

Original Research

Comparative evaluation of antimicrobial potential of different Single drug delivery formulations for the root canal disinfection in Regenerative Endodontics- An In-vitro study

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ABSTRACT:

Context: Triple antibiotic paste (1mg/ml) or calcium hydroxide is commonly recommended intracanal medicaments for regenerative endodontics, but their clinical application is still questionable. **Aim:** The present study aimed to evaluate the antibacterial and antifungal activity of Ofloxacin-Ornidazole and Nitrofurantoin as substitutes for conventional medicaments, i.e., either triple antibiotic mix (TAP) or Double antibiotic mix (DAP) or Ca(OH)₂ against *E. faecalis* and *C. albicans*. **Materials and methods:** *E. faecalis* (MTCC 439) and *C. Albicans* (MTCC 183) were inoculated onto Mueller Hinton Agar (MHA) agar and Sabouraud Dextrose Agar (SDA) plates, respectively. The test group medicaments were placed into the respective agar wells in Petri plates and incubated at 37°C aerobically for 24h, and growth inhibition zones were measured. The readings obtained were statistically analyzed using ANOVA. **Results:** Mean zone of inhibition against *E. faecalis* was found greater with DAP followed by Ofloxacin-Ornidazole, Nitrofurantoin, TAP, and Ca(OH)₂, and for *C. albicans*, it was found greater with TAP followed by DAP, Ofloxacin-Ornidazole, Nitrofurantoin, and Ca(OH)₂ with highly significant difference ($P = 0.000$). **Conclusion:** Ofloxacin-Ornidazole and Nitrofurantoin were found to have antimicrobial activity, effective against *E. faecalis* and *C. albicans*, comparable to the conventional medicaments. Hence, these new formulations could be suggested as alternative antimicrobial agents instead of traditional medicaments for endodontic disinfection.

Keywords: Ofloxacin-Ornidazole, Nitrofurantoin, TAP, DAP, Ca(OH)₂

Key Message: Mono-therapeutic disinfection of the root canals with either a single effective antibiotic medicament "Nitrofurantoin gel" or synergistic drug combination in a single formulation "Ofloxacin-Ornidazole gel" at 1mg/ml aids to overcome the unwanted drug interactions of multidrug combinations and chances of antagonism thus, minimizing the risk of adverse effects on stem cells.

Received: 10 August, 2022

Accepted: 14 September, 2022

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This article may be cited as: Dadala PRS, Pandranki J, Vanga NRV. Comparative evaluation of antimicrobial potential of different Single drug delivery formulations for the root canal disinfection in Regenerative Endodontics- An In-vitro study. J Adv Med Dent Scie Res 2022;10(10):48-54.

INTRODUCTION

In regeneration endodontics, chemical disinfection plays a crucial role in reducing the microbial load within the necrotic canal space before regeneration. American Association of Endodontics (AAE), 2021 has recommended using either calcium hydroxide or 1-5mg/ml concentrated triple antibiotic paste (Ciprofloxacin, Metronidazole, Minocycline) or Double antibiotic paste (Ciprofloxacin, Metronidazole) for disinfecting root canals in regenerative endodontics.^[1] The reduced concentrations of combination antibiotics (1-5mg/ml)

to maintain viable stem cells and lack of mechanical debridement question the disinfection efficacy of the current regenerative protocol. Clinical limitations of topical antibiotic pastes include development of bacterial resistance and sensitization^[2], reduction in dentinal strength and fracture resistance^[3], tooth discoloration^[4] etc. The existing clinical evidence is insufficient to support triple or double antibiotic paste for regenerative endodontics.

Mixing two or more antibiotics is time-consuming, associated with technical issues, unwarranted drug interactions, and combination therapy is of no

significant interest to pharmaceutical companies (Paul M, Leibovici L, 2009).^[5] It is often associated with superinfections, increased risk for toxicity and higher costs (Tängdén T, 2014).^[6] Hence, it is better to de-escalate combination antibiotic medicament to a single antibiotic efficient enough to combat the developing bacterial resistance.

