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Article

Propylene Glycol Potentiates the Inhibitory Action of CTZ Paste on Antibiotic-Resistant *Enterococcus faecalis* Isolated from the Root Canal: An *In Vitro* Study

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Abstract: This study aimed to evaluate if the change of vehicle for CTZ (chloramphenicol, tetracycline, zinc oxide, and eugenol) paste improves the inhibition of *Enterococcus faecalis in vitro*. The vehicles evaluated alone and mixed with CTZ were eugenol, propylene glycol (PG), chitosan, super-oxidized solution (SOS), grapefruit-seed extract (GSE), and as a negative control, 0.9% saline solution. A clinical isolated of *E. faecalis* was morphological and biochemical characterized, including antimicrobial susceptibility was tested using 20 antimicrobial agents. Once characterized, the clinical isolated was cultivated to performed Kirby–Bauer disc diffusion method with paper discs embedded with the different vehicles mixed or used alone, incubating at 37 ºC for 24 h. Data were analyzed by One-way ANOVA and the means were compared using Tukey test with a significance level of $p<0.05$. For vehicles used alone, GSE presented the greatest inhibition showing a statistically significant difference with the rest of the vehicles. When vehicles were mixed with the CTZ paste, PG showed a greater inhibition with a statistically significant difference from the rest of the vehicles. In conclusion, the vehicle used to mix the CTZ paste plays an important role in the inhibition of *E. faecalis in vitro*, therefore we consider that this can be an important factor to achieve success in the use of this technique.

Keywords: CTZ paste; grapefruit-seed extract; propylene glycol; antibiotic resistance; *E. faecalis*

1. Introduction

Dental caries, is an important disease in children, and being the most prevalent disease in oral cavity at this stage of life [1]. Nowadays, it has been reported in some countries with a prevalence greater than 90% in 6-year-old population therefore that the authors have considered to be a public health crisis [2].

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This disease destroys dental hard tissue affecting pulp vitality with a high risk of development of pulp and periapical lesions [3]. To eradicate this polymicrobial infection with aerobic and anaerobic bacteria [4-5], it has been proposed the non-instrumentation endodontic treatment (NIET) using a mixture of antibacterial drugs placed over the pulp floor [6], with the objective to prevent over instrumentation of root canals and irritation of periapical tissue decreasing chair time to only one visit [7], since endodontic treatment of primary teeth could be a challenge to the presence of root resorption and the successor tooth [6].

Antibiotic pastes have been proposed in endodontic therapy by their antimicrobial capacity and low cost [8]. Since, if infected primary teeth are not effectively treated, premature tooth extraction could be necessary, affecting masticatory function, maxillofacial and systemic growth [9] and increasing the risk of malocclusions [10]. Several antibiotics have been used in the preparation of these pastes, such as ciprofloxacin, metronidazole, minocycline, clindamycin [11], ornidazole [12] or rifampicin [13]. Interestingly, a mixture of chloramphenicol 500 mg, tetracycline 500 mg, zinc oxide 1000 mg and one drop of eugenol (CTZ) in a ratio of 1:1:2 was introduced for Cappiello in 1964 showing promising results [14]. The success of this paste is due to its composition, since, chloramphenicol is a broad spectrum antibiotic [15] with high solubility and bacteriostatic effect against gram positive anaerobic and gram negative aerobic bacteria [16]; the tetracycline is a broad-spectrum bacteriostatic agent that inhibits bacterial protein synthesis [17], and also the antimicrobial capacity of the zinc oxide supposedly effected by the production of hydrogen peroxide [18], however, some authors it has been reported that the zinc oxide alone has no inhibitory effect and it is the eugenol which has the antimicrobial action [19-20].

In the clinic are used several other types of paste to disinfection of the root canal system and also to create a favorable environment for endodontic regeneration such as the Triple Antibiotic Paste (TAP) which is composed by ciprofloxacin, metronidazole, and minocycline [21-22] . Herein, vehicles are well-known to be capable of interfering in several properties of endodontic materials, including cytotoxicity [23-25], specifically in TAP, it has been highlighted the importance of the vehicle since the use of the combination of Polyethylene Glycol and Propylene Glycol (PG) mixed as vehicle exhibit less cytotoxicity than when water is used [26]. In addition, the change of vehicle can interfere in the antimicrobial capabilities of the TAP, since some of them can improve this property against some microorganism [27].

Actually. it has been reported that antibiotic resistance decreases the antibacterial properties of endodontic filling pastes for root canal treatment [13], which highlights the importance of looking for vehicles that enhance the antimicrobial effects of these pastes. In the CTZ paste the conventional vehicle recommended by Cappiello it is the eugenol [14], however other type of substance can be tested to achieve this aim such as PG, a dihydric alcohol [28], which has bactericidal activity [29], and as a vehicle has demonstrated to improve the diffusing property of drugs [30]. Interestingly, chitosan has regeneration-inducting as well as antibacterial capabilities makes it a promising biomaterial with several applications on dentistry such as drug delivery vehicles [31]. Also, Grapefruit-seed extract (GSE) is a natural product obtained from *Citrus paradisi*, grinding their seeds, pulp and white membranes mixing them with glycerin [32]. This extract has shown a powerful antimicrobial activity [33] mainly attributed to the presence of polyphenolic compounds, flavonoids, citric and ascorbic acid, tocopherol and limonoid [34]. Interestingly, super-oxidized solution (SOS) which is an electrochemically processed solution made from water and NaCl present antimicrobial capacity, since has been proposed to clean of root canal walls and including is able to eradicate *Enterococcus faecalis* (*E. faecalis*) [35-37].

Several microorganisms, such as *E. faecalis* are present in endodontic infections, this one, has been widely studied due to its resistance to conventional endodontic treatment [38], being one of the most important bacteria on the root canal [39]. *E. faecalis* is a gram-positive facultative anaerobe cocci bacteria that that inhabits the human oral cavity, gastrointestinal tract, and vagina [40]. This bacteria has the capacity to invade dentinal tubules due to the presence of a collagen-binding protein (Ace) [41], and resist nutritional deprivation [42], as well as, delayed penetration of antimicrobial agents by the presence of its enterococcal surface protein (*Esp*), producing a biofilm of polystyrene [43]. Also survive in environments of 10° to 60° of temperature, avoiding the action of lymphocytes [44], with the ability

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to grow in high pH ambient [45], even forming a biofilm in the presence of $Ca(OH)_2$ solutions [46], mostly for this facility to attachment to abiotic surfaces [47] and other bacteria, serum, collagen and dentin [48]. Hence the virulence factors of *E. faecalis* as adhesion and colonization, resistance to host defense, inhibition on other bacteria, tissue damage and induction of inflammation summarized by Kayaoglu et al. [48], emphasize on the necessity to find strategies to manage the elimination of this microorganism.

