotics (3-Mix) containing metronidazole, ciprofloxacin and minocycline is widely used in endodontics for root canal disinfection, particularly in regenerative procedures and for performing LSTR (Lesion Sterilization and Tissue Repair). According to numerous research, *Enterococcus faecalis* causes significantly persistent root canal infections and frequently demonstrates antibiotic tolerance. The present study aimed to evaluate and compare the antimicrobial activity of amoxicillin, ciprofloxacin and metronidazole individually and in dual and triple combinations against a specific pathogenic root canal bacteria namely *E. faecalis* (ATCC 29212) using broth macro dilution method by determining MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) value. Results showed that in terms of MIC value, amoxicillin has lowest value among the three antimicrobials against *E. faecalis* and there was no inhibitory effect with metronidazole. In present study it is found that the combination of 10 µg/ml amoxicillin and 5 µg/ml ciprofloxacin is the most effective for elimination of *E. faecalis* (99.98% killing capacity). The current study can be further improvised by performing an in-situ study or performing it on bacterial biofilm model.

### 1. Introduction

Treatment of immature necrotic teeth is clinically challenging. Treatment options include apexification procedures using calcium hydroxide  $\text{Ca}(\text{OH})_2$  to

induce formation of a calcific barrier at the apex or mineral trioxide aggregate plug (MTA). Both procedures had clinical success, but as they prevent further root development, the tooth becomes vulnerable to fracture. Following a successful

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disinfection of the root canal, regenerative endodontic treatment (RET) was introduced as an alternative therapeutic option that permits continuing root development and apical closure. For the proper disinfection of root canal, in 1988 the cariology research unit at the Nigata University School of Dentistry developed a concept known as Lesion Sterilization and Tissue Repair (LSTR) (also called NIET or Non-Instrumental Endodontic Treatment) [7]. It is a "new biologic method for the treatment of carious lesions with periapical involvement employing a mixture of 3 antibiotics (3-Mix)". The composition of (3-Mix) was Metronidazole, Ciprofloxacin and Minocycline. Since then, several studies are done but none of them has suggested the exact antimicrobial drug dosage [1,2,3].

Several studies based on culture and molecular biology have revealed that the most prevalent species in teeth that have undergone root canal therapy is *E. faecalis*, with prevalence rates up to 90% of cases [4]. Compared to primary infections, root canal-treated teeth are nearly nine times more likely to harbor *E. faecalis* [5]. Among all primary endodontic infection cases, *E. faecalis* was more likely to be associated with asymptomatic cases than with symptomatic ones and it may cause secondary infections that later become persistent [6].

Modified Triple Antibiotic Paste (MTAP) using a combination of 3 antimicrobial drugs namely Metronidazole, Ciprofloxacin and Amoxicillin have been used in the present study against *E. faecalis* (a facultative anaerobic gram-positive coccus). Metronidazole is effective against protozoa, gram positive and gram negative anaerobic organisms. Ciprofloxacin is effective against both gram negative and gram positive microorganisms (mainly aerobic). Amoxicillin is a beta-lactam antibiotic which has broad spectrum of activity. It is active against gram-positive cocci, some gram-negative organisms, gram-positive anaerobic organisms, and gram-negative anaerobic organisms.

When other tests produce conflicting results, the gold standard procedure in diagnostic laboratories is the MIC, which can confirm unusual resistance and provide a conclusive answer. It can be determined by-Broth dilution tests, Disk diffusion test. Re-culturing (subculturing) broth dilutions that inhibit bacterial

growth (i.e., those at or above the MIC), yields the minimum bactericidal concentration (MBC).

The present study would aid in determining the drug concentration of three antimicrobials namely Amoxicillin, Ciprofloxacin, Metronidazole individually and in dual and triple combinations to be used as an intracanal medicament for LSTR (Lesion Sterilization and Tissue Repair) required for the success of regenerative endodontic treatment of nonvital pulpally involved tooth.

