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ORIGINAL ARTICLE

Comparison of Antibacterial Efficacy of Triple Antibiotic-Loaded Hydrogel Versus Modified Triple Antibiotic-Loaded Hydrogel as Intracanal Medicament Against *Enterococcus faecalis*: An *In vitro* Study

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ABSTRACT

Objective: Triple antibiotic paste (TAP) is known to have an essential role in the success of endodontic treatment by eliminating pathogens from the root canal system. Unfortunately, it causes discolouration and cytotoxicity at high concentrations. The objective of this research was to assess and compare the antimicrobial effectiveness of various concentrations (1 mg, 5 mg, 10 mg) of TAP, TAP hydrogel (TAPH), M-TAP, and M-TAP hydrogel (MTAPH) against *Enterococcus faecalis*.

Methods: The agar well diffusion method was used to assess the antibiotic sensitivity of the following intracanal medicaments: TAP (ciprofloxacin, metronidazole, and minocycline) mixed in a ratio of 1:1:1; TAPH, M-TAP (ciprofloxacin, metronidazole, and amoxicillin), M-TAPH and plain hydrogel. Each tested medicament was individually evaluated for its antimicrobial activity against *Enterococcus faecalis*. Structural and topographical characterisation were analysed using a Scanning Electron Microscope (SEM) and interpreted using ImageJ software. A microdilution broth test was performed to examine the minimum inhibitory concentration and minimum bactericidal concentration (MBC) of M-TAP and TAP.

Results: Except for the plain hydrogel, M-TAP and hydrogel and TAP and hydrogel showed significantly varied inhibitory zones at different concentrations. M-TAPH showed the highest mean zone of inhibition of 21.6, 33.33 and 38.0 mm at a concentration of 1, 5, and 10 mg/mL when compared to TAPH, which showed a mean zone of inhibition of 3.3 mm,12.3 mm, 21.3 mm at the respective concentrations. The MIC study shows that more than 75% of *Enterococcus faecalis* growth was inhibited by M-TAP at a concentration of 5 µg/mL, whereas TAP showed inhibition at a concentration of 35 µg/mL. MBC results indicate that almost 99.9% of the bacterial population was killed at a concentration of 100 µg/mL (10^{-1}) for TAP and 10 µg/mL (10^{-2}) for M-TAP.

Conclusion: The antibacterial efficacy of M-TAP was significantly higher than TAP. Application of M-TAP at lower doses is advised to overcome the disadvantages seen with TAP.

Keywords: Agar well diffusion, antibiotic paste, Carrageenan, *enterococcus faecalis*, hydrogel, minimum bactericidal concentration, minimum inhibitory concentration

HIGHLIGHTS

- The modified triple antibiotic medicament, in both paste and hydrogel forms, demonstrated higher antibacterial efficacy than the traditional triple antibiotic medicament at all concentrations tested.
- Carrageenan shows excellent potential as a natural polymer for drug delivery and has sustained drug-release properties.
- It has been demonstrated that antibiotic combinations at 1 mg/mL have antibiofilm effects against endodontic pathogens without any cytotoxic effects on dental stem cells.