Until now, no studies have been found in the literature evaluating if the change of vehicle in this paste can modified the drug delivery and potentiate their action. For the above, the aim of this study was to evaluate if these products inhibit *E. faecalis* by themselves or have the capacity to potentiate the antimicrobial effect of CTZ paste.

2. Materials and Methods

Materials

The evaluated vehicles were PG (Harleco, Mexico), chitosan (Sigma-Aldrich, USA), SOS (Esteripharma, México) and grapefruit-seed extract (Nutribiotic, Mexico) and as positive control and negative control were used eugenol (Viarden, Mexico) and 0.9% saline solution (S.S.) (PISA, Mexico), respectively. The CTZ (Farmacia Galenico, Mexico) was commercially obtained to avoid the variation in the formulation and the presence of excipients. The bacteria *E. faecalis* was donated by the Research Department of Endodontics of the Stomatology Faculty of the Autonomus University of San Luis Potosí, where was isolated from the root canal of patients with secondary endodontic infections employing a sterile paper tips, and subsequently cultivated in thioglycolate tubes that were incubated in an anaerobic chamber [49].

E. faecalis characterization

E. faecalis was characterized by gram staining for the determination of cell morphology and to classify in gram-positive or gram-negative bacteria using an optical microscopy. Then, biochemical test with 26 substrates and antibiotic resistance of 20 drugs were determinated using the kit Microscan pos combo panel type 33 (Beckman coulter Cat. #B1017-211) following the manufacturer's instructions.

Growth kinetic of E. faecalis

E. faecalis was cultivated in Tryptic Soy Broth (TSB) medium evaluating absorbance at 600 nm (Optizen pop, Mecasys, South Korea) every hour for 12 h to establish growth kinetics. Additionally, serial dilutions were made in trypto-casein soy agar plates (TSA) to evaluate colony forming units (CFU), evaluating cell viability during the growth kinetics.

Drugs preparation

500 mg of CTZ was mixed with the vehicles, using a different amount of each to obtain a paste with a firm and adhesive consistency (Table 1). The chitosan powder was incorporated with 0.4% of acetic acid in glycerol to obtain a hydrogel, for this was weight 1.34 g and mixed with 3 mL of saline solution to get a paste with the same consistency of the other groups.

Table 1. Amount and vehicle concentration.

Vehicle	Amount	Concentration
Eugenol	200 µL	37.50%
Electrolyzed super oxidation solution	200	0.00%
Grapefruit seed extract	600 µL	46%
Propylene glycol	400 µL	99.50%
Chitosan	1.34 g with 3 mL of S.s.	44.60%
Saline solution (S.s.)	400 µL	0.90%

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Kirby–Bauer disc diffusion method

The *E. faecalis* was adjusted equal to 0.5 McFarland standard by adding sterile distilled water, which corresponds to ~1.5x10^8 cells/mL. The bacterial suspension of 100 µL was distributed throughout to the plate with Müller Hilton agar (Becton Dickinson, USA) to place a triplicate of absorbent paper discs of 5 mm in diameter impregnated of each mixture or a triplicate of absorbent paper disc impregnated only with 5 µL of each different vehicles and as a control, discs impregnated with S.S. was employed. All plates were incubated 24 h at 37 ºC. After incubation the plates were observed and the inhibition zone was measured with a digital vernier (CALDI-6MP, Truper).

Statistical analysis

For the analysis of the data, means and standard deviation of the inhibition zone size were used. One-way ANOVA and Tukey test were used with a significance level of $p<0.05$ using GraphPad prism v.8.

3. Results

E. faecalis exhibit resistance behavior to antibiotics

The clinical isolated of E. faecalis, showed the typical cell morphology of this bacteria as Gram-positive cocci in pairs and short chains on Gram stain (Figure 1A). Also, the biochemical characterization showed that the clinical isolated has a 99.9% of correspondence with E. faecalis (Table 2). Furthermore, this characterization was complemented by resistance to antibiotics analysis (Table 3), observing that this bacterium was sensitive to Ampicillin, Ciprofloxacin, Daptomycin, Gentamicin, Penicillin, Rifampicin, and intermediate resistance to Linezolid. Interestingly, the clinical isolated was resistance to Erythromycin, Streptomycin and Tetracyclin, the latter being one of the main components of CTZ paste, highlighting the importance to improving the antimicrobial effect of CTZ paste.