## 2. Materials and Methods

**Collection of the study sample:** Freeze dried specimens of *Enterococcus faecalis* (ATCC 29212) were procured from Himedia Laboratories. All the three antimicrobials were procured from Gluconate Health LTD.

**Preparation of stock solutions of antibiotics:** 8000 µg/ml stock solutions in sterile distilled water were prepared for each antibiotic. Required concentrations were prepared from this stock solution by using dilution technique in a labelled eppendorf tubes.

**Distribution of study sample:** Individual antibiotic and their different combinations are classified as following: **Group A:** Metronidazole, **Group B:** Ciprofloxacin, **Group C:** Amoxicillin, **Group AC:** Metronidazole + Amoxicillin, **Group BC:** Ciprofloxacin+ Amoxicillin, **Group AB:** Metronidazole + Ciprofloxacin, **Group ABC:** Metronidazole + Ciprofloxacin+ Amoxicillin

**Study technique:** BHI broth media was prepared in a flask, blocked with a cotton plug and autoclaved. In the presence of an active flame, the cotton plug was removed from the mouth of the flask and one loopful of the freeze-dried *Enterococcus faecalis* (ATCC 29212) was taken in an inoculation loop which was sterilized by passing it over red heat and inoculated into the culture media. The mouth of the flask was then secured using a cotton plug, placed in an incubator (Unicorn Scientific Services) at 37°C and the culture media was allowed to stand for 48 hours. 9.7 ml of sterile BHI broth is transferred to the labeled test tube. 0.1 ml volume of antibiotic solutions was transferred at working dilution to test samples (except positive, negative culture and blank). 0.2 ml aliquots of test organism containing  $2.1 \times 10^5$  CFU/ml bacteria was added to the test tubes (except negative

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culture and blank). Only 10ml of BHI broth media was added to blank. All of them were incubated at 35°-37°C for 24 h, then MIC was determined which is defined as the lowest concentration of drug that will inhibit the visible growth of an organism after overnight incubation.

For MBC determination, the MIC dilution along with at least two of the more concentrated test product dilutions are plated using sterile swab sticks to obtain a lawn culture of the bacteria and enumerated to determine viable CFU/ml.

**Disposal of used instrument/material:** All instruments which had been used for the microbiological study were autoclaved and the waste (discarded cultures and stock solutions) was then discarded in properly labeled yellow disposal bags whereas used gloves, towels etc were discarded in labeled red disposal bags.

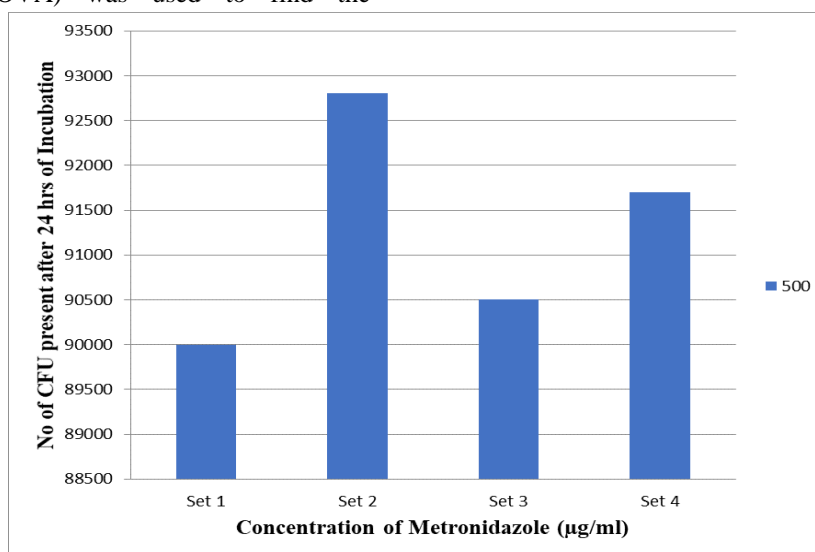
**Recording, storage and analysis of data:** Analysis of variance (ANOVA) was used to find the

significance of study parameters within and between the groups (Intra & Inter group analysis) ( $p=0.05$ ). Post-Hoc Tukey test were used to compare the difference in antimicrobial efficacy between all groups ( $p<0.001$ ).