Ofloxacin-Ornidazole combination antibiotic commercially available in single formulation had a broad spectrum, pharmacokinetic profile, tolerance, and dosing characteristics.^[7] Nitrofurantoin is a synthetic nitrofuran compound with hydantoin added side chain. It was the drug of choice for treating uncomplicated lower urinary tract infection in the 1970s and was replaced by trimethoprim-sulfamethoxazole and newer beta-lactam antibiotics.^[8] Increasing prevalence of beta-lactamase-producing bacteria and developing bacterial resistance has routed to the resurgence of Nitrofurantoin usage.^[9]

Ofloxacin-Ornidazole and Nitrofurantoin at a lower concentration (1mg/ml) individually have not yet been explored as mono-therapeutic drugs for root canal disinfection in a biocompatible vehicle. Scarce literature exists regarding their antimicrobial efficacy compared with the standard intracanal medicaments used today: Triple Antibiotic Paste (TAP), Double Antibiotic Paste (DAP), and Calcium Hydroxide. The present study aimed to evaluate the antimicrobial effect of two newer mono-therapeutic antibiotics at lower concentrations of 1mg/ml against *E.faecalis* and *C.albicans* and was compared with conventional intracanal medicaments used in regenerative endodontics. The null hypothesis tested was that all the medicaments would have a similar antimicrobial effect.

METHODOLOGY

The present study was conducted in the Department of Pedodontics and Preventive Dentistry, GITAM Dental College and Hospital, collaborating with the Department of Microbiology, GITAM Institute of Medical Sciences and Research, Visakhapatnam, Andhra Pradesh, India.

GEL PREPARATION

United States Pharmacopeia (USP) graded antibiotic powders were obtained from pharmaceutical companies (Yarrow Chem Products, India and M/s Mascot Health Series Pvt Limited, India). Compounded TAP powder was prepared by mixing Ciprofloxacin, Metronidazole, and Minocycline 1:1:1 ratio. DAP is prepared by mixing a 1:1 ratio of Ciprofloxacin and Metronidazole.^[10] Ofloxacin and Ornidazole were mixed in a fixed drug dosage ratio of 1:2.5 to obtain a substitute for Double Antibiotic paste (DAP-S). 1 mg/ml concentration of intracanal medicament was prepared by dissolving 50 mg of compounded antibiotic powder in 50 ml of sterile water. 1mg/ml 50mg of Nitrofurantoin powder was mixed in 50ml of pure powder to formulate a Single

antibiotic paste (SAP). Then, 4 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St Louis, MO, USA) was added to each mixture and stirred for 2 hours at room temperature using a magnetic stirrer to obtain a homogeneous antibiotic gel.^[11,12] The gel was left to stand still for an additional 2 hours to ensure the complete disappearance of all foam from the mixture. An antibiotic-free placebo gel (CMC) composed of sterile water and methylcellulose was prepared as a control.

PREPARATION OF TEST MICROORGANISM

E. faecalis (MTCC 439) and *C. albicans* (MTCC 183) were obtained and inoculated to the nutrient broth and incubated at 37°C for 24 hrs. The microbial growth turbidity was confirmed using 0.5 McFarland opacity standards, which can be comparable with a microbial suspension of 1.5×10^8 CFU(colony-forming unit)/ml. ^[13]

AGAR WELL DIFFUSION ASSAY

E.faecalis and *C.albicans* inoculations were used to make the lawn culture in a laminar airflow chamber using sterile cotton swabs on specified agar, i.e., Mueller Hinton Agar (MHA) agar and *Sabouraud Dextrose Agar* (SDA), respectively. The wells were punched in the cultivated agar Petri plates (4mm in-depth, 6mm diameter). A total of 84 wells, three wells in each of 28 Petri plates (14 plates for MHA and 14 plates for SDA), were prepared. 50µl of each intracanal medicament was dispensed into the assigned well using a micropipette. The plates were incubated under appropriate atmospheric conditions at 37°C for 24 hrs. ^[14]