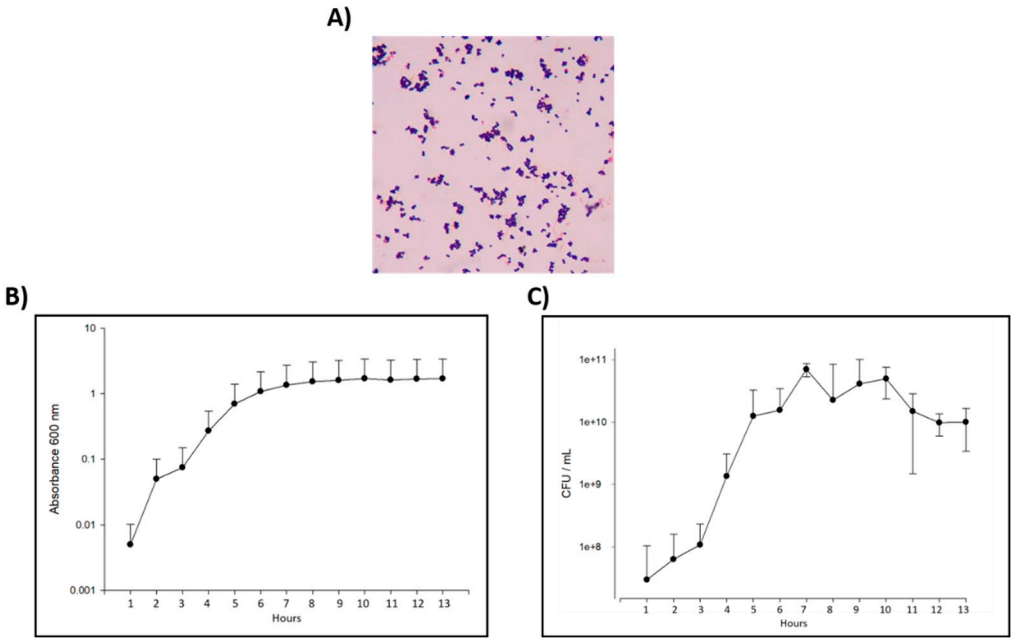

Figure 1. Morphology analysis, growth kinetics and viability of E. faecalis. A) Morphology analysis by Gram stainning of E. faecalis using optical microscopy, B) Growth kinetics establishment of E. faecalis by evaluating the absorbance at 600 nm, C) Cell viability analysis of E. faecalis by colony-forming unit (CFU).

Table 2. Biochemical test characterization of E. faecalis.

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Substrates	Result
Crystal violet	+
Micrococcus screen	+
Nitrate	-
Novobiocin	+
PNP-β-D-Glucuronide	-
Indoxyl phosphatase	-
Voges-Proskaure	-
Optochin	+
Phosphatase	+
40 % Bils esculin	+
L-Pyrrolidonyl-β-naphtothylamide	+
Arginins	+
PNP-β-D-galactopyranoside	+
Urea	-
Mannitol	+
Lactose	+
Trehalose	+
Mannose	+
Sodium Chloride 6.5 %	+
Sorbitol	+
Arabinose	-
Ribose	+
Inulin	-
Raffinose	-
Bacitracin	+
Pyruvate	+

Table 3. Antibiotics resistance test performed to E. Faecalis. *at the discretion of medical interpretation.

Antimicrobial agent	MIC (µg/mL)	Interpretation
Amoxicillin clavulanic acid	<=4/2	*
Ampicillin sulbactam	<=8/4	*
Ampicillin	<=2	Sensitive
Ceftriaxone	>32	*
Ciprofloxacin	<=1	Sensitive
Clindamicina	>4	*
Daptomycin	2	Sensitive
Erythromycin	>4	Resistance
Erythromycin synergy	>1000	Resistance
Gentamicin synergy	<=500	Sensitive
Gentamicin	8	*
Levofloxacin	2	Sensitive
Linezolid	4	Intermediate
Moxifloxacin	<=0.5	*
Nitrofurantoin	<=32	*
Oxacillin	>2	*
Penicillin	2	Sensitive
Rifampicin	<=1	Sensitive
Tetracycline	>8	Resistance
Trimethoprim sulfamethoxazole	>2/38	*

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E. faecalis has a conventional pattern of growth

The results of the bacterial calibration curve using OD showed that E. faecalis have a lag phase duration of 3 h, the logarithmic growth starts at hour 3 and continue for 6 more hours when the bacteria entrance in to the stationary phase (Figure 1B). Viability evaluation was perform counting the CFU each hour in a period of 13 h, observing that the growth peak for E. faecalis starts at hour 4 (Figure 1C), so at this time the bacteria was take it to carry out the antibiogram test with the CTZ paste mixed with different vehicles.

PG potentiates CTZ paste effect on E. faecalis

The results of the bacterial growth inhibition by CTZ paste mixed with different vehicles after 24 h showed that the bacterial growth of the paste it was not potentiated when was mixed with SOS (32.6±2.0 mm, p= 0.2000) and GSE (26.8±2.5 mm, p= 0.9891), showing no significant difference to positive control Eugenol (30.8±0.1), however, PG did show greater inhibition of bacterial growth (36.9±1.0 mm, p= 0.0021) compared to the positive control (Figure 3A). Interestingly, no significant difference was found when eugenol was compared to negative control S.S. (26.8±2.5 mm, p= 0.1542), meaning that this vehicle no promotes a real antibacterial capacity of the CTZ paste.

GSE has a potential antibacterial effect used alone

To evaluated if the vehicles used to enhance the effect of the CTZ have antimicrobial capacity when is used alone, Kirby–Bauer disc diffusion method was employed finding that S.S and Chitosan have a null antibacterial effect, however, PG (13.8±0.8, p= 0.9987) has a similar effect of Eugenol on E. faecalis (14.2±0.3), and interestingly a major inhibition was found by SOS (20.3±1.5, p= 0.0023) and GSE (30.9±3.6, p< 0.0001), been the last one the vehicle with the highest antibacterial effect (Figure 3B), proposing GSE as a solution that could be used alone as an irrigant.

GSE extract does not need antibiotics to improve its bactericidal capacity

The comparison of the inhibition of the bacterial growth of the vehicle alone and mixed with CTZ showed that the antibacterial effect shown by the Chitosan (p< 0.0001) and the S.s (p< 0.0011) mixed with the paste is due to their components, since they used alone did not show any inhibition in the growth of E. faecalis (Figure 2C). Nevertheless, both the conventional vehicle Eugenol from the CTZ (p< 0.0001), as well as SOS (p= 0.0011) and PG (p< 0.0001) increase their antibacterial potential once mixed with the paste. Interestingly, no significant differences were found when compared GSE used alone to mixed to the paste (p= 0.9762), therefore, antibiotics are not necessary to improve its bactericidal capacity, could be used alone as an irrigant solution.