The Statistical software IBM SPSS statistics 20.0 (IBM Corporation, Armonk, NY, USA) was used for the analyses of the data and Microsoft word and Excel were used to generate graphs, tables etc.

### 3. Results and Discussion:

Metronidazole (Group A) showed very less amount of bacterial growth inhibition after 24 hrs of incubation [Graph 1]. In a study, conducted by T. Alam *et al.* in 2005 revealed similar result as our study where Metronidazole did not have any inhibitory effect on any of the eight enterococcal isolates tested [8]. In a study, conducted by Triveni M Nalawade *et al.* (in 2016) found that the MIC value of Metronidazole against *E. faecalis* was 500µg/ml [9].

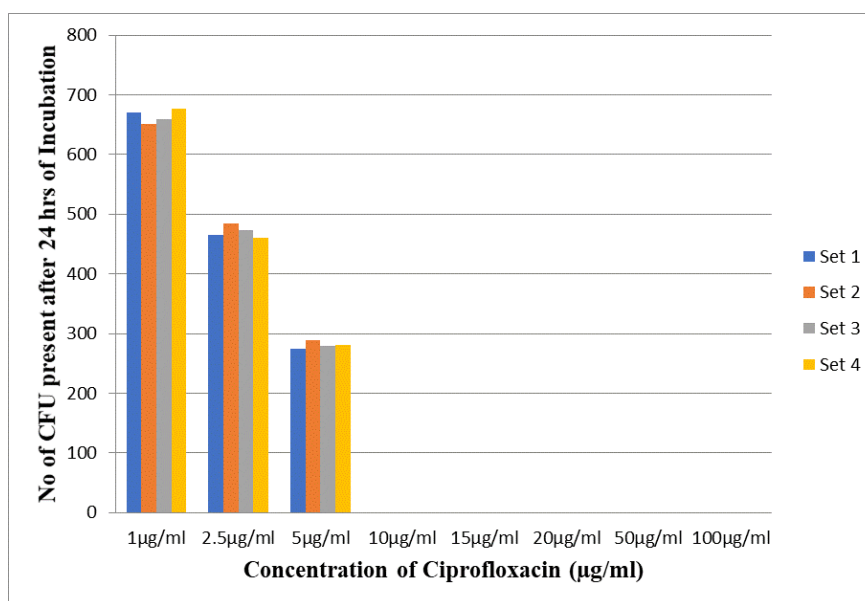


**Graph 1:** Comparison of antibacterial efficacy of different concentrations of metronidazole (Group A) against *E. faecalis*

The lowest concentration of Ciprofloxacin (Group B) that shows the inhibitory effect on *E. faecalis* i.e., MIC value is 5µg/ml [Graph 2] and MBC value is 10µg/ml. In a study, conducted by T. Alam *et al.* in 2005 found similar result as our study where the MIC value of Ciprofloxacin against seven isolates of *E. faecalis* was 5µg/ml [8]. In another study, conducted by Triveni M Nalawade *et al.* in 2016 revealed that

the MIC value of Ciprofloxacin against *E. faecalis* was 1.95µg/ml [9]. In another study, Barbosa-Ribeiro *et al.* in 2016 found that the MIC value of Ciprofloxacin against *Enterococcus faecalis* is 1.83 µg/ml [10]. Pinheiro *et al.* reported that the MIC value of Ciprofloxacin against *E. faecalis* isolates from canals of root filled teeth with periapical lesions ranges from 0.38-2.0 µg/ml [11].

