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Phenolic acids-loaded thermosensitive hydrogel for intracanal biofilm management

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ABSTRACT

Objective: In search of an inter-appointment intracanal medication capable of promoting root canal disinfection while preserving the viability of periapical cells, this study synthesized and characterized chitosan-poloxamer hydrogels (CPH) containing phenolic acids and evaluated their effects on multispecies biofilms and cell viability for potential endodontic use.

Methods: Cinnamic acid (CI), caffeic acid (CA) and controls (calcium hydroxide [CH] and chlorhexidine [CHX]) were incorporated into the CPH matrix. The hydrogels were characterized by flow and oscillatory rheometry, sol-gel transition temperature, compounds release profile and scanning electron microscopy analysis. The effect of hydrogels on multispecies biofilms formed in radicular dentin specimens was evaluated by confocal laser scanning microscopy, while cytotoxicity of CPH containing or not the compounds was assessed using resazurin assays on fibroblasts and macrophages cultures. Statistical analysis was performed with significance determined at $p < 0.05$.

Results: CPHs demonstrated pseudoplastic flow behavior and established a strong gel network at 37° C. Furthermore, hydrogels exhibited thermoresponsive behavior and sustained released of incorporated compounds. All formulations reduced bacterial loads in dentinal multispecies biofilms in dentin tubules, notably, CPH+CA (77.8 %) and CPH+CI (73.2 %) outperformed CPH+CH (53.6 %) and CPH+CHX (39.9 %). Overall, all CP hydrogels were cytocompatible when diluted at ratios over 1:4. CPH + CI showed lower cytotoxicity compared to CPH + CA, for both cell lines analyzed.

Conclusion: CPH demonstrated suitable thermoreversible and physicochemical characteristics to be applied as an injectable temporary medication. When particularly combined with cinnamic acid, it markedly reduced intraradicular multispecies biofilms and exhibited better cytocompatibility.

Clinical significance: Cinnamic-acid loaded chitosan-poloxamer hydrogel could be an effective intracanal medication for the management of infected root canals in endodontics.

1. Introduction

Conventional endodontic treatment markedly reduces the microbiota load within infected root canals. Nevertheless, the persistence of microorganisms, attributed to the anatomical complexity of the root canal system and their resistance to chemical-mechanical procedures, can lead to persistent or secondary infections [1]. The maintenance or development of periapical lesions following endodontic therapy has been associated to the presence and virulence of resistant microorganisms, particularly Gram-positive anaerobes such as *Streptococcus* spp., *Lactobacillus* spp., *Actinomyces* spp., *Enterococcus faecalis*, as well as Gram-negative anaerobes such as *Fusobacterium nucleatum*, within the

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root canal system and/or periapical tissues [2,3].

Disinfection of root canals in teeth with necrotic pulp and open apices is especially challenging. In immature teeth, the wide dentinal tubules and large canal space favor bacterial penetration, biofilm adhesion and growth. Moreover, extensive chemo-mechanical instrumentation, although effective in disrupting microbial biofilms and removing infected dentin, is not recommended due to increased risk of further root weaking and fracture, as well as potential cytotoxic effects on cells essential for continued root development and periapical tissue repair [4,5]. Although studies investigating the endodontic microbiome of incompletely developed teeth are limited, available data indicated a bacterial diversity comparable to that of mature teeth, with many detected genera, such as *Streptococcus, Actinobacteria, Enterococcus* and *Fusobacterium* also being associated with persistent infections [5,6]

Intracanal medicaments have been recommended as interappointment dressing to eradicate residual microorganisms, reduce inflammation and postoperative pain, eliminate apical exudate, control inflammatory root resorption, and prevent contamination from temporary restorations [7,8]. Calcium hydroxide (CH) remains the most recommended intracanal medicament due to of its antimicrobial and ability to stimulate mineralization, particularly in long-term clinical treatment such as dental trauma management and treatment of immature teeth with open apices [7]. Nonetheless, CH shows limited efficacy against multispecies biofilms has been shown inefficient results in multispecies biofilms, possibly by the buffering action of dentin, hydroxyapatite, and remnants of necrotic pulp tissue as well as inflammatory exudate [9,10]. Chlorhexidine (CHX), a cationic bisbiguanide, exhibits broad-spectrum antimicrobial activity, especially within a pH range of 5–7 [8]. As an intracanal medicament, CHX has demonstrated greater efficacy than CH in eliminating E. faecalis from dentinal tubules [11,12]. However, CHX has presented cytotoxicity when in contact to host cells even at low concentrations [13,14].

In this context, phytochemicals have gained attention as promising alternatives because they are eco-friendly, relatively inexpensive, and exhibit broad-spectrum antimicrobial activity with comparatively low cytotoxicity. Their multiple mechanisms of action may also reduce the risk of bacterial resistance [15]. Natural phenolic compounds found in plants range from simple molecules, such as phenolic acids (e.g., hydroxybenzoic and hydroxycinnamic acids), to complex, highly polymerized structures such as lignins and flavonoids. Hydroxycinnamic acids (cinnamic acid and their derivatives) most commonly found in nature - such as caffeic acid, ferulic acid, sinapic acid and *p*-coumaric acid - have been studied for their antioxidant, anti-inflammatory and antimicrobial properties, which are attributed to the presence of one or more hydroxyl groups in their structure [16]. Among them, cinnamic acid and caffeic acid have shown broad-spectrum activity against Gram-positive and Gram-negative bacteria, including species of *Enterococcus* and *Streptococcus, Escherichia coli* and fungi such as *Candida albicans* [16–19]. However, studies evaluating the antimicrobial effect of caffeic and cinnamic acids on oral bacteria remain relatively limited [17,18] and are particularly scarce when these bacteria are grown as biofilms [18,20].

The clinical application of phenolic acids as therapeutic agents is constrained by their poor stability, low water solubility, and low bioavailability [21]. To overcome these limitations, natural and synthetic polymers have been employed as drug carriers to enhance solubility, control release kinetics, and extend biological effect of phytomedicines [21]. Hydrogels, three-dimensional polymeric networks with high water-retention capacity, have attracted interest in biomedical and environmental applications [22]. Due to their structural similarity to the extracellular matrix, coupled with favorable chemical, physical, and biological properties, hydrogels have been widely investigated for drug delivery systems and tissue engineering, including for bone and dental regeneration [22–25].

Hydrogels can be developed from natural polymers, including chitosan or synthetic polymers, such as poloxamer-407. Chitosan, a polysaccharide derived from the chitin of crustaceans exoskeleton, is capable of forming hydrogels under suitable conditions. However, chitosan-based hydrogels have limited mechanical strength and poor swelling in aqueous environments [25–27]. Poloxamer 407, a synthetic triblock copolymer of ethylene oxide and propylene oxide have been largely employed as drug delivery systems, especially in combination with chitosan due to their advantages such as thermoreversible gelation, low toxicity, biocompatibility, simple gel preparation and compatibility with diverse biomolecules, including polyphenols. These properties promote enhanced solubilization and prolonged release profile of bioactive compounds for biomedical applications [28–30].

Recent investigations have demonstrated that conjugation or incorporation of cinnamic acids into chitosan-based hydrogels not only beneficially alters the structural and physicochemical properties of chitosan, but also notably enhances the antioxidant, antimicrobial, anti-inflammatory properties of the cinnamic acid [31–33]. Considering the persistence of microorganisms after conventional endodontic treatments and the lack of effective agents against multispecies biofilms formed within root canals, this study aimed to synthesize and characterize the physicochemical properties of chitosan-poloxamer hydrogels containing phenolic acids, as well as evaluate their effects on multispecies biofilms and cell viability, with the intent of developing a medication suitable for endodontic purposes.