DETERMINATION OF MICROBIAL INHIBITION

The zones of inhibition obtained around the agar wells with test medicaments were recorded in millimeters by measuring the shortest distance between the outer margin of the well and initial microbial growth with the help of HiAntibioticZone Scale™-C (HiMedia).^[14] The readings were tabulated and statistically analyzed using One Way Analysis of Variance (SPSS 23.0 version) for multiple group comparison. Tukey's post hoc analysis was used for the group-wise comparison.

RESULTS

Descriptive statistics with mean inhibitory zone values and standard deviation of *E. faecalis* and *C. albicans* after 24 hours was summarized in table 1, figure 1, and group-wise comparison between intracanal medicaments against each microorganism was summarized in table 2.

The range of inhibitory values between medicaments varied broadly for both microorganisms, and statistically highly significant differences were observed ($P < 0.001$).

Among the tested medicaments against *E.faecalis* (figure 2a), DAP- the combination of Ciprofloxacin and Metronidazole and Ofloxacin-Ornidazole (DAP-S) had the largest zone of inhibition measuring about $27.93 \pm 1.99\text{mm}$ and 25.41 ± 1.78 respectively, with no significant difference ($P > 0.05$). TAP and Nitrofurantoin showed similar inhibitory zones (mean difference of 0.229mm) with no significant difference ($P > 0.05$), but less than DAP and DAP-S. A highly significant difference is observed in other group-wise comparisons of medicaments ($P < 0.05$).

In *C.albicans* agar well diffusion assay (figure 2b), the inhibitory zone was largest with TAP

($22.38 \pm 1.93\text{mm}$) followed by DAP ($22.14 \pm 1.70\text{mm}$), DAP-S ($20.05 \pm 1.36\text{mm}$), and Nitrofurantoin ($19.43 \pm 0.76\text{mm}$). Pair-wise comparison within TAP, DAP, DAP-S, and SAP groups showed no significant difference between their efficacy ($P > 0.05$) except between TAP and SAP ($P < 0.05$).

$\text{Ca}(\text{OH})_2/\text{Saline}$ showed a minimal range of inhibitory against both *E. faecalis* ($14.59 \pm 1.73\text{mm}$) and *C.albicans* ($10.14 \pm 2.91\text{mm}$), almost similar to placebo CMC gel ($P > 0.05$). A highly significant difference in the antimicrobial efficacy exists between calcium hydroxide and other medicament gel ($p < 0.001$).

Table 1: Comparison of Inhibition Zone measurements of various media against *E.Faecalis*

Enterococcus faecalis				Candida albicans			
Group	Mean	Std. Error	F ratio – 84.365 P Value <0.001**	Group	Mean	Std. Error	F ratio – 84.365 P Value <0.001**
TAP	18.20 ± 1.44	0.543		TAP	22.38 ± 1.93	0.730	
DAP	27.93 ± 1.99	0.754		DAP	22.14 ± 1.70	0.642	
DAPS	25.41 ± 1.78	0.674		DAPS	20.05 ± 1.36	0.512	
SAP	18.43 ± 0.65	0.246		SAP	19.43 ± 0.76	0.289	
$\text{Ca}(\text{OH})_2$	14.59 ± 1.73	0.654	P Value <0.001**	$\text{Ca}(\text{OH})_2$	10.14 ± 2.91	1.100	P Value <0.001**
CMC	15.17 ± 1.53	0.579		CMC	8.28 ± 1.60	0.606	