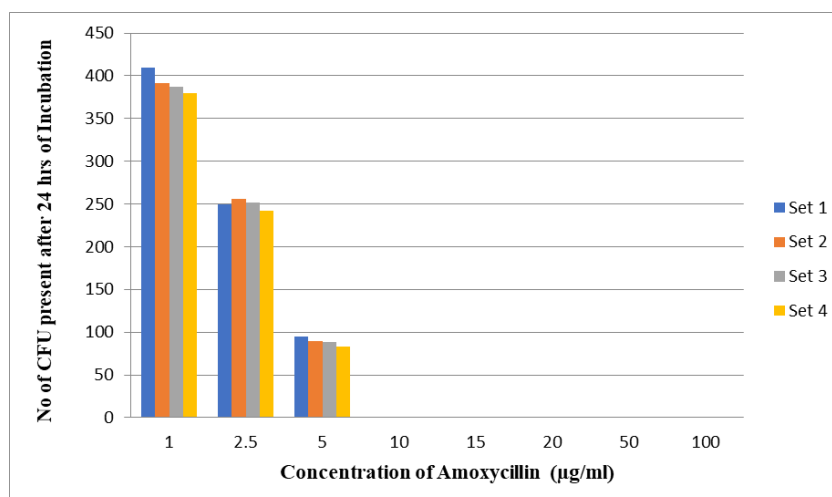




**Graph 2:** Comparison of anti bacterial efficacy of different concentrations of ciprofloxacin (Group B) against *E. faecalis*

In the present study, the lowest concentration of Amoxicillin (Group C) that shows the inhibitory effect on *E. faecalis* i.e. MIC value is 2.5µg/ml [Graph 3], MBC value is 10µg/ml. In a study, conducted by Triveni M Nalawade *et al.* in 2016 found the MIC value of Amoxicillin against *E. faecalis* was 15.625µg/ml [9]. In another study,

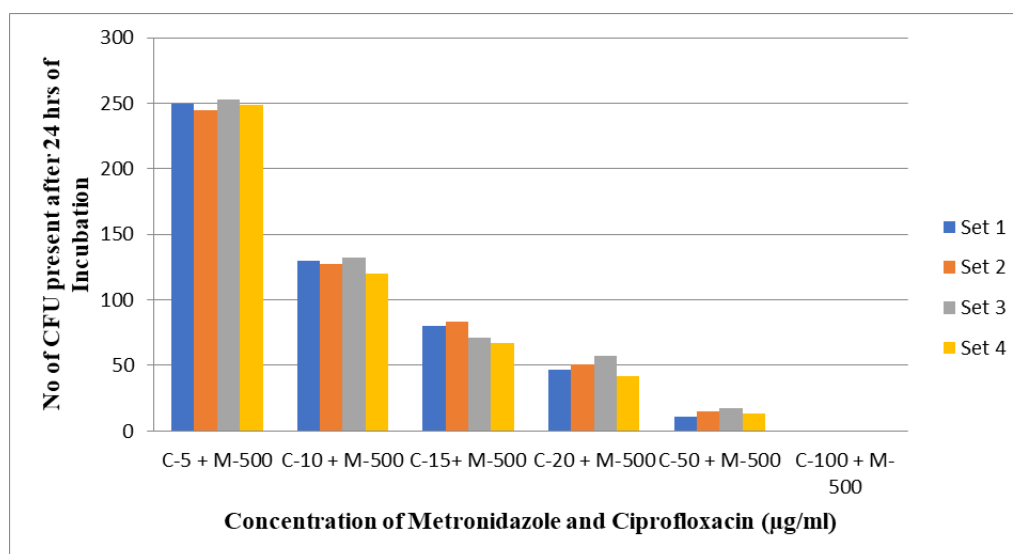
Barbosa-Ribeiro *et al.* found that the MIC value of Amoxicillin against the bacteria is 0.5µg/ml [10]. Conceição *et al.* found that the MIC values ranged from 0.5 to 2µg/ml for Amoxicillin. Pinheiro *et al.* reported that the MIC value of Amoxicillin against *E. faecalis* isolates from canals of root filled teeth with periapical lesions ranges from 0.25-0.75µg/ml [12].



