Vanessa Rodrigues dos Santos

EFEITO ANTIMICROBIANO E ANTI-INFLAMATÓRIO DE ÁCIDOS FENÓLICOS ISOLADOS, COMBINADOS E INCORPORADOS EM HIDROGÉIS DE QUITOSANA PARA APLICAÇÃO ENDODÔNTICA

Araçatuba – SP
2022

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Orientadora: Profª Drª Cristiane Duque

Coorientador: Prof. Dr Antonio Hernandes Chaves Neto

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Epígrafe

"Consagre ao Senhor tudo o que você faz, e os seus planos serão bemsucedidos."

Provérbios 16:3

Dedicatória

À Deus,

Pois acredito que cada vitória e conquista em nossas vidas acontece por sua permissão e graça. Reconheço o Seu cuidado durante todos os dias da minha vida inclusive durante essa trajetória. Por muitas vezes precisei de forças e ânimo para chegar até aqui e foi a Sua mão que me sustentou. Ao Senhor dedico esta conquista!

"Seja forte e corajoso! Não tenha medo, nem fique assustado, porque o Senhor, seu Deus, estará com você por onde quer que você andar." Josué 1:9

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"...honra a teu pai e a tua mãe, como o SENHOR, teu Deus, te ordenou, para que se prolonguem os teus dias e para que te vá bem na terra que o SENHOR, teu Deus, te dá." Deuteronômio 5:16

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Resumo Geral

Santos VR. Efeito antimicrobiano e anti-inflamatório de ácidos fenólicos isolados, combinados e incorporados em hidrogéis de quitosana para aplicação endodôntica [Tese]. Doutorado em Ciência Odontológica, área Saude bucal da criança, Araçatuba: Universidade Estadual Paulista; 2022.

Resumo Geral

O objetivo do tratamento endodôntico é manter a integridade da raiz, bem como, prevenir ou resolver doenças periapicais, pela erradicação dos microrganismos e de suas fontes de nutrientes provenientes do sistema de canais radiculares. A complexidade da anatomia dos canais radiculares e dos biofilmes multiespécies aumenta a dificuldade em eliminar os microrganismos e controlar a inflamação por procedimentos químico-mecânicos convencionais, o que justifica o uso de medicações intracanais. Novos compostos com amplo efeito antimicrobiano e potencial antiinflamatório, como os ácidos fenólicos, poderiam ser explorados como princípios ativos de medicamentos intracanais. Entretanto, para aumentar a solubilidade, controlar a liberação e estender os efeitos biológicos dos ácidos fenólicos, seria interessante incorporá-los em carreadores de medicamentos como os hidrogéis de quitosana. Este estudo foi dividido em dois capítulos que apresentaram como objetivos: 1) avaliar as atividades antimicrobiana, antibiofilme e antiinflamatória e a citotoxicidade do ácido cinâmico e seus derivados; 2) sintetizar e caracterizar as propriedades químicas e físico-mecânicas de hidrogéis termossensíveis de quitosana e poloxamer contendo ácidos fenólicos, e avaliar o efeito desses hidrogéis sobre biofilmes multiespécies e na viabilidade de macrófagos e fibroblastos. No capítulo 1, a atividade antimicrobiana do ácido cinâmico (CI) e seus derivados ácido cumárico (CO), ácido cafeico (CA), ácido ferúlico (FE) e ácido sinápico (SI) foi avaliada pela determinação da concentração inibitória e bactericida mínima (CIM/CBM) e Concentração Inibitória Fracionada (CIF) sobre *Enterococcus faecalis*, *Streptococcus mutans*, *Lactobacillus casei*, *Actinomyces israelii* e *Fusobacterium nucleatum*. Os ácidos fenólicos foram selecionados e seu efeito em biofilmes dual-espécies e multiespécies com as mesmas cepas padrão ou cepas clínicas foram avaliados por contagem bacteriana, microscopia eletrônica de varredura e microscopia confocal. A viabilidade de fibroblastos L929 e macrófagos RAW 264.7 na presença desses ácidos fenólicos foi