** -Highly Significant ($p < 0.001$)

Figure 1:- Graphical representation of Antimicrobial efficacy

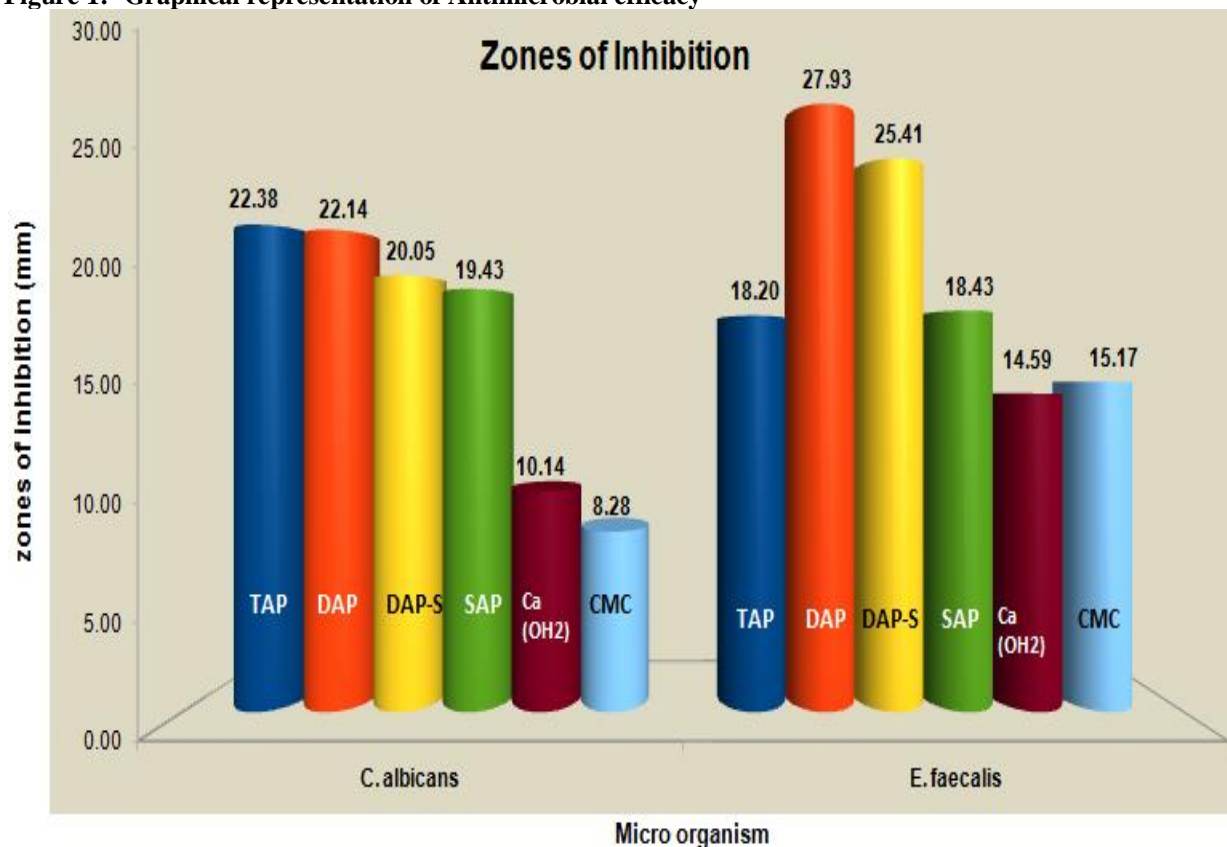
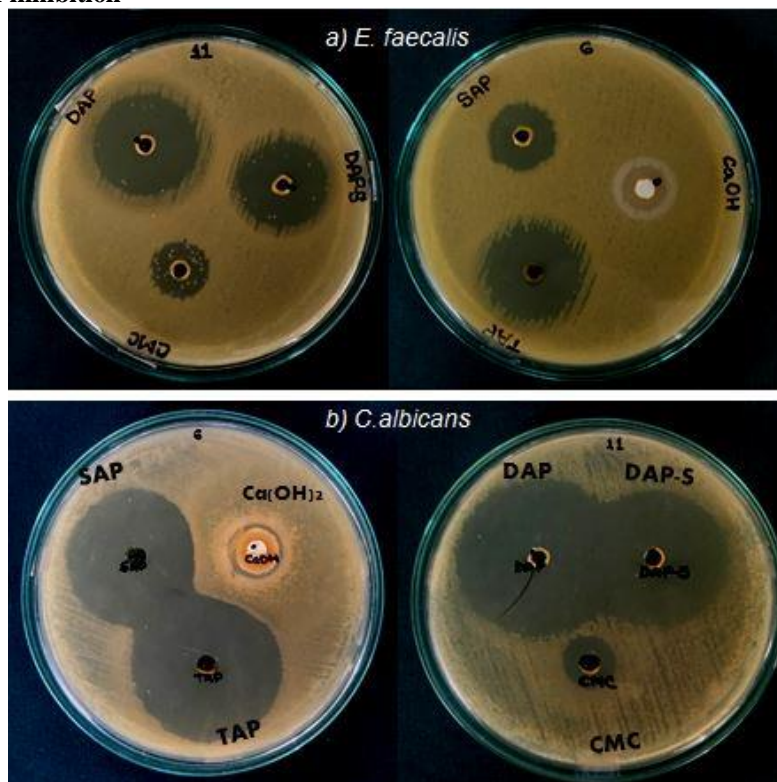