2. Materials and methods

This study was approved by Ethical Committee on the Use of Animals (protocol 00,709/2019) and the manuscript of this laboratory study was written in compliance with Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines.

2.1. Phenolic acids and controls

Unless stated otherwise, all compounds and reagents were purchased from Sigma-Aldrich® (St. Louis, MO, USA), bacterial culture media were obtained from Difco® (Sparks, MD, USA) and cell culture media/supplements were acquired from ThermoFisher Scientific® (Waltham, MA, USA). Stock solutions of cinnamic acid (CI, #C80857) and caffeic acid (CA, #C0625) were prepared in dimethylsulfoxide –DMSO and stored frozen. For biological assays, calcium hydroxide PA (CH, Biodynamics, PR, BR) and chlorhexidine digluconate (CHX, #C9394) served as positive controls, while the negative control consisted of untreated culture media. All solutions were sterilized by filtration through a 0.22 μm membrane filter. Stock solutions containing DMSO were diluted 100-fold to minimize the potential effects of this solvent on both bacterial and cellular growth.

2.2. Chitosan-poloxamer hydrogel (CPH) preparation

Initially, low molecular weight chitosan (50–190 Da; 75–85 % deacetylated) at 1 % wt/vol was dissolved in acetic acid solution (1 % wt/vol) (Synth®, Brazil) using mechanical stirrer set at 100 rpm overnight. Once fully dissolved, the chitosan dispersion was then refrigerated (4° C) and subsequently used as a solvent for the Poloxamer 407 (HP-407) dispersion. HP-407 (18 % wt/vol) was then added to the chitosan dispersion and stored at 4 °C for approximately 24 h [34,35]. The hydrogels (without antimicrobial agents) were previously sterilized via ultraviolet radiation in laminar flow hood for 30 min. For antibiofilm and cytotoxity assays, the hydrogels were combined with phenolic acids and controls as follows: CPH + CI at 5 mg/mL, CPH + CA at 5 mg/mL, CPH + CH at 1 mg/mL, CPH + CHX at 0.5 mg/mL. All the concentrations were selected based on previous microbiological assays conducted by this research group. Prior the use, CP hydrogels were agitated for 1 h and then incubated for 24 h at 5 °C to achieve total solubilization.

2.3. Physicochemical characterization of hydrogels

Flow Rheometry

Rheological measurements were conducted using an AR2000 pressure-controlled rheometer (TA Instruments, New Castle, DE, USA) with cone/plate geometry (40 mm diameter at a 52 μm gap). Continuous shear analysis of the hydrogels was performed in triplicate at 37±0.1 °C. Formulations were carefully applied to the lower plate and allowed to equilibrate for at least 1 min prior to testing [36]. Flow curves were obtained by varying the shear rates from 0 to 100 s^{-1} (increased over a period of 120 s, maintained at the upper limit for 10 s, and decreased over another period of 120 s). The consistency index and the flow index were determined using Eq. 1 [36] for a quantitative analysis of flow behavior: (1) $\tau = K.\gamma^{\eta}$ where τ is the shear stress (Pa), k is the consistency index [(Pa s) n], γ denotes the rate of shear ($s - 1$), and n is the flow behavior index (dimensionless).

2.4. Oscillatory rheometry

Oscillatory analysis of CPH was carried out after the linear viscoelastic region in which stress is directly proportional to strain, and the storage modulus remains constant was identified. Frequency sweep analysis was carried out at the frequency range of 1–10 Hz at a constant stress of 1 Pa [36]. Hydrogels were carefully applied to the plate as described previously. After determination of the linear viscoelastic region of hydrogels, frequency sweep analysis was evaluated from 1 to 10.0 Hz. Oscillatory analysis of hydrogels was performed in triplicate at 37 ± 0.1 °C.

2.5. Sol–gel transition temperature

The sol-gel transition temperature (Tsol-gel) of hydrogels was determined via oscillatory mode with temperature ramp, using cone-plate as previously described. Temperature sweep analysis was performed in triplicate across a range of 18–50 °C at a fixed frequency of 1.0 Hz and a heating rate of 3 °C/min under controlled stress conditions [36].

2.6. Compounds release from hydrogels

For these assays, 1 mL of CPH loaded with CI, CA, CH and CHX was incubated in 24-well microplates at 37 °C overnight. Subsequently, deionized water was added over the hydrogel and incubation continued for 7 days. The supernatant ($n = 3$) was collected after 48 h and again at 7 days for analysis of compounds release from the hydrogels. Quantification of phenolic compounds (CI and CA) was performed using a high-performance liquid chromatograph (SCL-10AVp, Shimadzu, Japan) coupled to a diode array detector (SPD-M10AVp, Shimadzu, Japan). Each sample was diluted and filtered and an aliquot of 20 μL was injected onto a C18 reversed-phase column (250×4.6 mm x 5 μm; Agilent). The mobile phase consisted of Phase A (Water: Formic Acid 99.75:0.25) and Phase B (Acetonitrile: Formic Acid 99.75:0.25), whose separation gradient started with 30 % of phase B, increasing to 40 % in 15 min; 50 % in 30 min and returning to 30 % in 10 min, under a flow rate of 1 mL/min. Chromatograms were analyzed using a Class-VP software with CI and CA being detected at 276 and 323 nm, respectively. Release of CHX was assessed using a UV–vis spectrometer at 255 nm, with reference to a standard curve. For CH detection, a colorimetric method was employed (Calcio Liquidform #90, Labtest, Lagoa Santa, Brazil), specifically adding 1 mL of arsenazo III to each aliquot, turning it into a purple solution. The calcium content was determined at a length of 600 nm in a UV–vis spectrometer [37].

2.7. Scanning electron microscopy

CPH was applied in coverslips for microscopic analysis. The hydrogel was dehydrated by washing in a series of ethanol (70 % for 10 min, 95 % for 10 min, and 100 % for 20 min) and air-dried in a desiccator. Afterwards, coverslips were mounted into aluminum stubs, sputter coated with gold, and analyzed in a scanning electron microscope (EVO LS15, Carl Zeiss) [37].

2.8. Effect of hydrogels on multispecies biofilms formed in root dentin tubules

Strain and growth conditions

The bacterial strains used in this study - *Streptococcus mutans* (ATCC 25,175), *Lactobacillus casei* (ATCC 393), *Actinomyces israelii* (ATCC 12,102), *Enterococcus faecalis* (ATCC 51,299) and *Fusobacterium nucleatum* (ATCC 25,586) - were donated by the Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil). Culture media selected for optimal growth of each bacterial species were as follows: Mitis Agar Salivarius Agar (Difco, Kansas City, MO, USA) with 0.2 U/mL bacitracin for *S. mutans*, MRS Rogosa Agar (Difco) for L. *casei*, Brain Heart Infusion Agar –BHIA (Difco) for *A. israelii* and *E. faecalis*. For *F. nucleatum*, BHIA enriched with 5mg/L of hemin, 5mg/mL of menadione and 5 % of defibrinated sheep blood was used. All plates were incubated at 37 °C under anaerobic conditions using AnaeroGen packs (Oxoid, Hampshire, UK) [37].