avaliada por ensaios de resazurina. Além disso, os níveis de mRNA dos marcadores pró-inflamatórios TNF-α, IL-1β, iNOS e COX-2 foram determinados por PCR quantitativo TaqMan após exposição de macrófagos aos ácidos fenólicos e ao lipopolissacarídeo (LPS). No capítulo 2, foi realizada a síntese e caracterização físico-mecânica de hidrogéis de quitosana-poloxamer (CPH) contendo ácidos fenólicos e avaliado seus efeitos no biofilme multiespécies e na viabilidade de macrófagos e fibroblastos. Os dados foram analisados estatisticamente considerando $p<0,05$. O ácido cinâmico e o ácido cafeico apresentaram efeito inibitório e bactericida contra todas as espécies bacterianas testadas, com os menores valores de CIM e CBM. Entretanto, não houve efeito sinérgico entre eles (FICI>0,5). Ambos os compostos (5x a CIM mais alta) foram eficazes na eliminação de biofilmes dual-espécies e na redução significativa de biofilmes multiespécies, especialmente o ácido cinâmico. O ácido cinâmico causou toxicidade mínima para ambas as culturas celulares nas concentrações de CIM e o ácido cafeico não foi citotóxico em concentrações abaixo de 0,125 mg/mL. Ambos os compostos reduziram significativamente TNF-α, IL-1β, iNOS e COX-2, de maneira dose-dependente. Os CPH foram caracterizados como termorreversíveis e com propriedades mecânicas e bioadesivas desejáveis. O efeito dos hidrogéis CPH+CA (77,8%) e CPH+CI (73,2%) em reduzir os biofilmes multiespécies foi superior ao CPH+ hidroxido de cálcio (CH) (53,6%) e CPH+ clorexidina (CHX) (39,9%). Em geral, CPH + CI causou menor citotoxicidade quando comparado a CPH + CA, para ambas as linhagens celulares. Conclui-se que o ácido cinâmico e ácido cafeico apresentaram efeito bactericida e contra biofilmes formados por bactérias associadas com infecções endodônticas, causando baixa citotoxicidade. Ambos os compostos apresentaram efeito antiinflamatório, inibindo a expressão de marcadores pró-inflamatórios em macrófagos estimulados por LPS. Os hidrogéis de quitosana-poloxamer foram termorreversíveis e apresentaram adequadas propriedades mecânicas e adesivas para aplicação clínica, e quando combinados principalmente com ácido cinâmico, promoveram a redução de biofilmes multiespécies formados nos túbulos dentinários, causando baixa toxicidade em fibroblastos e macrófagos.

Palavras-chave: Cinamatos, Ácidos cafeicos, Anti-infecciosos, Biofilmes, Anti-inflamatórios, Quitosana

General Abstract

Santos VR. Antimicrobial and anti-inflammatory effect of phenolic acids, alone, combined and incorporated in chitosan hydrogels for endodontic application [Tese]. Doutorado em Ciência Odontológica, área Saude bucal da criança, Araçatuba: Universidade Estadual Paulista; 2022.