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INTRODUCTION

Enterococcus faecalis (*E. faecalis*) is a microorganism frequently present in the root canal and can penetrate the dentinal tubules and tolerate a variety of antimicrobials. It is a resilient microorganism that, even though it constitutes a minor fraction of the microbiota within untreated root canals, assumes a significant role in developing persistent periradicular lesions following root canal procedures (1). It is frequently detected in many root canal treatment failures, exhibiting the capability to persist within the root canal as an individual organism or as a predominant constituent of the microbial community (2). Intracanal medication is used to eradicate bacteria that could affect the treatment outcome. Several frequently utilised medicaments include calcium hydroxide, chlorhexidine di-gluconate, and triple antibiotic paste (TAP). Calcium hydroxide [$Ca(OH)_2$] exhibits broad-spectrum antimicrobial properties against typical endodontic pathogens, although its efficacy is relatively reduced when combating *Enterococcus faecalis* and Candida albicans (3). A recent study indicates that combining 2% CHX gel with $Ca(OH)_2$ as an intracanal medicament demonstrates comparable effectiveness to TAP (4). Local antibiotic administration is a more effective antibiotic delivery method in endodontic therapy. Because of the complexity of the root canal infection, any single antibiotic is unlikely to allow for adequate bacterial load reduction (5). To deal with the different microbiota found, a combination of approaches is more likely (6). The most commonly used intracanal medication, $Ca(OH)_2$, cannot easily penetrate dentinal tubules, making it incapable of eliminating *E. faecalis* (7). TAP which is a combination of ciprofloxacin, minocycline, and metronidazole) is frequently used in endodontic therapy and has a wide range of applications in clinical settings (8). It treats periapical lesions, manages external inflammatory root resorption, and addresses root fractures. Additionally, it finds applications in controlling flare-ups within the root canal, enhancing its versatility in endodontic procedures (5). Furthermore, it is used as an intracanal medicament when loaded onto a scaffold, making it a valuable component in endodontics (9).

However, some restrictions, such as tooth discolouration, are a concern. Minocycline and related tetracycline antibiotics are known for their slow release from dentine, primarily due to their binding to collagen. However, this binding can also cause tooth discolouration (10). When TAP is used in endodontic procedures, the dentine undergoes demineralisation, exposing collagen. Over time, Minocycline oxidises and forms a black substance called Quinone, which darkens the root dentine. This black discolouration is a notable consequence of these antibiotics' actions in endodontics (11). The dentine bonding agent effectively diminished the intensity of the discolouration, but it was unable to prevent it completely (12). It was also observed that employing elevated concentrations of DAP or TAP for an extended period resulted in a significant reduction in dentine microhardness (13). As a result, a TAP modification, known as Modified triple antibiotic pate (M-TAP), has been proposed to eliminate the limitations.

Studies have indicated that clindamycin or additional broad-spectrum antibiotics, like amoxicillin or cefaclor, could be substituted for minocycline in TAP (14). A recent study found that tooth discolouration was less pronounced when using TAP formulations containing amoxicillin than those containing minocycline, doxycycline, or cefaclor (15). In another study, a favourable treatment outcome was achieved by using a triple antibiotic paste containing amoxicillin as a medicament for treating an infected immature premolar, and notably, no discolouration was observed (16).

According to recent studies, stem cells and pulp fibroblasts are directly and indirectly cytotoxic when antibiotics are administered at standard dosages (17). A study by Faria et al. (18) revealed that the cytotoxicity of TAP is attributable to the influence of both the vehicle and antibiotic formulations, which in turn impact the pH of TAP. It has been demonstrated that combinations of antibiotics at 1 mg/mL have antibiofilm actions against endodontic pathogens without having cytotoxic effects on apical papilla stem cells (19). The American Association of Endodontics (AAE) has also suggested that this should be the highest antibiotic concentration that can be used to disinfect the root canal during endodontic regeneration (20, 21). Clinicians have used this antibacterial paste for seven to 28 days. Only a few researchers have looked at the right consistency of this mixture to eradicate microbes. As a result, its exact consistency is uncertain (22). Research investigations have explored the effect of methylcellulose-based hydrogel on the antibacterial properties of different medicaments, such as double antibiotic paste containing metronidazole and ciprofloxacin and $Ca(OH)_2$. They concluded that methylcellulose hydrogel holds promise as a biocompatible and injectable medium for the controlled delivery of low-concentration antibiotics as intracanal medicaments (23). Injectable hydrogels offer the advantage of sustained release of medicaments (24). A prior study has shown that permeability becomes more pronounced as the pore size increases (25).