2.9. Teeth preparation, treatments and confocal analysis

After ethical approval, bovine teeth were selected, cleaned and prepared in accordance with Ma et al. [38]. and dos Santos et al. [39]. In brief, 4 mm cylindrical specimens were obtained from horizontally sectioned bovine dental roots using diamond discs, and the root canals were enlarged with a #6 bur. Each specimen was then sectioned into two cylindrical halves ($n = 6$), washed with distilled water and ultrasonically cleaned with 17 % EDTA for 3 min, followed by deionized water for 5 min. Following autoclaving, the specimens were transferred into microtubes and incubated in BHI broth containing a mixed culture of *E. faecalis, A. israelii, S. mutans*, L. *casei*, and *F. nucleatum*. Bacteria were mixed in equal aliquots at the same concentration (1–5 × 10^3 CFU/mL) in supplemented BHI broth containing 1 % glucose. The microtubes were centrifuged at 1400 x g, 2000 x g, 3600 x g and 5600 x g, twice each, for 5 min to promote dentin infection, replacing the culture medium every centrifugation. Microtubes were incubated at 37 °C in BHI with 1 % glucose for 2 weeks under 5 % CO_2, replacing the medium every 48 h. Subsequently, the specimens were rinsed with sterile water and exposed to 1 mL CPH with phenolic acids (CA and CI at 5mg/mL), controls (CH at 1mg/mL, CHX at 0.5mg/mL), or sterile water for 48 h. They were then washed for 1 min at 37 °C, halved, stained with LIVE/DEAD BacLight (ThermoFisher) for 5 min, and analyzed using Confocal Scanning Laser Microscopy (CLSM, Leica TCS SPE). Ten-micrometer-deep scans were taken at two random spots per dentin specimen using LAS AF Leica software. Red-to-green fluorescence ratios were quantified with Image J 1.48, and data were expressed as the proportion of dead cells (red) to total cells (red + green) for each treatment.

2.10. Cytotoxicity of hydrogels

The cytotoxicity of hydrogels was evaluated using L3T3-L1 fibroblast cultures (CL-173TM) and RAW 264.7 macrophage cultures, with experiments performed in triplicate over three independent days. Initially, hydrogel extracts were prepared by adding 1 mL each of CPH, CPH+CA, CPH+CI, CPH+CH, and CPH+CHX to 24-well plates, followed by incubation for 24 h. Subsequently, Dulbecco's Modified Eagle Medium (DMEM) was applied to the hydrogels and incubated for either 48 h or 7 days. Cells were maintained in DMEM supplemented with 10 % fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine within an incubator set at 5 % CO_2 and 95 % air at 37

°C. Cultures were seeded at a density of 1 × 10^4 cells/well in 96-well plates and allowed to adhere for 24 h. After incubation, the DMEM was removed by aspiration, and serial dilutions of hydrogel extracts in DMEM, ranging from 1:2 to 1:32, were subsequently added to the cell cultures. Control groups consisted of cells cultured in DMEM without any extract. After a 24 h treatment period, the extracts were removed and resazurin solution added for 4 h of incubation. Absorbance was measured at 570 and 600 nm using a spectrophotometer (Biotek, Winooski, VT). The metabolic activity of non-treated cells was established as the 100 % reference point, and all data were reported as a percentage relative to this baseline [39].

2.11. Statistical analysis

Data from cytocompatibility and microbiological assays were expressed in means/standard deviations and submitted to One-Way or Two-Way ANOVA and Tukey tests. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis.

3. RESULTS

3.1. Physicochemical characterization of hydrogels

Flow rheometry

Fig. 1. A-E display the flow rheograms of CPH and CPH containing CI at 5 mg/mL, CA at 5 mg/mL, CH at 1 mg/mL, or CHX at 0.5 mg/mL. All the formulations exhibited comparable flow characteristics, regardless of the compound that was loaded. Based on the analysis, CPH demonstrated non-Newtonian pseudoplastic behavior due to non-linear relationship between the shear rate (Pa) and the shear stress (1/s). Furthermore, the overlapping ascending and descending curves indicates that the CPH possesses thixotropic flow behavior where viscosity decreases with increasing shear rate and subsequently increases as the shear rate is reduced. This thixotropy reflects reversible changes in the hydrogel's internal structure, occurring in a time-dependent manner.

3.2. Oscillatory rheometry

Fig. 2 (A-E) presents the oscillatory rheometry results for CPH and CPH formulations containing CI at 5 mg/mL, CA at 5 mg/mL, CH at 1 mg/mL, or CHX at 0.5 mg/mL. Analysis indicates that all hydrogels, regardless of the loaded compound, demonstrated viscoelastic properties at 25 °C, characterized by a storage modulus lower than the loss modulus. Additionally, frequency-dependent behavior was observed. At this temperature, predominance of the loss modulus suggests that poloxamer micelles are loosely organized, resulting in a less structured system. In contrast, at 37 °C, a rapid increase in storage modulus demonstrates the formation of a strong gel network, with the storage modulus exceeding the loss modulus across all oscillatory frequencies.

3.3. Sol–gel transition temperature

The sol-gel transition temperature (Tsol-gel) of the hydrogels was assessed in oscillatory mode with a temperature ramp using a cone plate, as previously described. Temperature scan analysis was conducted between 18 °C and 50 °C at a defined frequency of 1.0 Hz and a heating rate of 3 °C/min under controlled voltage conditions. As depicted in Fig. 3 (A-E), the Tsol-gel is characterized by three distinct phases. Initially, prior to gelation, the loss modulus remains lower than the storage modulus, demonstrating viscoelastic behavior consistent with oscillatory rheology measurements. The second phase marks the onset of gelation around 24 °C, where the storage modulus surpasses the loss modulus. Additionally, the phase transition is highlighted by an abrupt change in tan(δ), with values below 1, indicating a dominance of storage modulus over loss modulus. Subsequently, CPH typically exhibit a solid-like structure while retaining some fluid-like properties, depending on the temperature. Rheological analyses further confirmed that both CPH with and without phenolic acids, as well as control samples, displayed thermosensitive and thermoreversible characteristics.

Data from Flow Behavior (n), Consistency Index (K), crossover (Sol-gel transition) temperatures for the formulations are presented in