General Abstract

The objective of endodontic treatment is to maintain the integrity of the root, as well as to prevent or resolve periapical diseases, by eradicating microorganisms and their sources of nutrients from the root canal system. The complexity of root canal anatomy and multispecies biofilms increases the difficulty in eliminating microorganisms and controlling inflammation by conventional chemical-mechanical procedures, which justifies the use of intracanal medications. New compounds with broad antimicrobial effect and anti-inflammatory potential, such as phenolic acids, could be explored as active principles of intracanal medications. However, to increase the solubility, control the release and extend the biological effects of phenolic acids, it would be interesting to incorporate them into drug carriers such as chitosan hydrogels. This study was divided into two chapters with the following objectives: 1) to evaluate the antimicrobial, antibiofilm and anti-inflammatory activities and the cytotoxicity of cinnamic acid and its derivatives; 2) synthesize and characterize chemical and physico-mecanical properties of thermosensitive chitosan and poloxamer hydrogels containing phenolic acids and evaluate the effect of these hydrogels on multispecies biofilms and on the viability of macrophages and fibroblasts. In chapter 1, the antimicrobial activity of cinnamic acid (CI) and its derivatives coumaric acid (CO), caffeic acid (CA), ferulic acid (FE) and sinapic acid (SI) was evaluated by determining the minimum inhibitory and bactericidal concentration (MIC/MBC) and Fractional Inhibitory Concentration (FIC) on *Enterococcus faecalis, Streptococcus mutans, Lactobacillus casei, Actinomyces israelii* and *Fusobacterium nucleatum.* Phenolic acids were selected and their effect on bispecies and multispecies biofilms with the same standard or clinical strains were evaluated by bacterial counts, scanning electron microscopy and confocal microscopy. The viability of L929 fibroblasts and RAW 264.7 macrophages in the presence of these phenolic acids was evaluated by resazurin assays. In addition, mRNA levels of the pro-inflammatory markers TNF-α, IL-1β, iNOS and COX-2 were determined by quantitative

TaqMan PCR after macrophage exposure to phenolic acids and lipopolysaccharide (LPS). In chapter 2, the synthesis and physical-mechanical characterization of chitosan-poloxamer (CPH) hydrogels containing phenolic acids were performed and their effects on multispecies biofilm and on the viability of macrophages and fibroblasts were evaluated. Data were statistically analyzed considering $p<0.05$. Cinnamic acid and caffeic acid showed an inhibitory and bactericidal effect against all bacterial species tested, with the lowest MIC and MBC values. However, no synergistic effect was observed between the compounds (FICI>0.5). Both compounds (at 5x the highest MIC) were effective in eliminating dual-species biofilms and significantly decreasing multispecies biofilms, especially cinnamic acid. Cinnamic acid caused minimal toxicity to both cell cultures at MIC concentrations and caffeic acid was not cytotoxic at concentrations below 0.125 mg/mL. Both compounds significantly reduced TNF-α, IL-1β, iNOS and COX-2, in a dose-dependent manner. CPH were characterized as thermoreversible and with adequate mechanical and bioadhesive properties. The effect of CPH+CA (77.8%) and CPH+CI (73.2%) hydrogels against multispecies biofilms was superior to CPH + calcium hydroxide (CH) (53.6%) and CPH + chlorhexidine (CHX) (39.9%). In general, CPH + CI caused less cytotoxicity when compared to CPH + CA, for both cell lines. In conclusion, cinnamic acid and caffeic acid showed bactericidal effect and against biofilms of bacteria associated with endodontic infections, causing minimal cytotoxicity. In addition, both compounds showed an anti-inflammatory effect, inhibiting the expression of pro-inflammatory markers in LPS-stimulated macrophages. The chitosan-poloxamer hydrogels were thermoreversible and presented adequate mechanical and bioadhesive properties for clinical application, and when combined specially with cinnamic acid, they promoted the reduction of multispecies biofilms formed in the dentinal tubules, causing low toxicity to fibroblasts and macrophages.

Keywords: Cinnamates, Caffeic Acids, Anti-infectives, Biofilms, Anti-inflammatories, Chitosan.