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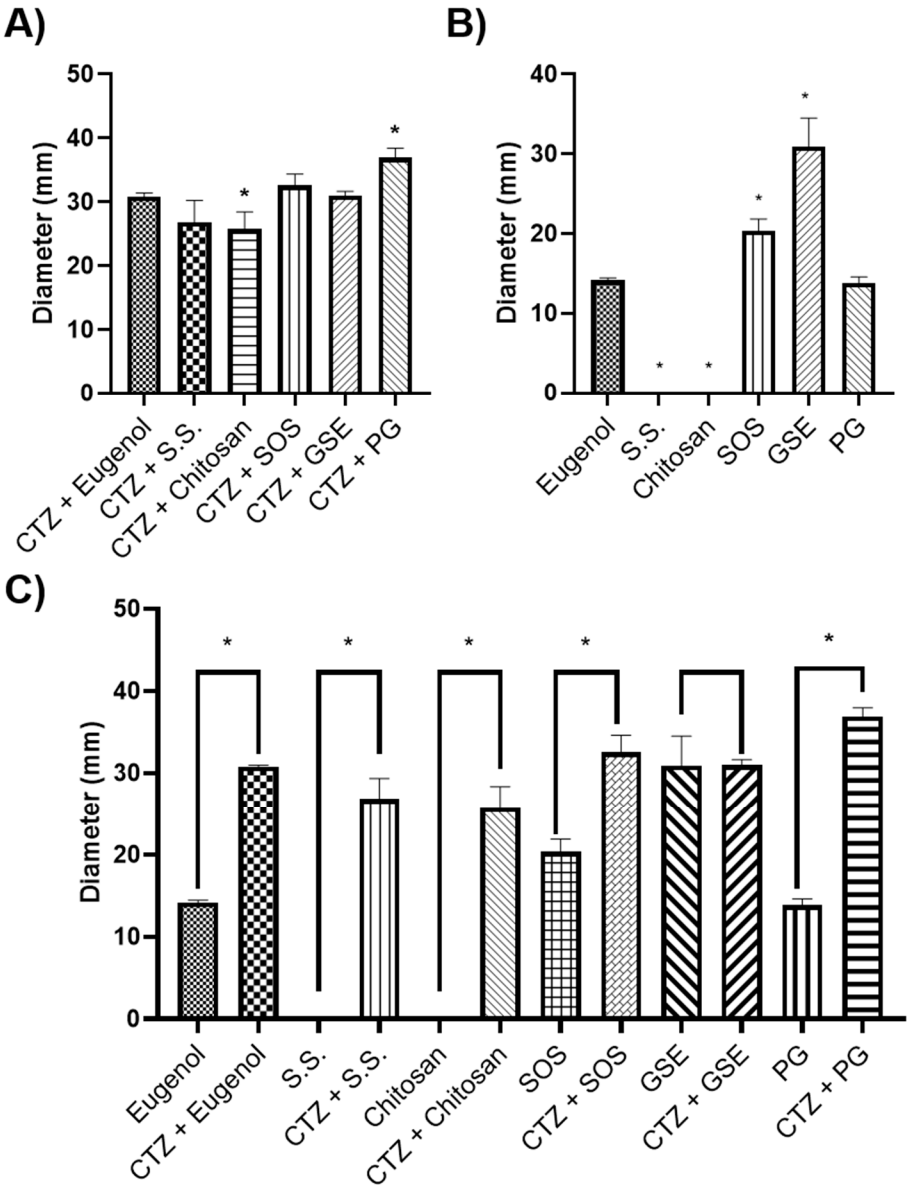

Figure 2. Effects evaluation of different vehicles mixed with CTZ paste or alone on E. faecalis. A) Analysis of the inhibition of E. faecalis using the mixes of CTZ paste with the different vehicles, B) Analysis of the inhibition of E. faecalis using the different vehicles, C) Comparison analysis of E. faecalis inhibition using the mixes of CTZ paste with the different vehicles and the vehicles used alone. * means $p < 0.05$.

4. Discussion

It has been reported that the presence of *E. faecalis* is similar in temporary and permanent teeth [50], so it is important to look for alternatives that allow the clinician to eliminate this microorganism. Currently, the use of a mixture of broad spectrum antibiotics has been introduced as a treatment for pulp therapy called CTZ paste [51], which have shown *in vitro* capacity to eliminate *E. faecalis* with the use of eugenol as a vehicle [15, 52-53], however, it has been reported that the change in the vehicle can improve the diffusion and release of this drugs [54], thus this study aimed to assess if the change of vehicle for CTZ paste improves the inhibition of *E. faecalis in vitro.*

Isolated strain in this study was examined in terms of antimicrobial susceptibility by a wide range of antibiotics and showed resistance to a large number of them such as Erythromycin, Streptomycin and Tetracyclin, however some of them being effective against *E. faecalis* such as to

Ampicillin, Ciprofloxacin, Daptomycin, Gentamicin, Penicillin, Rifampicin. The above result differ to Pazhouhnia et al. [55], since they reported a different pattern of antibiotics resistance in several *E. faecalis* isolated strains from root canal of individuals with periodontitis, interestingly, agreeing that both the strains isolated by them and our clinical isolate of *E. faecalis* are resistant to tetracycline, one of the main components of CTZ paste. This highlights the variability in pathogenicity that can be found in different clinical isolates.

The bactericidal capacity of eugenol is due its ability to cause hydrophobicity, this alters the cell membrane making it more permeable [20], which leads to the extreme loss of molecules and ions and finally the cell death [56]. Despite of this, in our results eugenol shows a medium inhibition alone and mixed with CTZ compare with the rest of the vehicles. The above results match with De Sales Reis et al. [53], who reported a similar inhibition to us of *E. faecalis* with CTZ paste mixed with eugenol. In the case of de Oliveira et al.[15], they reported a major inhibition of *E. faecalis*, however they employed a clinical isolated as we mentioned above, different isolates may differ in their pathogenicity.

Currently, chitosan is one of the most important drug delivery system [57]. Additionally, it has been reported that drug-free chitosan has antimicrobial activity against both Gram-negative and Gram-positive bacteria [58], including against *E. faecalis* [59], however, despite of the above reports, in our results we did not find any effect of chitosan used alone or as a vehicle for CTZ paste. We consider that this result was related with our high chitosan concentration since the dense consistency did not allow the release of the active principle.