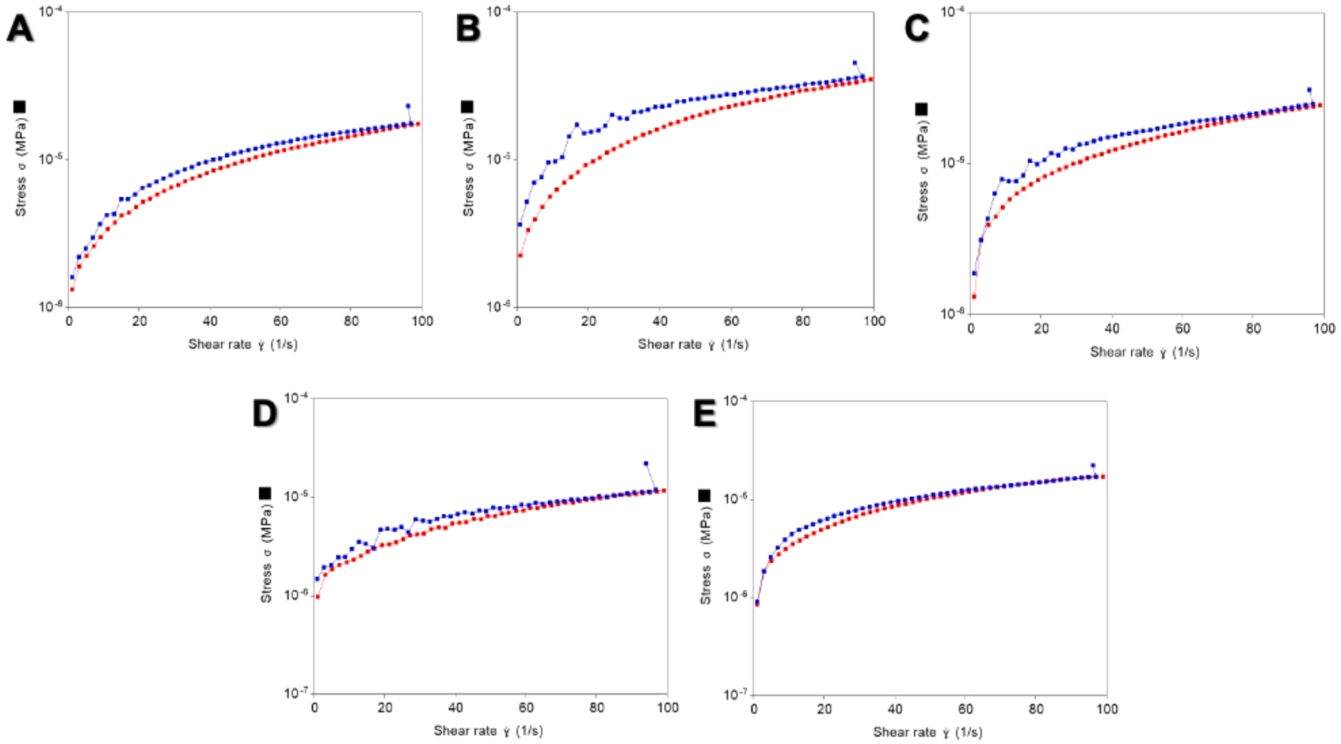

Fig. 1. Flow rheograms of chitosan-poloxamer hydrogels: A: CPH + CI; B:CPH + CA, C: CPH + CH, D: CPH + CHX, E:CPH. Red symbols represent the upward curve, and blue symbols represent the downward curve. Analysis was performed at 37 °C.

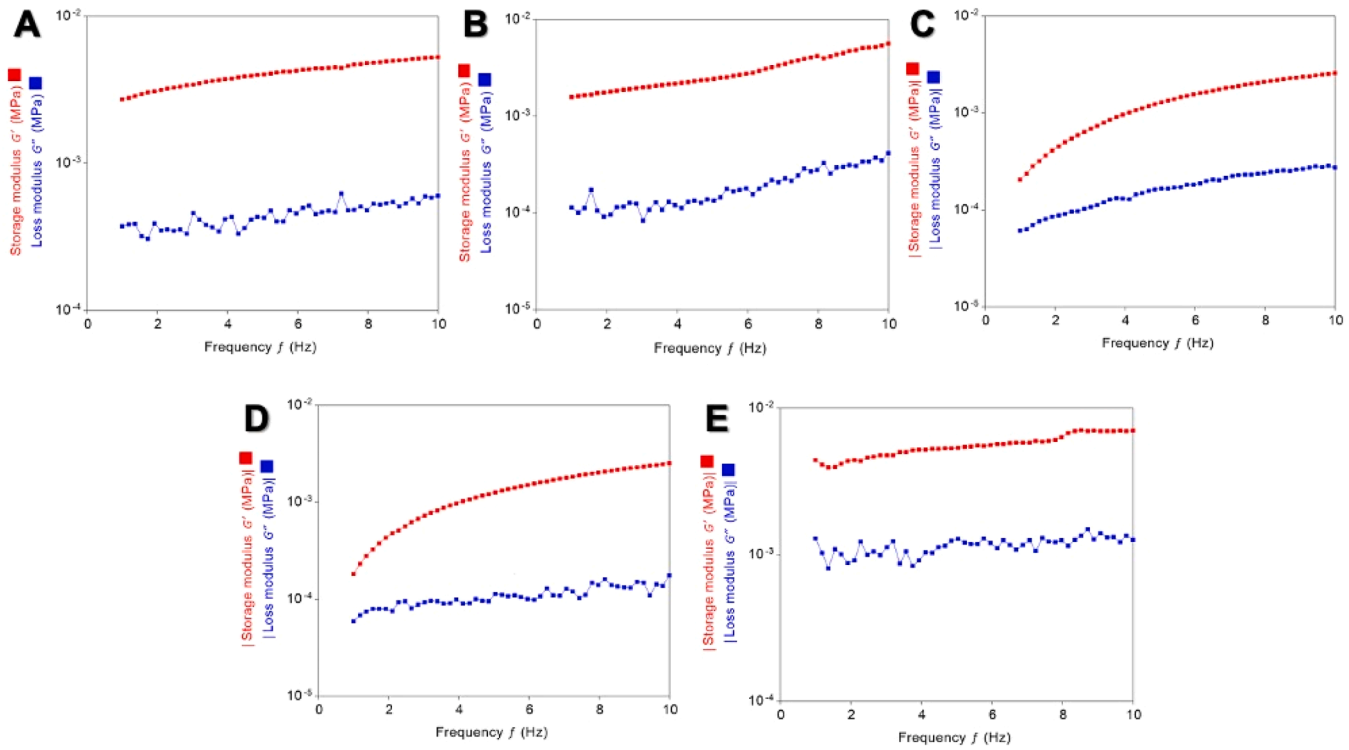

Fig. 2. Oscillatory analysis of chitosan-poloxamer hydrogels - A: CPH + CI; B:CPH + CA, C: CPH + CH, D: CPH + CHX, E:CPH. Analyzes performed at 37 °C. Storage modulus G' (red symbols) and loss modulus G'' (blue symbols).