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Capítulo 1

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Lista de abreviaturas

A. israelii	*Actinomyces israelii*
A. odontolyticus	*Actinomyces odontolyticus*
L. casei	*Lactobacillus casei*
S. mutans	*Streptococcus mutans*
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. sobrinus	*Streptococcus sobrinus*
L. monocytogenes	*Listeria monocytogenes*
M. luteus	*Micrococcus luteus*
B. cereus	*Bacillus cereus*
P. mirabilis	*Proteus mirabilis*
E. coli	*Escherichia coli*
S. marcescens	*Serratia marcescens*
K. pneumoniae	*Klebsiella pneumoniae*
C. albicans	*Candida albicans*
P. aeruginosa	*Pseudomonas aeruginosa*
S. pyogenes	*Staphylococcus pyogenes*
E. faecalis	*Enterococcus faecalis*
F. nucleatum	*Fusobacterium nucleatum*
CFU	Colony Forming Unit
µm	Micrometro
LPS	Lipopolissacarídeo
iNOS	Inducible nitric oxide synthase
NO	Óxido nitrico
NF-kB	Fator nuclear-kB
min	minuto
s	segundo
TNF-α	Fator de necrose tumoral

h	hora
mM	Micromolar
BHIA	Brain Heart Infusion Agar
µg	Microgramа
mm	Milimetro
mg	Miligrama
ng	Nanograma
g	Grama
BHI	Brain Heart Infusion
MH	Miller Hinton
mL	Mililitro
IL-6	Interleucina 6
IL-1β	Interleucina 1β
CHX	Chlorexidine digluconate
ROS	Reactive oxygen species
p-CA	Ácido p-cumárico
CI	Cinnamic acid
CA	Caffeic acid
FE	Ferulic acid
SI	Sinapic acid
CO	p-Coumaric acid
MBC	Minimum Bactericidal Concentration
CH	Calcium hydroxide
DEX	Dexamethasone
MIC	Minimum Inhibitory Concentration
DMSO	Dimethylsulfoxide
qPCR	quantitative PCR
PGE2	Prostaglandinas
L-929	fibroblasts
RAW 264.7	macrophages

Sumário

Sumário

Introdução Geral

O tratamento endodôntico convencional envolve procedimentos de preparo biomecânico (instrumentação associada a irrigantes antimicrobianos) e aplicação de medicação intracanal entre as consultas, seguida pela obturação do canal e restauração do dente (Jena et al., 2015; Haapasalo et al., 2014). O objetivo desse tratamento é manter a integridade da raiz, bem como, prevenir ou resolver doenças periapicais, pela erradicação dos microrganismos e de suas fontes de nutrientes provenientes do sistema de canais radiculares (Young et al., 2007). Embora o tratamento endodôntico convencional reduza significativamente a microbiota presente no interior dos canais radiculares (Siqueira et al., 2007), a permanência de microrganismos devido à complexidade anatômica do sistema de canais radiculares e a resistência destes ao tratamento químico-mecânico pode ocasionar infecções persistentes ou secundárias (Siqueira e Rôças, 2009).

A manutenção ou o desenvolvimento de lesões periapicais, após o tratamento endodôntico tem sido atribuído a fatores relacionados com a virulência e capacidade de sobrevivência de algumas espécies de microrganismos no sistema de canais radiculares e/ou tecidos periapicais (Sundqvist et al., 1998; Hancock et al., 2001; Nair et al., 1999; Estrela et al., 2014). Estudos tem revelado que as bactérias Gram-positivas anaeróbias facultativas são mais frequentes nesses casos, incluindo *Streptococcus* spp., *Actinomyces* spp. (*A. israelii* e *A. odontolyticus*), *Propionibacterium* spp., *Lactobacilos* spp., *Enterococcus faecalis*, entre outros (Siqueira e Rôças, 2009). Espécies bacterianas Gram-negativas, comuns nas infecções primárias, também podem permanecer após o tratamento químico-mecânico, como *Fusobacterium nucleatum*, ou ainda liberar a endotoxina lipopolissacarídeo (LPS) da sua membrana externa (Sakamoto et al., 2008).