The objectives of this study include formulation of the TAPH and M-TAPH using marine biopolymers as an intracanal medicament to destroy *Enterococcus faecalis* (*E.faecalis*), followed by an assessment of the antibacterial efficacy of TAP and M-TAP using the Agar well diffusion test. Its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also measured to assess the difference between its paste-like consistency and the minimum concentration that can inhibit the growth of microorganisms, as well as the lowest concentration of an antimicrobial agent that eradicates the microorganism. An additional objective was to demonstrate through a Scanning Electron Microscope (SEM) the morphology of the Carrageenan scaffold when used to release antibiotics. No prior studies have investigated the M-TAP and its antibacterial effectiveness when utilising marine-derived polymers, such as Carrageenan, as a vehicle medium. Therefore, this study aims to investigate the antimicrobial efficacy of a Carrageenan-based M-TAP at different concentrations, particularly in combating *E. faecalis*.

MATERIALS AND METHODS

All the experimental protocols were approved by the proceedings of the Scientific Review Board with reference number SRB/SDC/ENDO-2103/22/147. This study was conducted following the Declaration of Helsinki.

Preparation of Antibiotic Hydrogel

In this study, low doses of MTAP at 1, 5, and 10 mg/mL were meticulously prepared using a combination of Carrageenan and Xanthan gum, following a well-established protocol documented in previous research (26). Corresponding equivalents of individual antibiotics at 1 mg/mL is 3.3 mg, 5 mg/mL is 16.6 mg, and 10 mg/mL is 33.3 mg. A single operator carried out the preparation of the hydrogel.

Equivalent quantities of antibiotic powder were used to ensure uniformity across the antibiotic groups. Specifically, the TAP group consisted of ciprofloxacin, metronidazole, and minocycline, while the M-TAP group comprised ciprofloxacin, metronidazole, and amoxicillin. A stock solution of each antibiotic, with a concentration of 10 mg/mL, was meticulously prepared by dissolving the antibiotic powder in distilled water.

The subsequent step involved mixing the antibiotic solution, whether TAP or M-TAP, with a 0.5% solution of Carrageenan and Xanthan gum. This meticulous blending process was conducted under controlled conditions at a temperature of 37°C, facilitating the formation of hydrogels.

To generate M-TAP solutions with concentrations of 1, 5, and 10 mg/mL, precise amounts of amoxicillin, ciprofloxacin, and metronidazole were gradually combined in the respective volumes of sterile distilled water. This process occurred in a carefully sanitised environment and involved continuous stirring to ensure complete dissolution. Subsequently, Carrageenan and Xanthan gum powder were progressively introduced into each M-TAP solution with vigorous stirring, incrementally, until the desired gel-like consistency was attained at concentrations of 1, 5, and 10 mg/mL. This methodical approach was employed to ensure the study's accuracy and reproducibility of the M-TAP hydrogel formulations.

SEM Analysis

The samples were prepared for SEM analysis by lyophilising the hydrogel. The porosity and morphology of the lyophilised hydrogel were analysed using a SEM machine (FE-SEMIT800). The lyophilised hydrogel was sputter coated using platinum sputter coating, and the surface morphologies of plain Carrageenan hydrogel, TAP hydrogel (TAPH), and M-TAP hydrogel (MTAPH) were analysed at 250× under 5.00 kV. SEM images were analysed using ImageJ software (version 1.41, National Institute of Health, Bethesda, MD, USA) with the help of a scale bar provided along with the SEM image used as a reference to evaluate both pore size and flake size. This assessment involved calculating the average dimensions for each of these structural features. Assessing the porosity and morphology of the hydrogel is imperative as it indicates the successful infusion of antibiotics into the Carrageenan-based hydrogel. Additionally, it sheds light on whether the introduction of antibiotics impacts the structural characteristics of the Carrageenan scaffold.

Agar Well Diffusion Method

The agar well diffusion method was performed according to the guidelines set forth by the Clinical and Laboratory Standards Institute to evaluate the antibacterial activity against *E. faecalis* (27). Using a sterile swab, equal amounts of *E. faecalis* strains were swabbed onto Mueller-Hinton Agar plates (Hi-Media, Mumbai, India.) For uniform dispersal, turn the plates roughly 60°C between streaks. Wells with a 5 mm diameter were punched into the agar plate with sterile punching equipment. TAP, TAPH, M-TAP, M-TAPH, and plain hydrogel were inoculated in the respective wells at a concentration of 1, 5, and 10 mg/mL, and the experiment was carried out in triplicates. The plates were read when the growth was confluent. The inhibition diameter was measured with vernier callipers to the nearest whole millimetre.