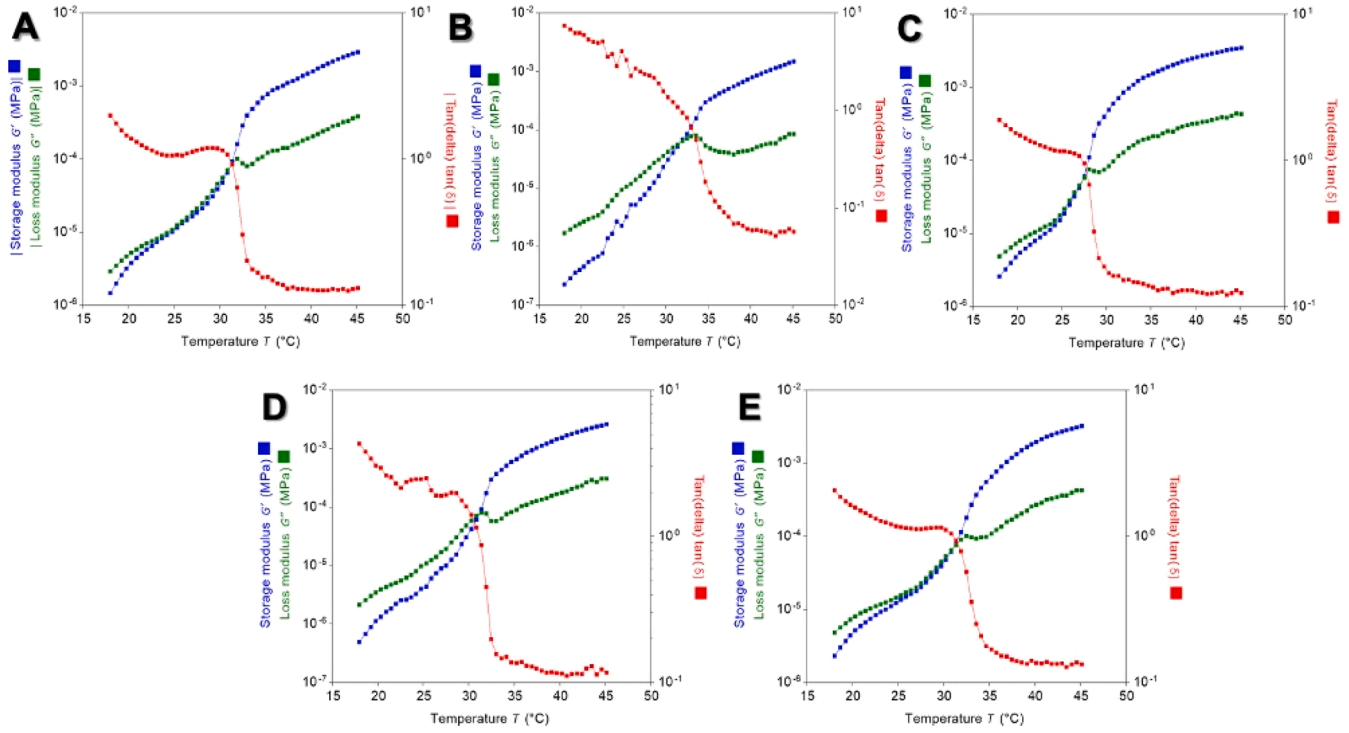

Fig. 3. Temperature sweep demonstrating storage modulus G' (blue symbol), loss modulus (green symbol) and loss tangent (tan d –red symbol) of chitosan-poloxamer hydrogels - A: CPH + CI; B:CPH + CA, C: CPH + CH, D: CPH + CHX, E:CPH. Analyzes performed at 37 °C.

Table 1. Eq. (1) confirms that the hydrogel displayed shear thinning behavior ($n < 1$) being characterized as pseudoplastic fluids. Formulations with pseudoplastic characteristics possess the capacity to restructure upon exposure to physiological conditions, accounting for the observed increase in the consistency index (K).

Table 1
Data from flow behavior (n), consistency index (k), crossover temperatures and compounds release (%) for the formulations.

	n	K ($Pa·s^n$)	Crossover temperatures (°C)	Compounds release (%) after 48h	Compounds release (%) after 7 days
CPH+CI	0.914	0.243	31.09	46.15	12.08
CPH+CA	0.900	0.534	31.69	43.25	30.72
CPH+CH	0.906	0.152	27.32	22.3	23.3
CPH+CHX	0.923	0.101	31.10	40.09	30.1
CPH	0.832	0.367	31.06	-	-

3.4. Compounds release from hydrogels

Except by CPH+CH (22.3 %), compound release from hydrogels at 48 h was comparable among the groups, averaging approximately 40 %. After 7days, the compounds release reduced for all groups (ranging from 10–34 % decrease), except by CPH+CH, which showed results comparable to those observed at 48 h (Table 1).

3.5. Scanning electron microscopy analysis

Fig. 4 illustrate the porous network architecture of the CP hydrogel, as well as the crosslinking interactions between chitosan and poloxamer 407 polymers, presented at three different magnifications.

3.6. Biological properties of hydrogels containing phenolic acids and controls

Effect on multispecies biofilms formed in root dentin and analysis by confocal microscopy

Fig. 5. A-F presents the findings from experiments in which 14-day multispecies biofilms comprising E. faecalis, S. mutans, A. israelli, L. casei and F. nucleatum formed inside the root canals were exposed to CPH containing phenolic acids and control CH and CHX for 48 h. Representative confocal images of CPH hydrogels are displayed in Fig. 5A-F comparing dead (red spots) and live (green spots) patterns among the groups. The antibiofilm effect of CPH+CA (77.8 %) and CPH+CI (73.2 %) hydrogels were notably higher than to CPH+CH (53.6 %) and CPH+CHX (39.9 %). No statistical difference was observed between CPH+CH and CPH+CHX. Additionally, CPH without antimicrobials agents exhibited minimal antibiofilm effect. These results are presented in Fig. 5G

3.7. Cytotoxicity of hydrogels

Fig. 6A-B illustrates the effects of 48-h and 7-day extracts of CPH hydrogels containing CI, CA, CH and CHX, or without antimicrobial agents on fibroblast cells after 24 h exposure. All CPH hydrogels (with or without antimicrobials) demonstrated cytotoxicity when diluted to 1/2, reducing cell viability below 80 % for both 48 h and 7days extracts, except by the of 7-day extract of CPH+CH. CPH + CI exhibited lower cytotoxicity compared to CPH + CA, particularly in the 48-h hydrogel extracts. In contrast, CPH + CHX was highly toxic to fibroblast cells, regardless the dilution.

Fig. 7A-B shows the effect of 48-h and 7-day extracts from CPH hydrogels containing CI, CA, CH and CHX, or without antimicrobial agents on macrophages viability after 24 h of exposure. Cell viability increased significantly with the higher dilutions, regardless the CPH hydrogel for both 48-h and 7-day extracts. For both timepoints, CPH, CPH+CI and CPH + CH were cytocompatible at a 1/8 dilution, while CPH + CA was cytocompatible at a 1/16 dilution, as evidenced by cell viability around 70 %. No cell viability was observed in macrophage cultures treated with CPH + CHX, independent on the dilution.

4. Discussion

In recent years, various therapeutic approaches have been developed by combining synthetic or natural compounds with chitosan-based hydrogels for the management of oral biofilm infections [23,25,27,40]. With the advance of the regenerative endodontics, new intracanal medications have been explored aiming to promote root canal disinfection while preserving the viability of remaining cells, especially for permanent teeth with open apices [7,24,41]. In the present study, chitosan-poloxamer hydrogels was characterized as pseudoplastic fluids capable of forming a strong gel network with storage modulus exceeding the loss modulus at 37° C. In addition, oscillatory rheology showed that hydrogels are thermoresponsive, since the change in temperature promotes an increase in viscosity and consequently induces the in situ gelification process at 37° C resulting in well-structured systems. The presence of the phenolic acids and controls (CH and CHX) did not interfere with the physico-chemical properties of CP hydrogels. Moreover, these hydrogels exhibited sustained release of their compounds over time.