Propylene glycol (1,2-propanediol), a dihydric alcohol [28], is a vehicle that has demonstrated to improve the diffusing property of drugs [30], which also have bactericidal activity [29]. In our results, this vehicle showed similar inhibition to eugenol against *E. faecalis* used alone, which differs from what was reported by Thomas et al. [60], who found that eugenol presented antibacterial properties against *E. faecalis*, meanwhile, PG did not, which coincides with other authors [61]. Conversely, other studies showed a bactericidal effect of 25 % against *E. faecalis* [29]. As vehicle, PG mixed with endodontic medicament has shown antimicrobial activity against several bacteria including *E. faecalis in vitro* [54]. Also, Pereira et al. evaluated this vehicle with tri-antibiotic pastes (TAP) against an ATCC of *E. faecalis in vitro* showing intratubular decontamination [61]. In our study, when this vehicle was mixed with CTZ achieved the highest halo of inhibition compared to the rest of the vehicles, this could be related with the hygroscopic nature and viscosity of PG which allow the sustained release of ions increasing the antibacterial properties of the drugs [62]. Our results showed that this vehicle allow the optimal release of the active principles.

In addition, super-oxidized solutions have been shown to be potent antimicrobial agents and disinfectants through oxidative damage [63], since electrolyzed water contains a mixture of inorganic oxidants, such as hypochlorous acid (HClO), hypochlorous acidic ion (ClO-), chlorine (Cl2), hydroxide (OH), and ozone (O3). Controversial results have been described to SOS antimicrobial capacity, since it may or may not be effective in eliminating *E. faecalis* from the root canal compared to NaOCl [36-37]. Despite the above, our result showed that SOS may have an intermediate antibacterial effect against our clinical isolated of *E. faecalis* used alone, however, its properties as a vehicle are not remarkable compared to the other materials evaluated.

Grapefruit seed extract is a natural product obtained from *Citrus paradisi*, grinding their seeds, pulp and white membranes mixing them with glycerine [64]. This extract has shown a powerful antimicrobial activity [65] mainly attributed to the presence of polyphenolic compounds, flavonoids, citric and ascorbic acid, tocopherol and limonoid[34]. This capacity has been evaluated against an ATCC reference strains of *E. faecalis*, showing from low [65] to high [66] antibacterial effect against this bacterium. In our study with *E. faecalis* clinical isolated, a great inhibition was observed, this could be related with the grapefruit seed extract concentration since in the previous studies were performed at concentration of 33%, meanwhile we employed a higher concentration of 46%. Although this extract by itself obtained the highest inhibition zone compared with the rest of the vehicles, the result was not the same when was mixed with CTZ showing a similar inhibition to eugenol, which is the recommend vehicle. Until now, no studies have evaluated this extract as a vehicle for CTZ, we observed that does not improve their antimicrobial activity, we consider that is not the ideal vehicle

for the application of these antibiotics, although, could be used alone as irrigation solution, since it has demonstrated antimicrobial activity against bacteria gram negative and positive and yeast [65]. It is very interesting since it could be used in primary and permanent endodontic procedures that includes periodontal tissues without side effects. Another putative advantage of this natural product, is that the bacteria does not show resistance to this product since it is a new substance, being the resistance an important persistent complication in endodontic treatment., and which have become in a global issue [13]. Therefore, it is promissory to use it as a single solution against *E. faecalis* and other bacterial strains present in the oral microbiome associated with active dental infections. However, further experiments need to be performed inside the root canal**.**

Green technology extracts could be an excellent option against the oral microbiome associated with active dental infections. Furthermore, it is an excellent option to use plant or fruit extracts to clean the root canal system from the irrigation phase; currently the use of blueberry and wild strawberry extracts [67] and enzymes from the peel of papaya orange and pineapple [68], has been proposed as an alternative to sodium hypochlorite (NaOCl), to avoid cause severe injuries that might occur with NaOCl accidents or in allergic patients [69], showing promising results in an *in vitro* study versus *E. faecalis*.

The effectiveness of GSE in the inhibition of biofilms by ***Porphyromonas endodontalis*** and ***Porphyromonas gingivalis*** it has been evaluated *in vitro* showing a promising antibiofilm activity, also a cytotoxicity assay with human gingival fibroblast was carried out observing high biocompatibility; missing characteristic in NaOCl, that can cause from a burning sensation in gingiva to periapical tissue necrosis [70].

5. Conclusions

The vehicle used to mix the CTZ paste plays an important role in the effect of inhibition against *E. faecalis in vitro*, although this is not the only bacteria that the clinician faces during pulp therapy, is one of the most resistant, therefore its eradication is extremely important. Our results showed that PG could be the best vehicle for the CTZ paste. Henceforth, it is necessary to evaluate if PG mixed with CTZ maintain a highest inhibition that eugenol against other microorganism related with pulpal pathologies.

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References

1. Kazeminia, M.; Abdi, A.; Shohaimi, S.; Jalali, R.; Vaisi-Raygani, A.; Salari, N.; Mohammadi, M., Dental caries in primary and permanent teeth in children's worldwide, 1995 to 2019: a systematic review and meta-analysis. *Head & face medicine* **2020,** *16* (1), 22.
2. Bagramian, R. A.; Garcia-Godoy, F.; Volpe, A. R., The global increase in dental caries. A pending public health crisis. *American journal of dentistry* **2009,** *22* (1), 3-8.
3. Organization, W. H., Sugars and dental caries. 2017
4. Reddy, S.; Ramakrishna, Y., Evaluation of antimicrobial efficacy of various root canal filling materials used in primary teeth: a microbiological study. *J Clin Pediatr Dent* **2007,** *31* (3), 193-198.