Determination of MIC and MBC of TAP and MTAP

This study calculated the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microdilution method. Briefly, different concentrations (1 mg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, 100 ng/mL, and 10 ng/mL) of TAP (minocycline) and M-TAP (amoxicillin) were added to *E. faecalis* (0.5 McFarland: 1.5×10^8 CFU/mL) cultured in nutrient broth. The MIC refers to the lowest concentration of a medicament, such as TAP (minocycline) or M-TAP (amoxicillin), that effectively prevents visible bacterial growth after 24 hours of incubation at 37°C. The MBC is determined by sub-culturing the MIC broths onto new nutrient agar plates. MBC is the lowest medicament concentration, killing 99.9% of the tested microorganisms. The findings of each experiment were run in triplicate, and the results were presented as mean with standard deviation (SD).

Statistical Analysis

The collected data were evaluated statistically using SPSS version 23 (SPSS 23.0, IBM, Chicago, USA). One-way ANOVA and a Post Hoc Test were done to compare each medicament's mean diameters of inhibition zones at different concentrations. The statistical significance was set at a p-value less than 0.05.

RESULTS

SEM Analysis

The morphology of the antibiotic Carrageenan scaffold was analysed using SEM at a resolution of 250x. The hydrogel demonstrated a flake-like structure with an interpenetrating network (Fig. 1). The pore size and flake size of the hydrogel were measured using ImageJ software. The average pore size of the plain hydrogel is 24.12 µm, while that of the TAPH is 24.33 µm, and the M-TAPH is 20.74 µm (Table 1). Thus, the pore size of the hydrogel was somewhat similar, which is known to help in the sustained release of the antibiotics. The average flake size of the plain hydrogel is 18.73 µm while that of TAPH is 73.16 µm and M-TAPH is 65.23 µm. It is seen that there is an increase in the flake size of the antibiotic-containing hydrogel when compared to the plain hydrogel. This indicates the infusion of the antibiotics into the Carrageenan-based hydrogel.

Agar Well Diffusion Test

The mean and standard deviation of the diameters of the growth inhibition zones were measured and presented in Table 2 below. M-TAPH showed the highest mean zone of inhibition of 21.6 mm, 33.33 mm, and 38.0 mm at a concentration of 1, 5, and 10 mg/mL when compared to TAPH, which showed a mean

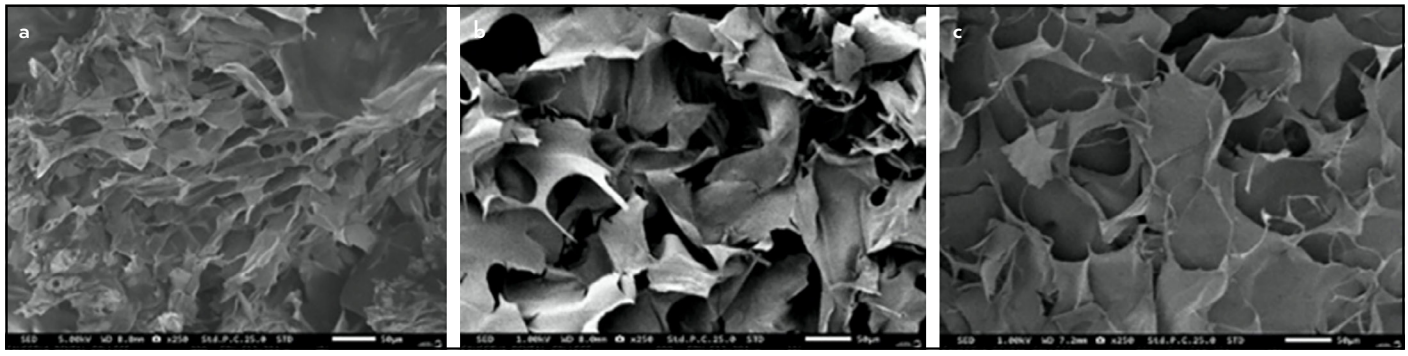

Figure 1. (a) SEM analysis demonstrating Plain hydrogel. (b) SEM analysis demonstrating TAP hydrogel. (c) SEM analysis demonstrating MTAP hydrogel