Thermosensitive hydrogels undergo sol-gel phase transition when exposed to temperature stimuli, which in this study occurred at around 30 °C. Chitosan has been widely applied in medical fields due to its low toxicity, biocompatibility, biodegradability, mucosal adhesion properties and antimicrobial activities [26,27]. Through physical and chemical modifications, chitosan formulations can be prepared to form hydrogels capable of controlled drug release [42,43], however, chitosan alone lacks thermoreversible properties. Gel formation in chitosan hydrogels typically results from non-covalent interactions, including electrostatic, hydrophobic, and hydrogen bonds [44], while drug release capacity depends on environmental factors such as pH, temperature, light, and ionic strength [45]. In the present study, in addition to the thermoreversible properties conferred by the hydrogel, the combination of chitosan and poloxamer enhanced viscosity and elasticity during continuous gelation, producing a strong gel network, as previously reported by Wunnoo et al. [46]. and Sankar et al. [47]. Poloxamer-407 is a copolymer composed of a polypropylene oxide chain flanked by polyethylene oxide units. Structurally, the hydrophobic core consists of a polypropylene oxide chain, whereas the hydrophilic corona is formed by polyethylene oxide chains [48,49]. At temperatures between 2 and 15 °C, polyethylene oxides are soluble; above 15 °C, micelle formation

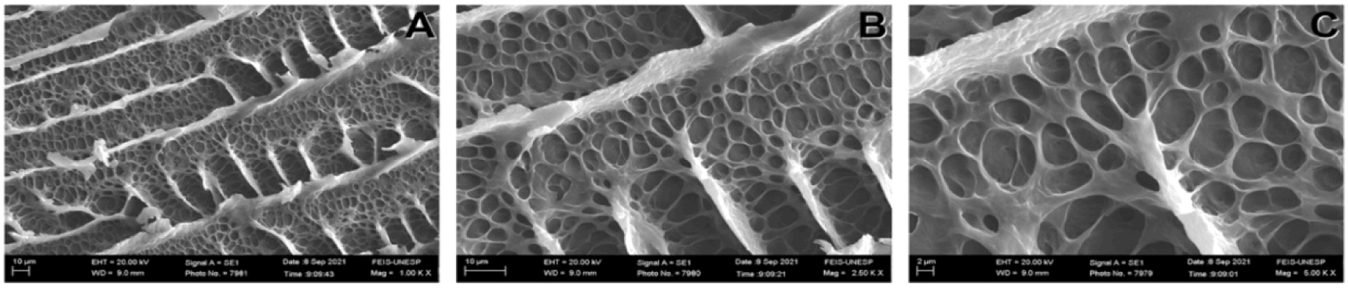

Fig. 4. Scanning electron microscopy representative images of chitosan-poloxamer hydrogels. A –1000x; B –2.500x and C –5000x.

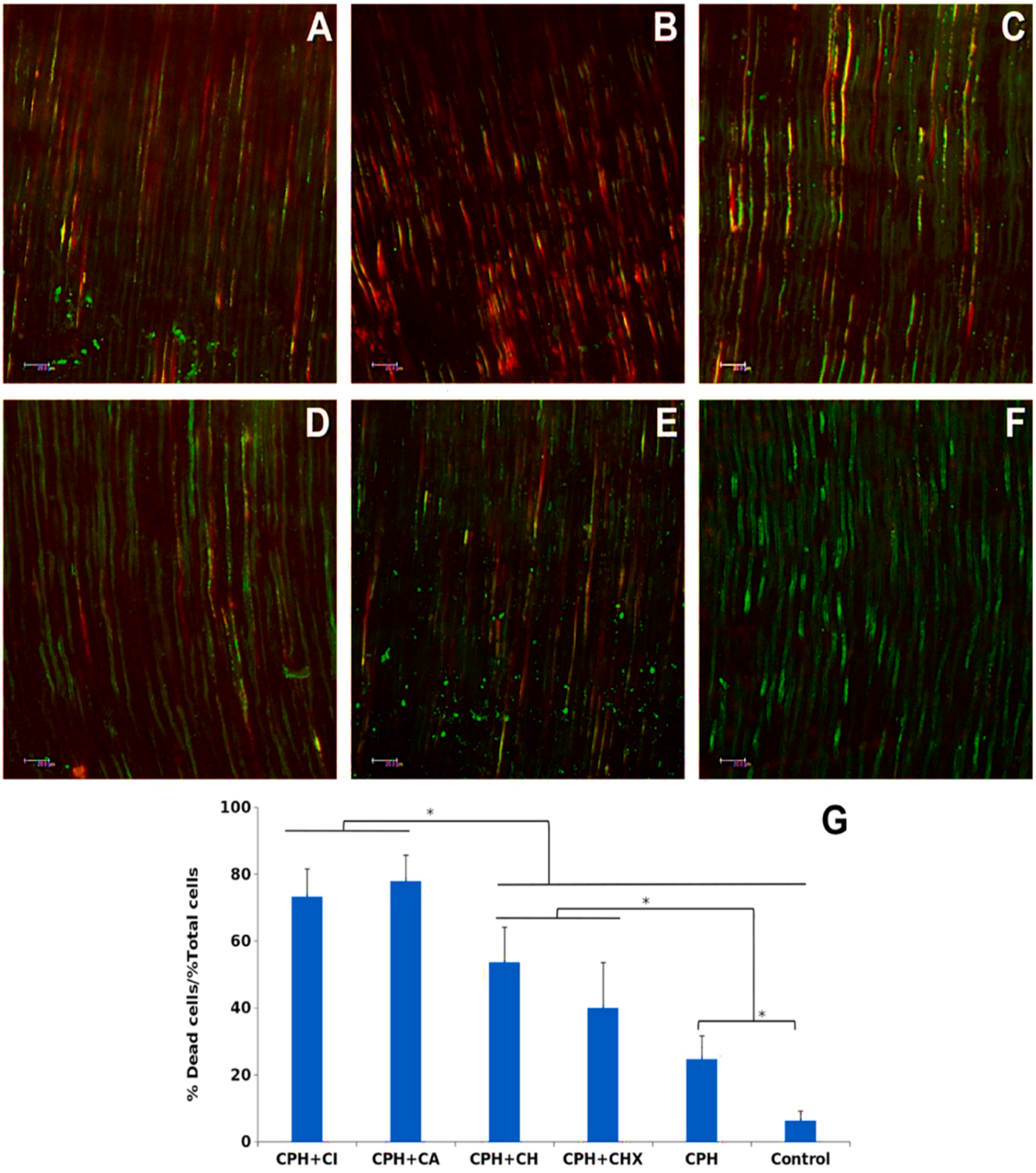

Fig. 5. Representative confocal microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms and treated for 48 h with the following groups with A. Hydrogel (CPH) with cinnamic acid (CI) at 5 mg/mL, B. CPH with caffeic acid (CA) at 5 mg/mL, C. CPH with calcium hydroxide (CH) at 1mg/mL, D.CPH with chlorhexidine (CHX) at 0.5 mg/mL, E. CPH without antimicrobial agents, F. Control –Bacterial growth without antimicrobial agents. G. Mean (SD) of the percentages of dead cells of multispecies biofilms after 48 h of treatment with hydrogels and controls. Bacterial counts were obtained by Image J analysis. *Statistical difference among the groups, according to One-Way ANOVA and Tukey test ($p < 0.05$).

occurs, followed by gelation due to dehydration [50].