5. Soto-Sainz, J. E.; Alarcón-Romero, P.; Gastélum-Rosales, G.; Castro-Salazar, Y.; Ramos-Payán, R.; Romero-Quintana, G.; Aguilar-Medina, M., Presence of red complex bacteria and Aggregatibacter actinomycetemcomitans in necrotic primary teeth with periapical abscess in children of Sinaloa, México. *Revista Biomédica* **2020,** *31* (2), 76-83.
6. Chouchene, F.; Masmoudi, F.; Baaziz, A.; Maatouk, F.; Ghedira, H., Antibiotic Mixtures in Noninstrumental Endodontic Treatment of Primary Teeth with Necrotic Pulps: A Systematic Review. *International journal of dentistry* **2021,** *2021*, 5518599.
7. Nanda, R.; Koul, M.; Srivastava, S.; Upadhyay, V.; Dwivedi, R., Clinical evaluation of 3 Mix and Other Mix in non-instrumental endodontic treatment of necrosed primary teeth. *J Oral Biol Craniofac Res* **2014,** *4* (2), 114-9.
8. Luengo-Fereira, J.; Ayala-Jimenez, S.; Carlos-Medrano, L. E.; Toscano-Garcia, I.; Anaya-Alvarez, M., Clinical and Radiographic Evaluation of Formocresol and Chloramphenicol, Tetracycline and Zinc Oxide-Eugenol Antibiotic Paste in Primary Teeth Pulpotomies: 24 month follow up. *J Clin Pediatr Dent* **2019,** *43* (1), 16-21.
9. Zou, J.; Meng, M.; Law, C. S.; Rao, Y.; Zhou, X., Common dental diseases in children and malocclusion. *Int J Oral Sci* **2018,** *10* (1), 1-7.
10. Bhujel, N.; Duggal, M. S.; Saini, P.; Day, P. F., The effect of premature extraction of primary teeth on the subsequent need for orthodontic treatment. *Eur Arch Paediatr Dent* **2016,** *17* (6), 423-434.
11. Raslan, N.; Mansour, O.; Assfoura, L., Evaluation of antibiotic mix in Non-instrumentation Endodontic Treatment of necrotic primary molars. *European journal of paediatric dentistry* **2017,** *18* (4), 285-290.
12. Sain, S.; J, R.; S, A.; George, S.; J, S. I.; S, A. J., Lesion Sterilization and Tissue Repair-Current Concepts and Practices. *Int J Clin Pediatr Dent* **2018,** *11* (5), 446-450.
13. Rivera-Albarrán, C. A.; Morales-Dorantes, V.; Ayala-Herrera, J. L.; Castillo-Aguillón, M.; Soto-Barreras, U.; Cabeza-Cabrera, C. V.; Domínguez-Pérez, R. A., Antibiotic Resistance Decreases the Efficacy of Endodontic Filling Pastes for Root Canal Treatment in Children's Teeth. *Children (Basel, Switzerland)* **2021,** *8* (8).
14. Cappiello, J., Tratamientos pulpares en incisivos primarios. *Rev Asoc Odontol Argent* **1964,** *52* (4), 139-45.
15. de Oliveira, S. C. M.; de Omena, A. L. C. S.; de Lucena Lira, G. A.; Ferreira, I. A.; Imparato, J. C. P.; Calvo, A. F. B., Do Different Proportions of Antibiotics in the CTZ Paste Interfere with the Antimicrobial Action? In Vitro Study. *Pesquisa Brasileira em Odontopediatria e Clínica Integrada* **2019,** *19*, 4801-4808.
16. Sanchez-Borges, M.; Thong, B.; Blanca, M.; Ensina, L. F.; Gonzalez-Diaz, S.; Greenberger, P. A.; Jares, E.; Jee, Y. K.; Kase-Tanno, L.; Khan, D.; Park, J. W.; Pichler, W.; Romano, A.; Jaen, M. J., Hypersensitivity reactions to non beta-lactam antimicrobial agents, a statement of the WAO special committee on drug allergy. *World Allergy Organ J* **2013,** *6* (1), 1-23.
17. Soares, G. M.; Figueiredo, L. C.; Faveri, M.; Cortelli, S. C.; Duarte, P. M.; Feres, M., Mechanisms of action of systemic antibiotics used in periodontal treatment and mechanisms of bacterial resistance to these drugs. *J Appl Oral Sci* **2012,** *20* (3), 295-309.
18. Seil, J. T.; Webster, T. J., Antimicrobial applications of nanotechnology: methods and literature. *International journal of nanomedicine* **2012,** *7*, 2767-81.
19. Harini Priya, M.; Bhat, S. S.; Sundeep Hegde, K., Comparative evaluation of bactericidal potential of four root canal filling materials against microflora of infected non-vital primary teeth. *J Clin Pediatr Dent* **2010,** *35* (1), 23-9.
20. Jaidka, S.; Somani, R.; Singh, D. J.; Sheikh, T.; Chaudhary, N.; Basheer, A., Herbal combat against E. faecalis - An in vitro study. *J Oral Biol Craniofac Res* **2017,** *7* (3), 178-181.
21. Sabrah, A. H. A.; Yassen, G. H.; Liu, W.-C.; Goebel, W. S.; Gregory, R. L.; Platt, J. A., The effect of diluted triple and double antibiotic pastes on dental pulp stem cells and established Enterococcus faecalis biofilm. *Clinical Oral Investigations* **2015,** *19* (8), 2059-2066.
22. Tawfik, H.; Abu-Seida, A. M.; Hashem, A. A.; Nagy, M. M., Regenerative potential following revascularization of immature permanent teeth with necrotic pulps. *International endodontic journal* **2013,** *46* (10), 910-922.
23. Leonardo, M. R.; Bezerra da Silva, L. A.; Leonardo, R. d. T.; Utrilla, L. S.; Assed, S., Histological evaluation of therapy using a calcium hydroxide dressing for teeth with incompletely formed apices and periapical lesions. *Journal of Endodontics* **1993,** *19* (7), 348-352.
24. Nelson Filho, P.; Silva, L. A.; Leonardo, M. R.; Utrilla, L. S.; Figueiredo, F., Connective tissue responses to calcium hydroxide-based root canal medicaments. *International endodontic journal* **1999,** *32* (4), 303-11.
25. Duarte, M. A. H.; Alves de Aguiar, K.; Zeferino, M. A.; Vivan, R. R.; Ordinola-Zapata, R.; Tanomaru-Filho, M.; Weckwerth, P. H.; Kuga, M. C., Evaluation of the propylene glycol association on some physical and chemical properties of mineral trioxide aggregate. *International endodontic journal* **2012,** *45* (6), 565-570.
26. Faria, G.; Rodrigues, E. M.; Coaguila-Llerena, H.; Gomes-Cornelio, A. L.; Neto Angeloco, R. R.; Swerts Pereira, M. S.; Tanomaru Filho, M., Influence of the Vehicle and Antibiotic Formulation on Cytotoxicity of Triple Antibiotic Paste. *J Endod* **2018,** *44* (12), 1812-1816.