SEM: Scanning Electron Microscope

zone of inhibition of 3.3 mm,12.3 mm,21.3 mm at the respective concentrations. Plain hydrogel showed a minimum mean zone of inhibition at 0.66 mm (Fig. 2). Overall, M-TAP and M-TAPH had the highest zone of inhibition in the agar diffusion well plates at all concentrations. TAP and TAPH showed minimal inhibitory effects compared to M-TAPH (Fig. 3). However, the plain hydrogel had minimal to no antibacterial properties. Results indicate significant differences between all the groups ($p<0.0001$).

Determination of MIC and MBC of TAP and MTAP

The minimal inhibitory concentration (MIC) study shows that the *E. faecalis* growth was inhibited (more than 75%) by M-TAP at a concentration of 5 µg/mL (range of 1 to 10 µg/mL), whereas TAP showed inhibition at a concentration of 35 µg/mL (range of 10 to 50 µg/mL) as illustrated below in Figure 4. This study confirms that M-TAP exhibits effective bactericidal activity, about 14 times higher, against E, faecalis, compared to the other tested drug TAP. After the MIC determination of both tested drugs (M-TAP and TAP), aliquots of 50 µL from all the MIC tubes (after 18 h) were seeded on nutrient agar plates by spread plate technique and incubated for 24 h at 37°C. As shown in Figure 5, the MBC results indicate that almost 99.9% of the bacterial population was killed at a concentration of 100 µg/mL (10^{-1}) for TAP and 10 µg/mL (10^{-2}) for M-TAP.

TABLE 1. Demonstrates average pore size (µm) and flake size (µm) of the Carrageenan-based hydrogels

	Pore size (µm)	Flake size (µm)
Plain hydrogel	24.12	18.73
TAP hydrogel	24.33	73.16
MTAP hydrogel	20.74	65.23

DISCUSSION

This study evaluated the effectiveness of various antibiotic combinations in eradicating *E. faecalis* at different concentrations in a gel-like consistency. The present study compared triple antibiotic and modified triple antibiotic medicament in paste and gel form at the low concentrations of 1, 5, and 10 mg/mL. Carrageenan was used as the carrier material to form the hydrogel as it contains sustained release properties. The results of the present study showed that modified triple antibiotic medicament in both forms had higher antibacterial efficacy when compared to that of triple antibiotic medicament at all concentrations. This study demonstrated that the antibacterial efficiency of antibiotics increased with an increase in antibiotic concentration. The results showed that TAP and M-TAP were effective in much lower concentrations, which correlates with the concentrations suggested by AAE clinical considerations for a regenerative procedure. A notable increase in the flake size was observed in the hydrogel-containing antibiotics compared to the plain hydrogel, indicating successful antibiotic infusion into the Carrageenan-based hydrogel. Furthermore, the plain hydrogel and the antibiotic-infused hydrogel exhibited similar properties, implying that adding antibiotics does not disrupt the morphology of the Carrageenan scaffold.

The research employed carrageenan, a sulfated polysaccharide, as a carrier material, highlighting a key strength of the study. Sulfated polysaccharides are chosen for their distinctive advantages over synthetic carrier agents in this context. These advantages may include improved biocompatibility, reduced toxicity, and potential enhanced therapeutic properties, making carrageenan an appealing choice for the formulation under investigation.