To the best of our knowledge, no previous studies have evaluated thermoreversible chitosan hydrogels containing cinnamic or caffeic acid as potential antibiofilm agents for endodontic applications. In this study, biofilms were formed by mixing five bacterial species, representative of the most important groups of bacteria involved in the endodontic persistent infections: *Enterococcus, Streptococcus, Lactobacillus, Actinomyces* and *Fusobacterium*. Despite some heterogeneous results regarding the mostly prevalent pathogens found in teeth with endodontic failures, some bacterial species or gender of bacteria are prevalent in endodontic

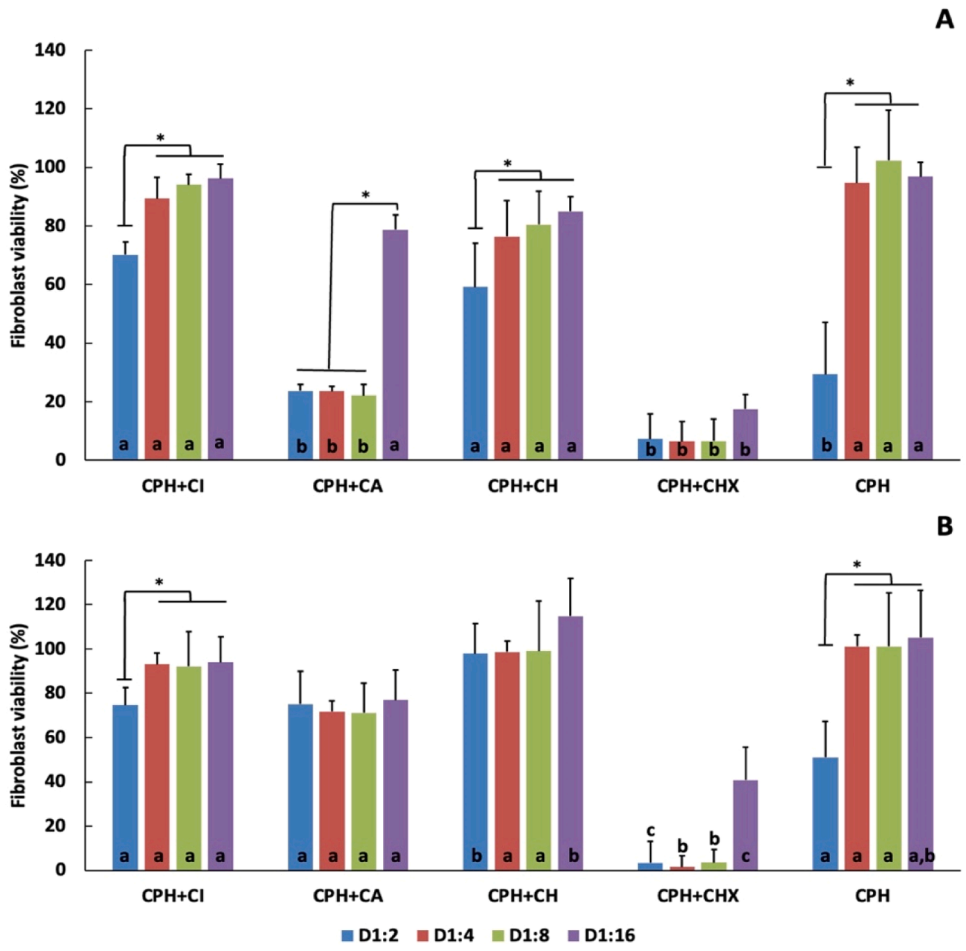

Fig. 6. Effect of the 48-h (A) and 7-day (B) extracts of chitosan-poloxamer hydrogels (CPH) containing cinnamic acid (CI) or caffeic acid (CA) and controls calcium hydroxide (CH) and chlorhexidine (CHX) on the viability of fibroblasts (3T3) after 24 h of exposure, using staining with resazurin. Hydrogel extracts were serially diluted in DMEM medium, ranging from a 1:2 to a 1:16 dilution. * statistical difference among the dilutions, considering each hydrogel separately. [a]different letters show statistical difference among the groups, considering each dilution separately. Two-Way ANOVA and Tukey test ($p < 0.05$).

infections [51]. Firmicutes (examples: streptococci and enterococci) and Bacteroidetes are the most abundant phyla detected in root canal infections, followed by Actinobacteria and Fusobacteria, independent on the type of endodontic infections [52]. *Enteroccocus faecalis* was the most prevalent bacteria (45.8 %) found in teeth with secondary infections, followed by *Streptococcus* (30 %), *Actinomyces* (13.3 %), *Fusobacterium* (6.7 %), *Lactobacillus* (6.7 %) spp. and others [53]. The persistence of members of the phylum Firmicutes, and the genera *Streptococcus* spp., *Lactobacillus* spp. and *Enterococcus* spp., was also observed in necrotic pulp of permanent teeth with incomplete root formation, after instrumentation and even after post-medication (calcium hydroxide or triantibiotic paste), as reported for mature permanent teeth [5]. Independent on the prevalence, these bacterial species are resistant to disinfection measures because they are able to grow in both aerobic and anaerobic conditions, to degrade alternative nutrients from residual necrotic pulp tissue or periodontal ligament, to escape from host's defenses, and have the ability to adhere, coaggregate and survive in biofilms inside dentin tubules and in extra-radicular areas [54–56].

The incorporation of phenolic acids into CPH enhanced their activity against multispecies biofilms, with no significant differences observed between cinnamic acid and caffeic acid formulations. Nonetheless, hydroxycinnamic acids conjugated with chitosan or incorporated into nanogels has been investigated to potentiate antimicrobial activity, supporting the promise of this combination [32,33,57–59]. For instance, chitosan conjugated with cinnamic acid exhibited greater antifungal activity against Pyricularia oryzae and Botrytis cinerea than chitosan alone, with one conjugate showing a 12-fold increase in activity against B. cinerea [58]. Similarly, chitosan conjugated with caffeic acid displayed enhanced antibacterial activity against 15 clinical isolates, methicillin-resistant Staphylococcus aureus strains, and several foodborne pathogens, compared to native chitosan [57]. Encapsulation of essential oils in cinnamic acid–grafted chitosan nanogels also improved antifungal activity against Microsporum canis, leading to complete inhibition of mycelial growth [32]. Chitosan–hydroxycinnamic acid conjugates demonstrated bactericidal activity against *S.* aureus and *E. coli* while increasing antioxidant activity compared to unmodified chitosan, with one caffeic acid conjugate showing 4000-fold higher activity than chitosan and greater potency than free caffeic acid [33]. In another study, gallic and trans-cinnamic acids embedded in a gelatin–chitosan matrix exhibited stronger antioxidant activity than films containing only trans-cinnamic acid, along with antibacterial effects against *E. coli* [58]. Cinnamic acid-conjugated hydroxypropyl chitosan derivatives demonstrated inhibitory effects on biofilms formed by *S. aureus* and *E. coli*, as evidenced by reduced biofilm thickness and decreased numbers of viable cells observed through confocal laser scanning microscopy [60]. Notably, in this study, chlorhexidine (CHX) incorporated into CPH exhibited the lowest antibiofilm efficacy in the dentin-block model. This unexpected finding could be associated to the lower concentration of CHX compared to the other antimicrobial agents or to a reduced release of the compound from the hydrogels. In addition, CHX has other reported limitations such as its low diffusibility, limited penetration into deep biofilm layers, reduced effect due to the buffering capacity of