27. Mandal, S. S.; Margasahayam, S. V.; Shenoy, V. U., A Comparative Evaluation of the Influence of Three Different Vehicles on the Antimicrobial Efficacy of Triple Antibiotic Paste against Enterococcus faecalis: An In vitro Study. *Contemporary clinical dentistry* **2020,** *11* (2), 150-157.
28. Cruz, E. V.; Kota, K.; Huque, J.; Iwaku, M.; Hoshino, E., Penetration of propylene glycol into dentine. *International endodontic journal* **2002,** *35* (4), 330-6.
29. Nalawade, T. M.; Bhat, K.; Sogi, S. H., Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms. *Journal of International Society of Preventive & Community Dentistry* **2015,** *5* (2), 114-9.
30. Chua, E. G.; Parolia, A.; Ahlawat, P.; Pau, A.; Amalraj, F. D., Antifungal effectiveness of various intracanal medicaments against Candida albicans: an ex-vivo study. *BMC Oral Health* **2014,** *14*, 53.
31. Wieckiewicz, M.; W Boening, K.; Grychowska, N.; Paradowska-Stolarz, A., Clinical application of chitosan in dental specialities. *Mini reviews in medicinal chemistry* **2017,** *17* (5), 401-409.
32. Bevilacqua, A.; Ficelo, S.; Corbo, M. R.; Sinigaglia, M., BIOACTIVITY OF GRAPEFRUIT SEED EXTRACT AGAINST PSEUDOMONAS SPP. *Journal of Food Processing and Preservation* **2010,** *34* (3), 495-507.
33. Cvetnić, Z.; Vladimir-Knezević, S., Antimicrobial activity of grapefruit seed and pulp ethanolic extract. *Acta Pharm* **2004,** *54* (3), 243-50.
34. Kanmani, P.; Rhim, J. W., Antimicrobial and physical-mechanical properties of agar-based films incorporated with grapefruit seed extract. *Carbohydr Polym* **2014,** *102*, 708-16.
35. Tour Savadkouhi, S.; Mohtasham Maram, M.; Purhaji Bagher, M.; Afkar, M.; Fazlyab, M., In Vitro Activity of Superoxide Water on Viability of Enterococcus faecalis Biofilm on Root Canal Wall. *Iranian endodontic journal* **2021,** *16* (3), 189-192.
36. Rossi-Fedele, G.; Figueiredo, J. A.; Steier, L.; Canullo, L.; Steier, G.; Roberts, A. P., Evaluation of the antimicrobial effect of super-oxidized water (Sterilox®) and sodium hypochlorite against Enterococcus faecalis in a bovine root canal model. *J Appl Oral Sci* **2010,** *18* (5), 498-502.
37. Zan, R.; Alacam, T.; Hubbezoglu, I.; Tunc, T.; Sumer, Z.; Alici, O., Antibacterial Efficacy of Super-Oxidized Water on Enterococcus faecalis Biofilms in Root Canal. *Jundishapur J Microbiol* **2016,** *9* (9), e30000.
38. Basmaci, F.; Oztan, M. D.; Kiyan, M., Ex vivo evaluation of various instrumentation techniques and irrigants in reducing E. faecalis within root canals. *International endodontic journal* **2013,** *46* (9), 823-30.
39. Asnaashari, M.; Eghbal, M. J.; Sahba Yaghmayi, A.; Shokri, M.; Azari-Marhabi, S., Comparison of Antibacterial Effects of Photodynamic Therapy, Modified Triple Antibiotic Paste and Calcium Hydroxide on Root Canals Infected With Enterococcus faecalis: An In Vitro Study. *J Lasers Med Sci* **2019,** *10* (Suppl 1), S23-S29.
40. Alghamdi, F.; Shakir, M., The Influence of Enterococcus faecalis as a Dental Root Canal Pathogen on Endodontic Treatment: A Systematic Review. *Cureus* **2020,** *12* (3), e7257.
41. Zhang, C.; Du, J.; Peng, Z., Correlation between Enterococcus faecalis and Persistent Intraradicular Infection Compared with Primary Intraradicular Infection: A Systematic Review. *J Endod* **2015,** *41* (8), 1207-13.
42. Stuart, C. H.; Schwartz, S. A.; Beeson, T. J.; Owatz, C. B., Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. *J Endod* **2006,** *32* (2), 93-8.
43. Chiniforush, N.; Pourhajibagher, M.; Parker, S.; Benedicenti, S.; Shahabi, S.; Bahador, A., The effect of sublethal photodynamic therapy on the expression of Enterococcal surface protein (esp) encoding gene in Enterococcus faecalis: Quantitative real-time PCR assessment. *Photodiagnosis Photodyn Ther* **2018,** *24*, 311-317.
44. Prada, I.; Micó-Muñoz, P.; Giner-Lluesma, T.; Micó-Martínez, P.; Collado-Castellano, N.; Manzano-Saiz, A., Influence of microbiology on endodontic failure. Literature review. *Medicina oral, patologia oral y cirugia bucal* **2019,** *24* (3), e364-e372.
45. Evans, M.; Davies, J. K.; Sundqvist, G.; Figdor, D., Mechanisms Involved In The Resistance Of Enterococcus Faecalis To Calcium Hydroxide. *Australian Endodontic Journal* **2001,** *27* (3), 115-115.
46. Jhajharia, K.; Parolia, A.; Shetty, K. V.; Mehta, L. K., Biofilm in endodontics: A review. *Journal of International Society of Preventive & Community Dentistry* **2015,** *5* (1), 1-12.
47. Toledo-Arana, A.; Valle, J.; Solano, C.; Arrizubieta, M. J.; Cucarella, C.; Lamata, M.; Amorena, B.; Leiva, J.; Penadés, J. R.; Lasa, I., The enterococcal surface protein, Esp, is involved in Enterococcus faecalis biofilm formation. *Applied and environmental microbiology* **2001,** *67* (10), 4538-45.
48. Kayaoglu, G.; Ørstavik, D., Virulence factors of Enterococcus faecalis: relationship to endodontic disease. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists* **2004,** *15* (5), 308-20.
49. Galván-Pacheco, J.; Vitales-Noyola, M.; González-Amaro, A. M.; Bujanda-Wong, H.; Aragón-Piña, A.; Méndez-González, V.; Pozos-Guillén, A., Evaluation of in vitro biofilm elimination of Enterococcus faecalis using a continuous ultrasonic irrigation device. *Journal of oral science* **2020,** *62* (4), 415-419.
50. Cogulu, D.; Uzel, A.; Oncag, O.; Aksoy, S. C.; Eronat, C., Detection of Enterococcus faecalis in Necrotic Teeth Root Canals by Culture and Polymerase Chain Reaction Methods. *Eur J Dent* **2007,** *1* (4), 216-21.