Figure 2: Zones of inhibition**Table 2:- Group-wise comparison of antimicrobial efficacy between medicaments.**

	Enterococcus faecalis		Candida albicans	
	Mean difference	Significance	Mean difference	Significance
TAP Vs DAP	-9.729	<0.001**	0.236	1 NS
TAP Vs DAP-S	-7.214	<0.001**	2.333	0.188NS
TAP Vs SAP	-0.229	1 NS	2.953	0.049*
DAP Vs SAP	9.500	<0.001**	2.717	0.084 NS
DAP Vs DAP-S	2.514	0.054 NS	2.097	0.288 NS
DAP-S Vs SAP	6.986	<0.001**	0.620	0.988 NS
TAP Vs Ca(OH) ₂	3.610	0.002*	12.240	<0.001**
DAP Vs Ca(OH) ₂	13.339	<0.001**	12.004	<0.001**
DAP-S Vs Ca(OH) ₂	10.834	<0.001**	9.907	<0.001**
SAP Vs Ca(OH) ₂	3.839	0.001**	9.287	<0.001**
DAP Vs CMC	12.757	<0.001**	13.859	<0.001**
TAP Vs CMC	3.029	0.012*	14.094	<0.001**
DAP-S Vs CMC	10.243	<0.001**	11.761	<0.001**
SAP Vs CMC	3.257	0.006*	11.141	<0.001**
Ca(OH) ₂ Vs CMC	-0.581	0.982 NS	1.854	0.421 NS

*-Significant (p<0.05), **-Highly Significant (p<0.001), NS – Not significant (P>0.05)

DISCUSSION

E.faecalis is a gram-positive facultative anaerobe considered as the most resistant endodontic pathogen, which can adapt to the harsh environment even with poor nutrition supply and resist high alkaline pH (11.1).^[15] *Candida albicans* is the most frequently associated fungal species with persistent or refractory endodontic infections. It invades dentinal tubules and survives sequestered within biofilms and intertubular dentin.^[16] Because of high collagenolytic activity, *C.albicans* uses dentin as a source of nutrition, which further promotes its colonization in

the root canal and increases its virulence. Hence, in the current study, *E.faecalis* and *C.albicans* were selected as target microorganisms to evaluate the antimicrobial effect of intracanal medicaments.