**Graph 3:** Comparison of anti bacterial efficacy of different concentrations of amoxicillin (Group C) against *E. faecalis*

In present study, the MIC value of combination of Metronidazole and Ciprofloxacin (Group AB) is 505 µg/ml (Ciprofloxacin 5 µg/ml + Metronidazole 500 µg/ml) [Graph 4] and MBC value is 600 µg/ml (Ciprofloxacin 100 µg/ml + Metronidazole 500

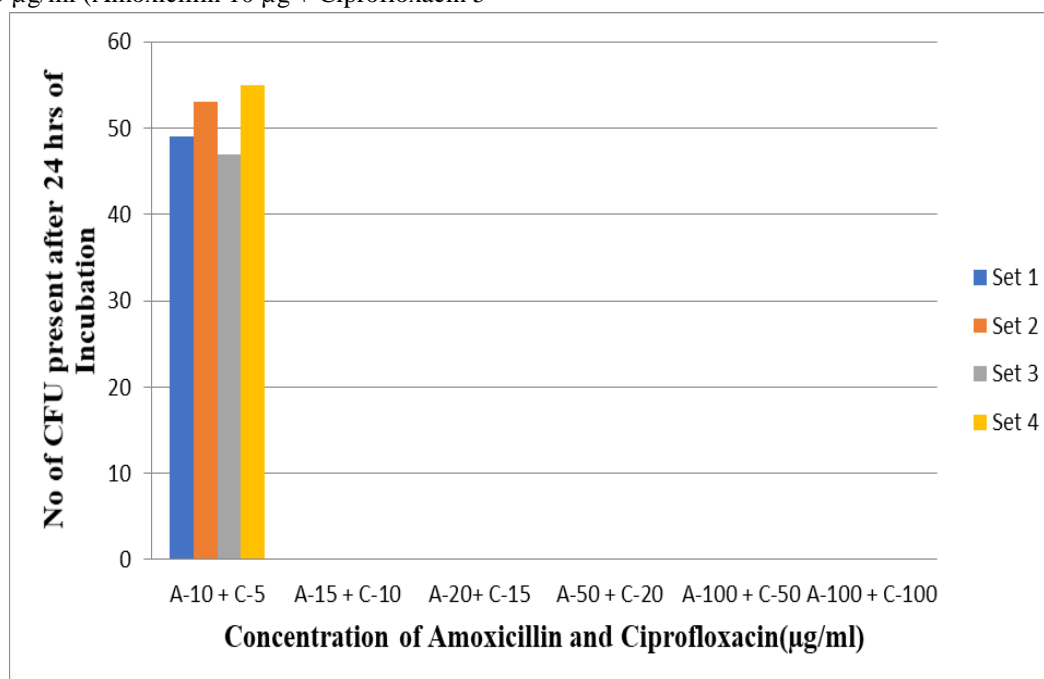
µg/ml). It showed that high Metronidazole concentration (i.e.500µg/ml) neither affected the bactericidal efficacy of Ciprofloxacin on *E. faecalis* which resonates with the result of a study done by R. Werk, L. Schneider in 1988 [13].



**Graph 4:** Comparison of anti bacterial efficacy of combinations of metronidazole and ciprofloxacin (Group AB) against *E. faecalis*

In present study, it is found that the MIC value of combination of Amoxicillin and Ciprofloxacin (Group BC) is 15 µg/ml (Amoxicillin 10 µg + Ciprofloxacin 5

µg) [Graph 5] and MBC value is 25 µg/ml (Amoxicillin 15 µg + Ciprofloxacin 10 µg).