TABLE 2. Comparison of mean diameters of growth inhibition zones (mm) ±SD of antibiotic hydrogels at different concentrations against *E. faecalis*

Groups	TAP	MTAP	TAP hydrogel	MTAP hydrogel	Plain hydrogel	ANOVA (p)
1mg	3.3333±0.5	22.8333±0.2	3.3333±0.5	21.6667±0.5	0.6667±0.5	p≤0.001
5mg	12.3333±1.5	32.6667±3.2	15.3333±1.1	33.3333±1.5	0.6667±0.5	p≤0.001
10 mg	21.3333±5.1	38.0000±1.7	20.6667±7.2	38.0000±2.6	0.6667±0.5	p≤0.001

ANOVA: Analysis of variance

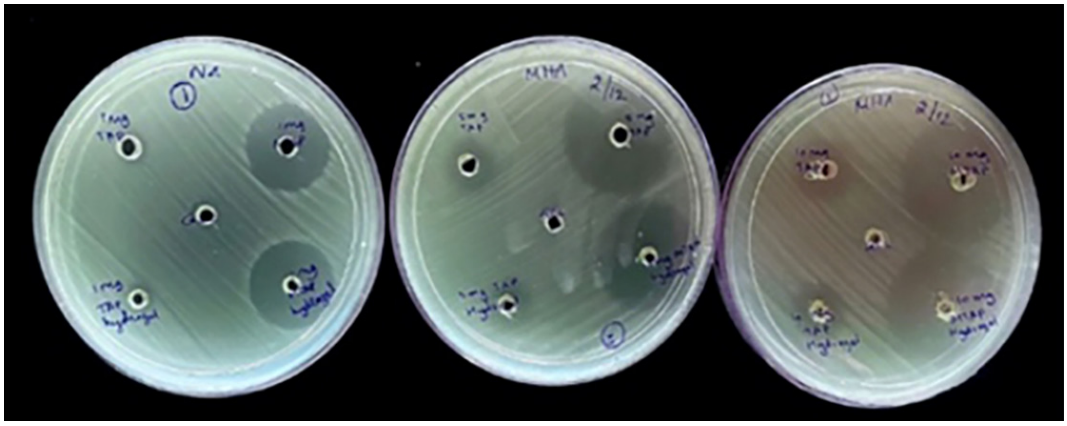

Figure 2. Zones of inhibition of growth of *E.faecalis* against antibiotic pastes and hydrogel

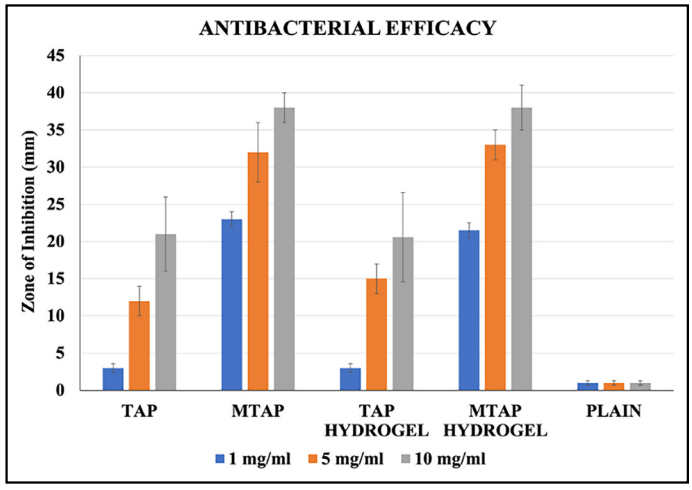

Figure 3. Assessment of antibacterial effect at different concentrations

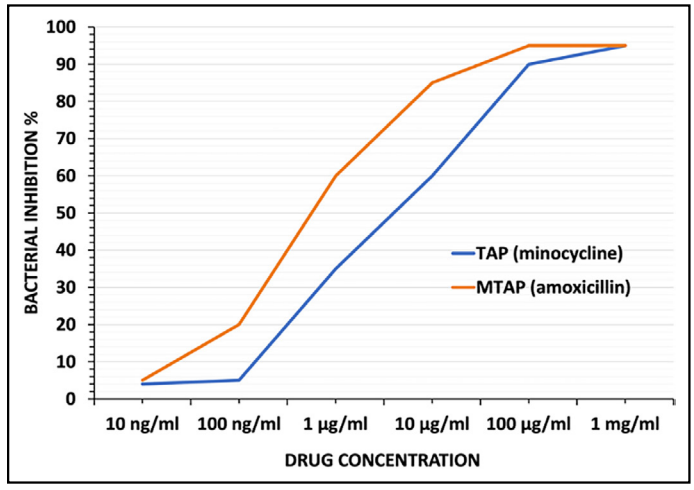

Figure 4. Minimum inhibitory concentration (MIC) tests for TAP (minocycline) and MTAP (amoxicillin) against *Enterococcus faecalis*.