Intracanal medicaments used in regenerative endodontic procedures have to be biocompatible towards mammalian cells, specifically stem cells of the apical papilla (SCAP). The toxicity of TAP and DAP to SCAP is concentration-dependent, and 1000mg/ml of clinical concentration for root canal disinfection was highly toxic to stem cells.^[17] American Academy of Endodontics (2021) suggested

using either Ca(OH)_2 or lower concentrations of Triple antibiotic paste (1-5mg/ml) as intracanal medicament.^[1] Ruparel NB and colleagues (2012) have observed only 33–56% survival rate of stem cells when exposed to 1 mg/ml TAP, and no adverse effect was observed on the viability of stem cells of the apical papilla at 0.1–0.01 mg/ml concentration.^[17] Ca(OH)_2 dressing even though biocompatible, shows the decreased antibacterial efficacy when it comes in contact with inflammatory exudation in the root canal, limiting its effectiveness against resistant species like *E.faecalis*.^[18] To overcome the limitations of conventional medicaments, the present research evaluates the antimicrobial efficacy of two newer formulations i.e., Ofloxacin-Ornidazole and Nitrofurantoin, which are immune to developing multidrug resistance. AAE has recommended using lower concentrations of antibiotics (1-5mg/ml) for disinfection of root canal.^[1] Still, preparing a homogenous mix of antibiotics with a 1mg/ml clinical concentration in a liquid vehicle is challenging.

Carboxy Methyl Cellulose (CMC) is an anionic, water-soluble, FDA-approved cellulose derivative used as a vehicle for the drug delivery in this study (Zennifer A,2021).^[19] It has been stated as a promising scaffold biomaterial due to its desirable properties such as ease of chemical modification, flexibility, stability, and pH sensitivity leading to applications in tissue engineering, 3D bioprinting, drug delivery, cosmetic fillers, and cancer therapy. The carboxylate group in CMC is responsible for in situ hydrophilic character, gellation, bioadhesion, sensitivity to environmental stimuli, and controlled drug release (Javanbakht S, Shaabani A,2019).^[20]

The antimicrobial activity of calcium hydroxide is attributed to the high pH with the release of hydroxyl ions, alkalizing the surrounding environment. But, Ca(OH)_2 showed a minor zone of inhibition against both the microorganisms. The least inhibitory zone against *E.faecalis* is due to its primary resistance mechanism in an alkaline environment. The resistance mechanism of *E. faecalis* is due to the proton pump in their plasma membrane that pumps protons into the cell to acidify the cytoplasm and maintain homeostasis in an alkaline environment (Siqueira JF Jr., Lopes HP 1999, Evan et al. 2002).^[21,22]

Triple antibiotic paste (TAP) gel combines Ciprofloxacin, Metronidazole, and Minocycline in equal proportions, and double antibiotic paste (DAP) gel constitutes Ciprofloxacin and Metronidazole. Ciprofloxacin has a broad spectrum of activity and acts against both Gram-negative and Gram-positive bacteria, inhibiting cell division and enzyme inactivation. Obligate anaerobes are predominantly present in the deep dentin of infected root canals, where Metronidazole acts effectively by disrupting their bacterial DNA. Minocycline works by inhibiting matrix metalloproteinase enzyme and enzyme inactivation (Athanassiadis B, 2007).^[23] But, Hoshino *et al.*(1996) have investigated the

antibacterial effect of Ciprofloxacin, Metronidazole, and minocycline and concluded that none of the drugs resulted in the complete elimination of bacteria. However, in combination, these drugs were able to consistently sterilize all samples.^[24] In the present study, TAP showed antifungal efficacy against *C.albicans* similar to DAP and comparatively showed broad inhibitory zones than other medicaments. But, the inhibitory zones of TAP against *E.faecalis* are smaller than DAP, suggestive of uneventful drug interactions, which might lead to decreased antibiotic susceptibility of *E.faecalis*.

The origin of resistant microbial species requires novel effective medicaments into the limelight. This research is the first study to explore the antimicrobial efficacy of new formulations, i.e., Ofloxacin-Ornidazole as a synergistic drug combination and Nitrofurantoin as a single drug formulation, tested against *E.faecalis* and *C.albicans* at the lowest concentration (1mg/ml).