51. Lokade, A.; Thakur, S.; Singhal, P.; Chauhan, D.; Jayam, C., Comparative evaluation of clinical and radiographic success of three different lesion sterilization and tissue repair techniques as treatment options in primary molars requiring pulpectomy: An in vivo study. *J Indian Soc Pedod Prev Dent* **2019,** *37* (2), 185-191.
52. Piva, F.; Italo, F.; Estrela, C., Antimicrobial activity of different root canal filling pastes used in deciduous teeth. *J. Mat. Res* **2008,** *11* (2), 171-173.
53. de Sales Reis, B.; Barbosa, C. C. N.; de Castro Soares, L.; Brum, S. C.; Cecilio, O. L.; Marques, M., Análise "in vitro" da atividade antimicrobiana da pasta ctz utilizada como material obturador na terapia pulpar de dentes decíduos. *Rev. Prouniversus* **2016,** *7* (3), 39-42.
54. Nalawade, T. M.; Bhat, K. G.; Sogi, S., Antimicrobial Activity of Endodontic Medicaments and Vehicles using Agar Well Diffusion Method on Facultative and Obligate Anaerobes. *Int J Clin Pediatr Dent* **2016,** *9* (4), 335-341.
55. Pazhouhnia, S.; Bouzari, M.; Arbabzadeh-Zavareh, F., Isolation, characterization and complete genome analysis of a novel bacteriophage vB_EfaS-SRH2 against Enterococcus faecalis isolated from periodontitis patients. *Scientific reports* **2022,** *12* (1), 13268.
56. Tantry, B. A.; Kumar, A.; Rahiman, S.; Tantry, M. N., Antibacterial evaluation and phytochemical screening of methanolic extract of Ocimum sanctum against some common microbial pathogens. *Glob Adv Res J Microbiol* **2016,** *5* (1), 1-6.
57. Desai, N.; Rana, D.; Salave, S.; Gupta, R.; Patel, P.; Karunakaran, B.; Sharma, A.; Giri, J.; Benival, D.; Kommineni, N., Chitosan: A Potential Biopolymer in Drug Delivery and Biomedical Applications. *Pharmaceutics* **2023,** *15* (4), 1313.
58. Li, Y.; Chi, Y.-Q.; Yu, C.-H.; Xie, Y.; Xia, M.-Y.; Zhang, C.-L.; Han, X.; Peng, Q., Drug-free and non-crosslinked chitosan scaffolds with efficient antibacterial activity against both Gram-negative and Gram-positive bacteria. *Carbohydrate Polymers* **2020,** *241*, 116386.
59. Wang, N.; Ji, Y.; Zhu, Y.; Wu, X.; Mei, L.; Zhang, H.; Deng, J.; Wang, S., Antibacterial effect of chitosan and its derivative on Enterococcus faecalis associated with endodontic infection. *Experimental and therapeutic medicine* **2020,** *19* (6), 3805-3813.
60. Thomas, P. A.; Bhat, K. S.; Kotian, K. M., Antibacterial properties of dilute formocresol and eugenol and propylene glycol. *Oral Surg Oral Med Oral Pathol* **1980,** *49* (2), 166-70.
61. Pereira, T. C.; Vasconcelos, L. R.; Graeff, M. S.; Duarte, M. A.; Bramante, C. M.; Andrade, F. B., Intratubular disinfection with tri-antibiotic and calcium hydroxide pastes. *Acta Odontol Scand* **2017,** *75* (2), 87-93.
62. Srinivas, S.; Jibhkate, N.; Baranwal, R.; Avinash, A.; Rathi, S., Propylene glycol: a new alternative for an intracanal medicament. *J Int Oral Health* **2016,** *8* (5), 611.
63. Huang, Y.-R.; Hung, Y.-C.; Hsu, S.-Y.; Huang, Y.-W.; Hwang, D.-F., Application of electrolyzed water in the food industry. *Food control* **2008,** *19* (4), 329-345.
64. Bevilacqua, A.; Ficelo, S.; Corbo, M. R.; Sinigaglia, M., Bioactivity of grapefruit seed extract against Pseudomonas spp. *J Food Process Pres* **2010,** *34* (3), 495-507.
65. Cvetnic, Z.; Vladimir-Knezevic, S., Antimicrobial activity of grapefruit seed and pulp ethanolic extract. *Acta Pharm* **2004,** *54* (3), 243-50.
66. Reagor, L.; Gusman, J.; McCoy, L.; Carino, E.; Heggers, J. P., The effectiveness of processed grapefruit-seed extract as an antibacterial agent: I. An in vitro agar assay. *J Altern Complement Med* **2002,** *8* (3), 325-32.
67. Davoudi, A.; Razavi, S. A.; Mosaddeghmehrjardi, M. H.; Tabrizizadeh, M., The Effect of Fragaria vesca Extract on Smear Layer Removal: A Scanning Electron Microscopic Evaluation. *Iranian endodontic journal* **2015,** *10* (3), 204-7.
68. Mavani, H. A. K.; Tew, I. M.; Wong, L.; Yew, H. Z.; Mahyuddin, A.; Ahmad Ghazali, R.; Pow, E. H. N., Antimicrobial Efficacy of Fruit Peels Eco-Enzyme against Enterococcus Faecalis: An In Vitro Study. *International journal of environmental research and public health* **2020,** *17* (14).
69. Kumar, A.; Ajitha, P.; Raghu, S.; Muralidaran, Comparative evaluation of antimicrobial activity of 3% sodium hypochlorite, 2% chlorhexidine, and 5% grape seed extract against Enterococcus faecalis and Candida albicans - An in vitro study. *Drug Invention Today* **2019,** *12*, 53-56.
70. Nurani Hayati, A. S. W., Boedi Oetomo Roeslan, Effectiveness of Grapefruit (Citrus Paradisi) And Lime (Citrus Aurantifolia) Against Pathogenic Root Canal Biofilms. *INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH* **2020,** *12* (3).