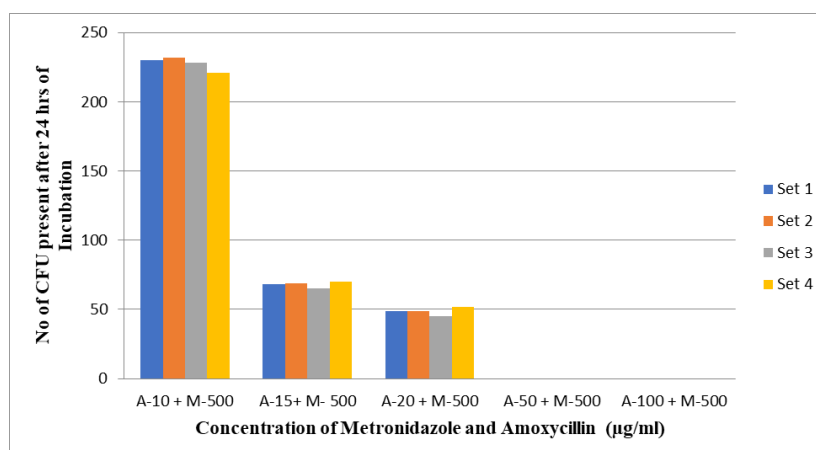


**Graph 5:** Comparison of anti bacterial efficacy of combinations of metronidazole and Amoxicillin (Group AC) against *E. faecalis*

In present study, it is found that the MIC value of combination of Amoxicillin and Metronidazole (Group AC) is 510 µg/ml (Amoxicillin 10 µg/ml +

Metronidazole 500 µg/ml) [Graph 6] and MBC value is 550 µg/ml (Amoxicillin 50 µg/ml + Metronidazole 500 µg/ml).

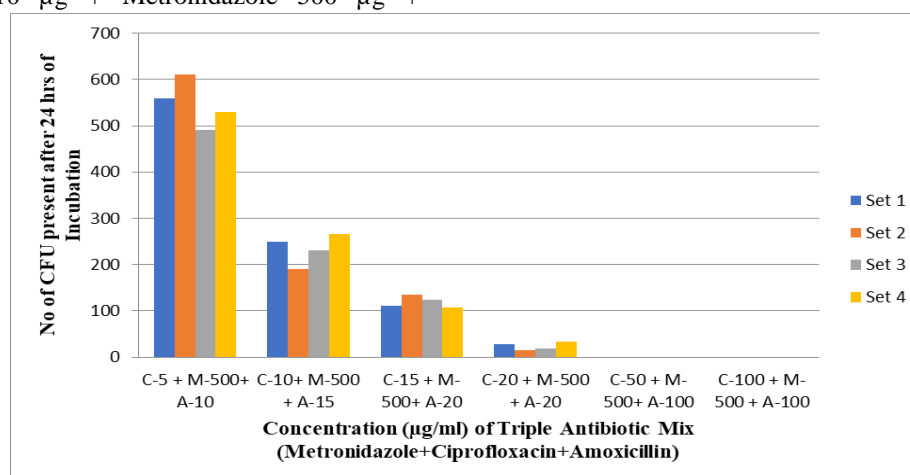
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**Graph 6:** Comparison of anti bacterial efficacy of combinations ciprofloxacin and amoxicillin (Group BC) against *E. faecalis*

In present study, it is found that the MIC value of combination of Amoxicillin and Ciprofloxacin and Metronidazole (Group ABC) is 515 µg/ml (Amoxicillin 10 µg + Metronidazole 500 µg +

Ciprofloxacin 5 µg) [Graph 7] and MBC value is 650 µg/ml (Amoxicillin 100 µg + Metronidazole 500 µg + Ciprofloxacin 50 µg).



**Graph 7:** Comparison of anti bacterial efficacy of combinations of amoxicillin, ciprofloxacin and metronidazole (Group ABC) against *E. faecalis*

**Table 1:** One way ANOVA test to determine antibacterial efficacy within and between the groups

	Sum of Squares	DF (Degree of freedom)	Mean Square	F	p-value
Between Groups	138279.833	5	27655.967	343.434	0.000
Within Groups	1449.500	18	80.528		
Total	139729.333	23			

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The p-value of one-way ANOVA for all the study groups is lower than 0.05, suggesting that the one or more antibiotic groups are significantly different (Table 1). Post-hoc Tukey HSD test showed

that the Gr C (Amoxicillin) and Gr BC (Amoxicillin + Ciprofloxacin) the most effective as it showed significant differences ( $<0.001$ ) with the all the other groups followed by Group B (Table 2).