However, it is crucial to note that the current study is considered preliminary, signifying the need for subsequent investigations to fully comprehend the hydrogel's properties and performance. The limitations of this study include the fact that *in vitro* studies may not comprehensively represent the clinical potential of the substance under investigation. Furthermore, it's worth noting that this study focused solely on single-species biofilm analysis. Nevertheless, a more comprehensive evaluation of the medicament's efficacy could have been achieved by including multi-species biofilms in the assessment. While carrageenan stands out as a favourable carrier, the hydrogel's formulation may necessitate further fine-tuning to optimise its characteristics and functionalities. This aspect, identified as a potential weakness of the study, emphasizes the ongoing nature of research and the necessity for continued exploration and refinement to enhance the hydrogel's effectiveness for its intended applications.

Recent research suggests that antibiotics, at the concentrations used in clinical practice, have both direct and indirect cytotoxic effects on stem cells and pulp fibroblasts (28). Various antibiotic combinations at 1 mg/mL have been shown to have antibiofilm effects against endodontic pathogens while having no indirect cytotoxic effects on apical papilla stem cells (29, 30). This is the recommended concentration by the American Association of Endodontists to be used in regenerative endodontics. In this study, the formulated hydrogel possesses sustained release properties attributed to the incorporation of carrageenan as a carrier agent. Furthermore, the hydrogel exhibits reduced toxicity, attributed to both the low concentration of antibiotics and the use of a biocompatible and biodegradable carrier agent. These characteristics may contribute to enhanced tissue healing and improved compatibility with tissues. Moreover, the pre-fabrication of these hydrogels in the appropriate concentration and gel-like consistency could enhance their ease of use.

Future studies assessing the effectiveness of the antibiotic hydrogel involving disk diffusion models with polymicrobial biofilm have to be undertaken at different periods (31). This research produced some information that could be applied to developing novel drug-loaded Carrageenan hydrogel for endodontic applications. The potential of drug-loaded hydrogels to penetrate and localise in dentinal tubules should be further investigated to create M-TAPH as a commercial product. The release of medication from hydrogels and its long-term antibacterial effect should also be considered in the future, as well as its antibacterial activity in the root canal model.

CONCLUSION

Based on the results of the present study, it can be concluded that M-TAP has significant antibacterial effects at low concentrations when compared to that of TAP. Considering the var-