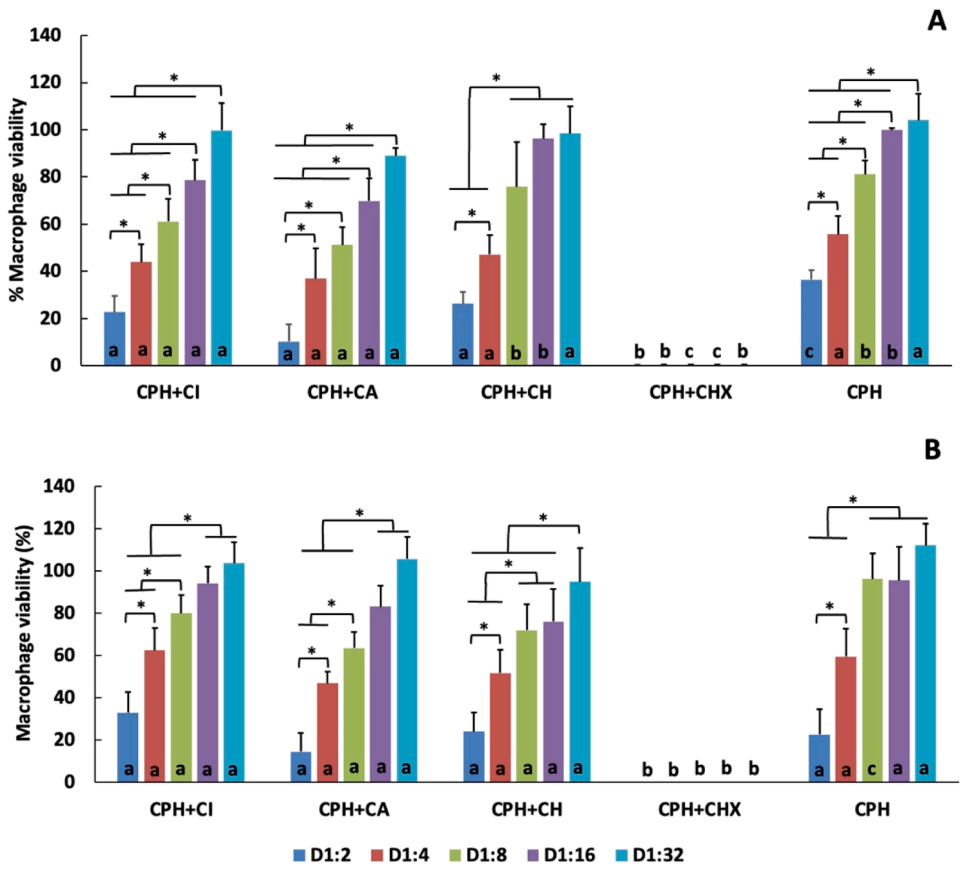

Fig. 7. Effect of the 48-h (A) and 7-day (B) extracts of chitosan-poloxamer hydrogels(CPH) containing cinnamic acid (CI) or caffeic acid (CA) and controls calcium hydroxide (CH) and chlorhexidine (CHX) on the viability of macrophage (RAW 264.7) after 24 h of exposure, using staining with resazurin. Hydrogel extracts were serially diluted in DMEM medium, ranging from a 1:2 to a 1:16 dilution. * Statistical difference among the dilutions, considering each hydrogel separately. [a]Different letters show statistical difference among the groups, considering each dilution separately. Two-Way ANOVA and Tukey test ($p < 0.05$).

dentin [10,61] and even bacterial resistance, previously reported for *Enterococcus* spp [62].

In the current study, the toxicity of the CPH, with or without antimicrobial agents, was evaluated in fibroblasts and macrophages cultures to represent the resident cell populations found in the apical area of the tooth. Overall, both the 48-h and 7-days extracts of the hydrogel matrix itself (CPH without antimicrobial agents) exhibited cytotoxicity when diluted at ratios of 1:2 (for fibroblasts) and 1:4 (for macrophages). These results should be noted and considered for further investigations. However, it is important to emphasize that data obtained from in vitro cytotoxicity models, in which cells are directly exposed to materials, cannot be directly extrapolated to in vivo conditions. Furthermore, in teeth affected by apical periodontitis, the intense inflammatory reaction and edema in the apical environment may further dilute toxic agents that could reach the region during chemo-mechanical treatment or even after intracanal medication. Additionally, intracanal medications placed into root canals are not expected to have direct contact with apical cells [63]. This study showed that cinnamic acid–containing hydrogels were more cytocompatible than those loaded with caffeic acid. This difference could be related to their chemical structure. Although the basic structure remains the same, the number and positions of the hydroxyl groups lead to change in the biological properties of the phenolic acids [64]. Corroborating with our study, in a previous study, caffeic acid reduced GN61 fibroblast viability at concentrations greater than 1.5 mM (270 μg/mL) and cinnamic acid only at 6.75 mM (1 mg/mL) [65]. Previous studies have shown that chitosan-based hydrogels generally exhibit low cytotoxicity in different cell lines [66–69]. For instance, water-soluble chitosan–ethylene glycol acrylate methacrylate (CS-EGAMA) and polyethylene glycol dimethacrylate (PEGDMA) formulations were pH-sensitive and non-cytotoxic to L929 and SW1353 cell lines [66]. Water-soluble phosphonium chitosan derivatives also displayed low cytotoxicity in L929 cells [67]. Curcumin-loaded chitosan phosphate nanoparticles were cytocompatible when tested in peripheral blood mononuclear cells and murine macrophage-like cell lines [68]. Gelatin–chitosan–polyvinyl alcohol hydrogels synthesized at varying polymer ratios via freeze-drying and sterilized by steam showed low cytotoxicity (>70 % cell viability) in the HT29-MTX-E12 cell line [69]. Cytotoxicity assays demonstrated that VERO CCL-81 cells maintained over 80 % viability following exposure to chitosan hydrogels crosslinked with curcumin, subsequent to dual encapsulation within poloxamer 407 and citric acid [70]. Other studies have indicated that poloxamer is considered safe as an in situ gelling agent for ocular, intranasal, and oral drug delivery [71,72].

It is important to note that, despite their many advantages, hydrogels also present limitations. Their poor mechanical properties, low long-term stability, lack of radiopacity, and uncontrolled degradation rates hinder their immediate clinical application in endodontics. Previous studies have explored chitosan-based systems for endodontic applications, particularly in bone repair [33,73,74]. However, to the best of our knowledge, this is the first study to evaluate the antibiofilm properties and cytotoxicity of chitosan–poloxamer hydrogels containing cinnamic and caffeic acids, compared to hydrogels containing calcium hydroxide and chlorhexidine, two widely used intracanal medications. Within the limitations of this in vitro study, CPH containing phenolic acids appear promising as intracanal medicaments for delivering antimicrobial agents, reducing microbial load, and supporting periapical regeneration. Nonetheless, further in vitro studies focusing on their mechanical properties, as well as in vivo investigations, are necessary to validate

their potential clinical application.

5. Conclusions

Chitosan-poloxamer hydrogel demonstrated suitable thermoreversible and physicochemical characteristics to be applied as an injectable temporary medication. When particularly combined with cinnamic acid, it markedly reduced intra-radicular multispecies biofilms and exhibited better cytocompatibility. Cinnamic-acid loaded hydrogel could be an effective intracanal medication for the management of infected root canals in endodontics.

CRediT authorship contribution statement

Vanessa Rodrigues dos Santos: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Karina Sampaio Caiaffa:** Writing – review & editing, Visualization, Methodology, Investigation. **Amanda Caselato Andolfato Souza:** Writing – review & editing, Visualization, Methodology, Investigation. **Jesse Augusto Pereira:** Writing – review & editing, Visualization, Methodology, Investigation. **Gabriel Flores Abuna:** Writing – review & editing, Visualization, Methodology, Investigation. **Tais de Cássia Ribeiro:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Marco C . Bottino:** Writing – review & editing, Writing – original draft, Conceptualization. **Marlus Chorili:** Writing – review & editing, Writing – original draft, Conceptualization. **Cristiane Duque:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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