A persistência de endotoxinas no sistema de canais radiculares pode levar a injúrias nos tecidos periapicais. Isso ocorre porque o LPS estimula a produção de enzimas que induzem o estresse oxidativo, como a iNOS (*inducible nitric oxide synthase*) que em macrófagos modula a produção de óxido nítrico (NO, *nitric oxide*). Grandes quantidades de NO produzida por iNOS tem sido diretamente relacionada com a patofisiologia de uma variedade de doenças e na inflamação (Marletta, 1993). Além disso, essas endotoxinas aumentam a atividade fagocítica de células polimorfonucleares e a secreção de citocinas pró-inflamatórias, tais como, fator de

necrose tumoral (TNF-α), interleucinas (IL-1β, IL-6, entre outras), prostaglandinas (PGE2). A enzima ciclooxigenase-2 (COX-2) também é induzida por estímulos inflamatórios, como endotoxinas bacterianas e citocinas e está elevada quando há lesão tecidual (Desai et al., 2018). Sabe-se que a resposta inflamatória é uma aliada ao processo de reparo, entretanto, a secreção descontrolada dessas citocinas induz a expressão de ligante do receptor de NF-κβ (RANKL) e a reabsorção óssea (Walsh et al., 2006).

Todas as dificuldades de controlar a infecção e a inflamação nos tecidos periapicais se complicam ainda mais no caso de dentes permanentes imaturos, pois a ampla abertura do forame apical promove ainda mais contato dos produtos bacterianos ou substâncias químicas de uso endodôntico com o tecido vital residual. Isso indubitavelmente terá impacto sobre a bainha epitelial de Hertwig que inibirá sua capacidade de estimular as células indiferenciadas da papila apical em se diferenciar em odontoblastos e continuar a formação radicular (Banchs e Trope, 2004). Embora os tratamentos convencionais frequentemente conduzam à resolução dos sinais e sintomas das patologias pulpares (Sheehy e Roberts, 1997; Bose et al., 2009; Parirokh e Torabinejad, 2010) em dentes permanentes maduros, promovem pouco ou nenhum benefício para a continuidade do desenvolvimento radicular, manutenção da nocicepção pulpar normal e defesa imune esperada para um dente permanente jovem (Diogenes et al., 2013, 2014). A pasta de hidróxido de cálcio é muito utilizada como medicação, em procedimentos de terapia pulpar vital como pulpotomia e em casos de pulpite irreversível/necrose pulpar. Essa medicação também é indicada para dentes permanentes jovens em procedimentos de apicificação. No entanto, a necessidade de múltiplas sessões de troca da pasta de hidróxido de cálcio leva ao aumento da citotoxicidade do medicamento que interfere na proliferação das células remanescentes e a completa formação das raízes. Como consequência, esses tratamentos convencionais aumentam o risco de reinfecções e de fraturas dentárias nos dentes imaturos (Khoshkhounejad et al., 2019). A clorexidina (CHX) tem sido recomendada como alternativa de medicação pelo seu amplo potencial antimicrobiano, geralmente associada ao hidróxido de cálcio, para casos de reabsorção radicular e perfuração radicular, porém seu uso é limitado devido à sua alta

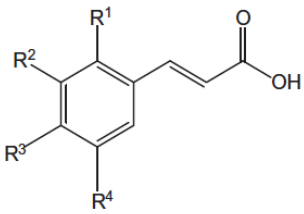
citotoxicidade (Gomes et al. 2003; Basrani et al. 2004; Lessa et al. 2010; Vasudeva et al. 2017).

Compostos fenólicos ou polifenólicos são metabólitos secundários produzidos por plantas de grande porte e possuem múltiplas funções essenciais na fisiologia da planta, como na sua defesa contra patógenos e na resposta a condições de estresses ambientais. Além disso, apresentam potencial terapêutico para o organismo humano, principalmente como agentes antimicrobianos, antioxidantes, antialérgicos, anti-inflamatórios, anticâncer, anti-hipertensivos, osteogênica e antiosteoclastogênica (Pietta, 2000; Tripoli et al., 2007; Daglia, 2012). Sua atividade protetora foi atribuída inicialmente às propriedades antioxidantes, neutralizantes de radicais livres, quelantes de metais e a habilidade de inibir ou reduzir a função de diferentes enzimas, como telomerases, ciclooxigenases, ou lipoxigenases, e recentemente, pela interação com receptores celulares e vias de transdução de sinais (Daglia, 2012).