Ofloxacin-Ornidazole is available as a fixed drug combination in the ratio of 1:2.5 for oral and parental infusion. Ofloxacin is the most evident quinolone effective against aerobic, Gram negative, and Gram positive bacteria. It has a long terminal half-life with favorable pharmacokinetics, resulting in a higher area under the concentration-time curve than Ciprofloxacin. Ornidazole, congener of metranidazole is a nitroimidazole derivative, effective against anaerobic and protozoal infection. (Shrivastava SM., Kumar S. and Chaudhary M., 2009).^[12] The combination of Ofloxacin-Ornidazole is highly effective and synergistic with bactericidal activity to combat antibiotic drug resistance of organisms. The combination is non-toxic even at the maximum prescribed individual drug level and has no evident drug interactions.^[25] In the current study, the antimicrobial efficacy of fixed drug combination, i.e., Ofloxacin-Ornidazole, showed almost similar efficacy as that of DAP against both microorganisms with no significant difference between them ($p>0.05$). Also, Ofloxacin-Ornidazole was found more effective than TAP to inhibit *E. faecalis*.

Nitrofurantoin has stood as a single solution to the ever-increasing menace of antibacterial resistance in Urinary tract infections. The presence of a hydantoin ring with a nitro-substituted furanyl side-chain caused the bactericidal action targeting multiple sites in a bacterial cell and at different levels. The mechanism of action includes inhibition of bacterial enzymes involved in carbohydrate synthesis, inhibits DNA synthesis, RNA synthesis, and total protein synthesis by the non-specific attack on ribosomal proteins. (McOsker CC 1994, Guay DR 2001).^[26,27] In the present study, Nitrofurantoin showed an inhibitory zone of 18.43mm, similar to TAP against *E.faecalis*. Its efficacy against *C.albicans* with an inhibitory zone of 19.43mm was considerable with other medicaments.

3% Nitrofurantoin gel showed better antibacterial efficacy with an inhibitory zone of 24.33 ± 0.10 mm than 2% Chlorhexidine gel.^[28] Alrahman MSA, Faraj BM, Dizaye KF (2020) noticed that Nitrofurantoin at 6.25 mg/ml has the ability to eradicate *E.faecalis* isolated from blood with sepsis. At 25mg/ml concentrations, it could effectively remove *E.faecalis* from dentinal chips and root canals comparable to 25mg/ml Modified TAP (Ciprofloxacin, Metronidazole, and Clindamycin).^[29] This shows that the antimicrobial efficacy of Nitrofurantoin is concentration-dependent. Both 12.5mg/ml and 25mg/ml were biocompatible with the rat subcutaneous connective tissue.^[30] Hence Nitrofurantoin, even at 25mg/ml concentration, could be a safe biological alternative to enhance the survival rate of stem cells of apical papillae. The present study being *in-vitro*, the results may not show the full clinical potential of the tested material as *in vivo* study.

CONCLUSION

Despite the study limitations, the results are promising. They indicate that 1mg/ml Ofloxacin-Ornidazole synergistic combination gel and 1mg/ml of Nitrofurantoin gel, a single drug formulation, were found effective against *E.faecalis* and *C.albicans*. "Ofloxacin-Ornidazole" as a synergistic combination and "Nitrofurantoin" as a single solution has proven their antimicrobial efficacy at 1mg/ml concentration. Thus, both are biologically safe alternatives to conventional intracanal medicaments (TAP, DAP, and Ca(OH)_2). Hence, using these effective monotherapeutic medicaments could overcome the unwanted drug interactions of multidrug combination, chances of antagonism, developing bacterial resistance, risk of adverse effects, systemic allergic reaction, superinfections, etc. Further studies need to be warranted to explore their application as a monotherapeutic agent in the field of regenerative endodontics.

SOURCE(S) OF SUPPORT

Nil

CONFLICTING INTEREST (IF PRESENT, GIVE MORE DETAILS)

Nil

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