**Table 2: INTERGROUP COMPARISON AMONG VARIOUS GROUPS OF ANTIMICROBIAL AGENTS**

Dependent Variable: **Mean number of *E. faecalis* colony count at MIC of different groups**

Tukey HSD

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group B	Group C	131.0000*	6.3454	.000	110.834	151.166
	Group AB	31.7500*	6.3454	.001	11.584	51.916
	Group AC	53.2500*	6.3454	.000	33.084	73.416
	Group BC	230.0000*	6.3454	.000	209.834	250.166
	Group ABC	66.0000*	6.3454	.000	45.834	86.166
Group C	Group B	-131.0000*	6.3454	.000	-151.166	-110.834
	Group AB	-99.2500*	6.3454	.000	-119.416	-79.084
	Group AC	-77.7500*	6.3454	.000	-97.916	-57.584
	Group BC	99.0000*	6.3454	.000	78.834	119.166
	Group ABC	-65.0000*	6.3454	.000	-85.166	-44.834
Group AB	Group B	-31.7500*	6.3454	.001	-51.916	-11.584
	Group C	99.2500*	6.3454	.000	79.084	119.416
	Group AC	21.5000*	6.3454	.033	1.334	41.666
	Group BC	198.2500*	6.3454	.000	178.084	218.416
	Group ABC	34.2500*	6.3454	.000	14.084	54.416
Group AC	Group B	-53.2500*	6.3454	.000	-73.416	-33.084
	Group C	77.7500*	6.3454	.000	57.584	97.916
	Group AC	-21.5000*	6.3454	.033	-41.666	-1.334
	Group BC	176.7500*	6.3454	.000	156.584	196.916
	Group ABC	12.7500	6.3454	.375	-7.416	32.916



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Group BC	Group B	-230.0000*	6.3454	.000	-250.166	-209.834
	Group C	-99.0000*	6.3454	.000	-119.166	-78.834
	Group AB	-198.2500*	6.3454	.000	-218.416	-178.084
	Group AC	-176.7500*	6.3454	.000	-196.916	-156.584
	Group ABC	-164.0000*	6.3454	.000	-184.166	-143.834
Group ABC	Group B	-66.0000*	6.3454	.000	-86.166	-45.834
	Group C	65.0000*	6.3454	.000	44.834	85.166
	Group AB	-34.2500*	6.3454	.000	-54.416	-14.084
	Group AC	-12.7500	6.3454	.375	-32.916	7.416
	Group BC	164.0000*	6.3454	.000	143.834	184.166

## 4. Summary and Conclusion:

Within the limitations of the present study, it can be concluded that in terms of MIC value, **Group C (Amoxicillin)** has lowest value among three antimicrobials against *E. faecalis*, followed by Group B (Ciprofloxacin), Group BC (Amoxicillin + Ciprofloxacin), Group AB (Metronidazole + Ciprofloxacin), Group AC (Metronidazole + Amoxicillin), Group ABC (Metronidazole + Ciprofloxacin+ Amoxicillin). Group A (Metronidazole) has no effect on the bacteria. In terms of killing capacity at their MIC value, the order of antimicrobial efficacy is – Group BC (Amoxicillin + Ciprofloxacin) > Group C (Amoxicillin) > Group AC (Metronidazole + Amoxicillin) & ABC (Metronidazole + Ciprofloxacin + Amoxicillin) > Group AB (Metronidazole + Ciprofloxacin) > Group B (Ciprofloxacin).

Recommended minimum concentration of triple antibiotic paste is 0.1-1 mg/ml. In present study it is found that the combination of 10 µg/ml amoxicillin and 5 µg/ml ciprofloxacin is the most effective for elimination of *E. faecalis* (99.98%). The current study can be further improvised by performing an in-situ study or performing it on bacterial biofilm model.

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