Dependendo da sua estrutura química, os compostos fenólicos e polifenólicos podem ser classificados em várias classes incluindo os ácidos fenólicos, flavonóides, estilbenos, cumarinas, ligninas e taninos (Shahidi & Yeo, 2018). Os ácidos fenólicos são encontrados em fontes naturais, como grãos, sementes, leguminosas e cereais, e formam pontes com macromoléculas como a celulose, hemicelulose e pectina para a construção das estruturas da parede celular da planta. Podem ocorrer em formas conjugadas ou livres. Eles são divididos em dois grupos, os ácidos hidroxibenzóicos, como os ácidos p-hidroxibenzóico, protocatecuico, vanílico, siríngico e gálico e os ácidos hidroxicinâmicos, incluindo os ácidos p-cumárico, cafeico, ferúlico e sinápico (Shahidi & Yeo, 2018). Os ácidos hidroxicinâmicos são também moléculas precursoras de polifenóis como estilbenos, chalconas, flavonoides, ligninas e antocianidinas (El-Seedi et al., 2012).

Dentro do grupo dos ácidos fenólicos, os ácidos cinâmicos e seus derivados têm recebido especial atenção dos pesquisadores devido ao seu potencial para aplicação médica, destacando seu amplo espectro antimicrobiano e antifúngico (Guzman, 2014). O termo “cinâmico” é proveniente da especiaria canela (*Cinnamomum zeilanicum)* que vem sendo usada desde a antiguidade como um agente flavorizante e por suas propriedades estimulantes, carminativas, antissépticas e inseticidas. A casca de várias espécies de *Cinnamomum* contém quantidades consideráveis de (E)-cinnamaldeído,

um aldeído volátil responsável pelo sabor pungente e doce da canela. Ácidos cinâmicos também são encontrados em grãos de café, chá, mate, cacau, maçãs e peras, bagas, frutas cítricas, uva, legumes, espinafre, beterraba, alcachofra, batata, tomate, aipo, fava e cereais. Ácidos cinâmicos frequentemente aparecem como conjugados de éster com ácido quínico, conhecidos como ácidos clorogênicos, mas também podem formar ésteres com outros ácidos, açúcares ou lipídios, ou formar amidas com aminas aromáticas e alifáticas (Guzman, 2014). Os derivados naturais dos ácidos cinâmicos mais abundantes na natureza e que tem apresentado considerável potencial antimicrobiano são: ácido p-cumárico, ácido cafeico, ácido ferúlico e ácido sinápico. A **figura 1** mostra a estrutura química dos principais derivados do ácido cinâmico ou ácidos hidroxicinâmicos.



Ácidos Fenólicos	R1	R2	R3	R4
Ácido cinâmico	H	H	H	H
Ácido p-cumárico	H	H	OH	H
Ácido cafeico	H	H	OH	OH
Ácido ferúlico	H	OCH_3	H	H
Ácido sinápico	H	OCH_3	OH	OCH_3

Figura 1. Estrutura química do ácido cinâmico e seus derivados hidroxicinâmicos

O ácido p-cumárico (p-CA, 4-hydroxycinnamic acid)) é um ácido fenólico da família dos ácidos hidroxicinâmicos, biologicamente sintetizado através da via do chiquimato com fenilalanina e tirosina como precursores (El-Seedi et al., 2012). O p-CA desempenha um papel central no metabolismo secundário, porque pode ser subsequentemente transformado em outros ácidos fenólicos, como por exemplo, ácido cafeico, ácido ferúlico, ácido clorogênico e ácido sinápico, em flavonoides, lignina e outros metabolitos secundários (El-Seedi et al., 2012). O p-CA livre ou ligado é amplamente distribuído em frutos, legumes e cereais. Pesquisas mostraram que esses