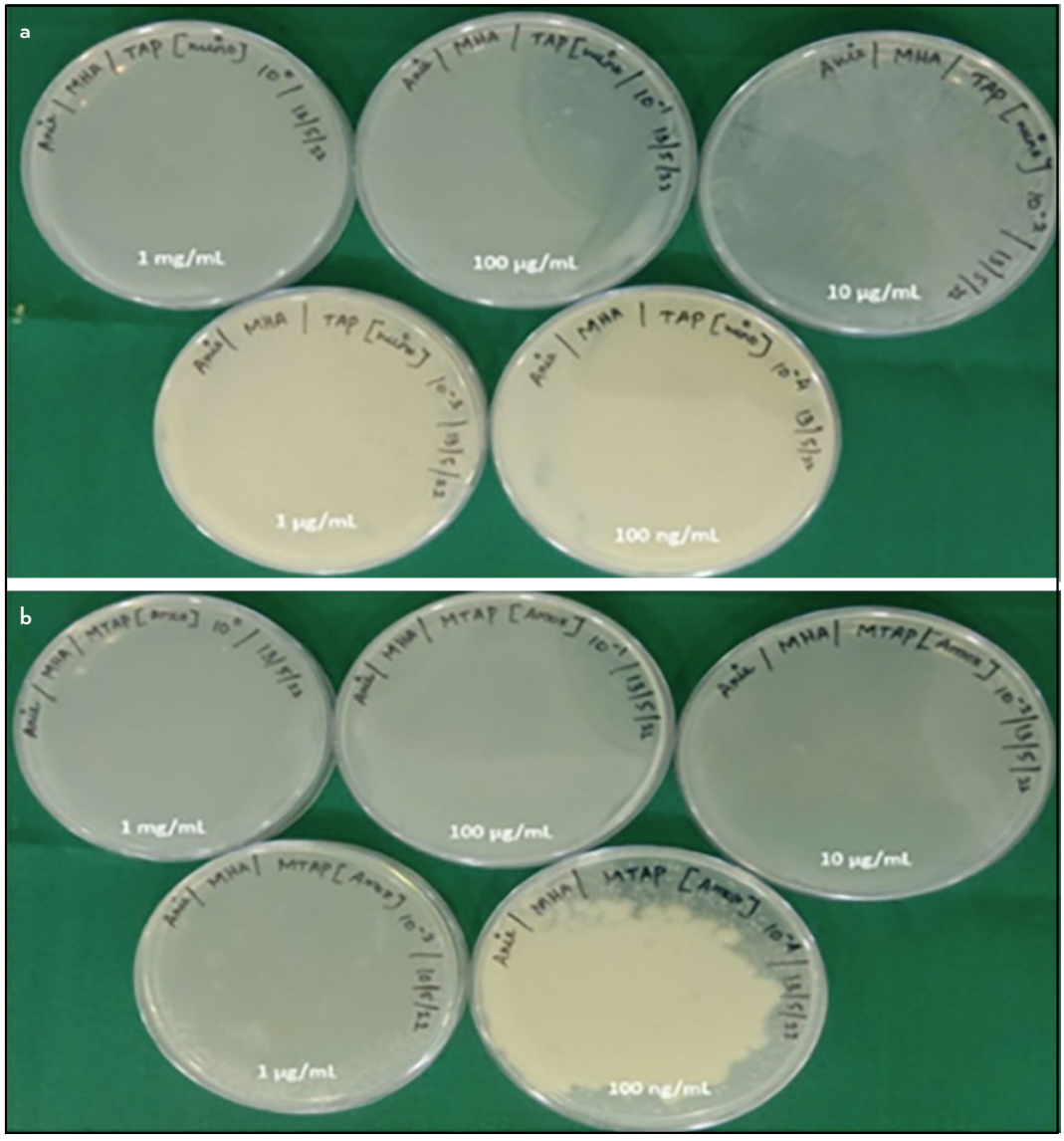

Figure 5. Minimal bactericidal activity (MBC) of (a) TAP (minocycline) and (b) MTAP (amoxicillin) against *Enterococcus faecalis*.

ious disadvantages of TAP in clinical practice, M-TAP can be regarded as a suitable substitute at lower concentrations. Furthermore, the utilisation of M-TAP incorporated into a marine biopolymer-based hydrogel is favoured due to its multitude of advantageous properties, including user-friendliness, sustained release capabilities, and biocompatibility. However, It is essential to emphasise the need for further clinical research, as the current study was conducted *in vitro* using a single microorganism. To provide a more comprehensive understanding of the medicament's efficacy and applicability in real clinical scenarios, extensive studies are strongly recommended, particularly for assessing its performance on polymicrobial biofilms.

Disclosures

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Ethics Committee Approval: The study was approved by the Saveetha Dental College and Hospital Ethics Committee (no: 2103/22/147, date: 16/03/2022).

Authorship Contributions: Concept – S.K.; Design – R.E.; Supervision – J.K.; Funding – A.S.V.; Materials – R.E.; Data collection and/or processing – A.S.V.; Data analysis and/or interpretation – R.E., A.S.V.; Literature search – N.M.S.; Writing – A.S.V.; Critical review – J.K.

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