ácidos (especialmente quando conjugados) exibem várias bioatividades, incluindo antioxidante, anti-inflamatória, antimutagênica, anti-úlcera, anti-plaquetária e atividades anticancerígenas (El-Seedi et al., 2012; Pragasam et al., 2013). O p-CA têm apresentado comparativamente maior potencial antibacteriano que o próprio ácido cinâmico, com amplo espectro de ação contra bactérias Gram positivas e Gram negativas. Estudo mostrou que p-CA rompe a membrana externa das bactérias Gram-negativas aumentando a permeabilidade, além de interagir com o DNA e, portanto, inibindo processos bioquímicos essenciais relacionados aos ácidos nucleicos (Lou et al., 2012).

O ácido cafeico (3,4-Dihydroxycinnamic acid, 3-(3,4-Dihydroxyphenyl) -2-propenoic acid) é um metabólito secundário vegetal da via fenilpropanóide, classificado como um ácido hidroxicinâmico contendo grupos funcionais fenólicos e acrílicos e seus derivados incluem amidas, ésteres, ésteres de açúcar e glicosídeos. Pode ser encontrado em muitos produtos vegetais como bebidas de café, aveia, trigo, arroz e azeite (Fu et al., 2010, Stojković et al., 2014). Tem sido relatado que o ácido cafeico mostrou potencial antimicrobiano e efeito sinérgico com antibióticos contra *S. aureus, S. epidermidis, K. pneumoniae, S. marcescens, P. mirabilis, E. coli, P. aeruginosa, B. cereus, M. luteus, L. monocytogenes* e *C. albicans* (Cushnie et al., 2005, Loes et al., 2014, dos Santos et al., 2018). A atividade antibacteriana do ácido cafeico contra diferentes cepas de *S. aureus* foi avaliada em um estudo de Kepa et al. (2018) e apresentou valores de CIM de 256 a 1024 µg/mL. O ácido cafeico não teve influência significativa no crescimento de fibroblastos para concentrações entre 0,06 e 1,26 mg/mL (Pinho et al.,2014). Também atua como um inibidor específico do fator nuclear-kB (NF-kB), um fator de transcrição heterodimérico que desempenha um papel central na inflamação (Liang et al., 2015)

O ácido ferúlico (trans-4-Hydroxy-3-methoxycinnamic acid) pertence ao grupo ácido fenólico comumente encontrado em tecidos vegetais (Mattila et al., 2002). O ácido ferúlico é mais comumente encontrado em grãos integrais, espinafre, salsa, uvas, ruibarbo e sementes de cereais, principalmente trigo, aveia, centeio e cevada. Tem apresentado efeito inibitório contra espécies como *S. aureus, S. pyogenes, P. aeruginosa, E. faecalis, C. albicans*, com valores de CIM entre 515 e 695 µM (Ergün et

al., 2011; Georgiev et al., 2012). A atividade anti-inflamatória do ácido ferúlico foi determinada, *in vitro*, em modelo de macrófagos THP-1, tanto em repouso (não estimulado) como em modelo inflamatório (por ação de fator de necrose tumoral ou lipopolissacarídeo). Os autores verificaram que o ácido ferúlico limita a formação de espécies reativas de oxigênio (ROS) e reduz a liberação de interleucina-1β e interleucina-6 pró-inflamatórias por macrófagos tratados com lipopolissacarídeos (Szulc-KielbikI et al., 2017).

O ácido sinápico (3,5-dimethoxy-4-hydroxycinnamic acid) pode ser encontrado em forma livre, como outros ácidos hidroxicinâmicos e também é encontrado na forma de ésteres. Ésteres hidroxicinâmicos são encontrados como glicosídeos (ésteres de açúcar) ou como ésteres de uma variedade de compostos orgânicos. O ácido sinápico é uma substância identificada em várias frutas, legumes, cereais, oleaginosas, algumas especiarias e plantas medicinais de uma variedade de compostos orgânicos. O ácido sinápico apresentou atividade inibitória contra diversas espécies bacterianas incluindo *S. aureus* e *S. pyogenes* (Georgiev et al., 2012). Também apresentou efeito anti-inflamatório inibindo ROS como radicais hidroxila, superóxido, NO e peroxinitrito (Niciforovic e Abramovic, 2014).

Diversos hidrogéis injetáveis biocompatíveis e biodegradáveis vêm sendo desenvolvidos para aplicações biomédicas com o objetivo de superar as limitações dos produtos naturais, especialmente como veículos terapêuticos. Hidrogéis apresentam uma transição do estado líquido para o gel pela formação de uma rede tridimensional em resposta a estímulo externo, como temperatura ou pH. Particularmente, os hidrogéis termossensíveis submetem-se a uma transição de estado em uma temperatura específica devido o balanço energético das interações hidrofílicas-hidrofóbicas entre as cadeias de polímeros e moléculas de água (Ponce-Vargas et al., 2013; Ramos et al., 2012). Hidrogéis injetáveis termossensíveis podem ser produzidos a partir de quitosana, um polissacarídeo de origem animal obtido da quitina do exoesqueleto de crustáceos, e indicados para aplicações biomédicas. Outro polímero, o poloxamer 407 (mistura de óxido de etileno e óxido de propileno), também foi adicionado ao hidrogel de quitosana, para promover propriedades termorreversíveis e aperfeiçoar a formulação do medicamento, como facilitar a preparação do gel, a solubilização de biomoléculas, prolongando assim o efeito farmacológico (Dumortier et

al., 2006; Giuliano et al., 2018; Yu et al., 2018). Estudos têm demostrado que a introdução de grupos fenólicos em estruturas de quitosana não somente altera as propriedades estruturais e físico-químicas (solubilidade, estabilidade térmica, cristalinas e propriedades reológicas) da quitosana, mas também aumentam notavelmente suas propriedades antioxidantes, antimicrobiana, antitumoral, anti-inflamatória, entre outras (Liu et al., 2013; Liu et al., 2017).

Sendo assim, o estudo de novas alternativas de tratamento endodôntico para dentes permanentes imaturos é primordial visando favorecer o aprimoramento e/ou substituição das técnicas e dos materiais já existentes. Dessa forma, esse estudo teve como objetivos avaliar *in vitro* 1) os efeitos antimicrobiano, antibiofilme, citotóxicico e anti-inflamatório de ácidos fenólicos derivados do ácido cinâmico e 2) o efeito antibiofilme e a citotoxicidade de hidrogéis termossensíveis de quitosana e poloxamer 407 contendo ácidos fenólicos como proposta de medicação para aplicação endodôntica.

Objetivos Gerais

Objetivos Gerais

Os objetivos deste estudo foram:

1) avaliar as atividades antimicrobiana, antibiofilme e antiinflamatória e a citotoxicidade do ácido cinâmico e seus derivados;

2) sintetizar e caracterizar as propriedades químicas e físico-mecânicas de hidrogéis termossensíveis de quitosana e poloxamer 407 contendo ácidos fenólicos, e avaliar o efeito desses hidrogéis sobre biofilmes multiespécies e na viabilidade celular de macrófagos e fibroblastos.

Capítulo 1

Antimicrobial, antibiofilm and antiinflamatory properties of cinnamic acid and cafeic acid for endodontic applications

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Abstract

The complexity of root canals anatomy and multispecies biofilms increases the difficulty in eliminating the microorganisms and control inflammation by conventional chemical-mechanical procedures. Then new compounds with wide-range antimicrobial effect and antiinflamatory potential, such as the phenolic acids, could be explored as active principle for intracanal medicaments. This study evaluated the antimicrobial activity of the cinnamic acid and its derivatives coumaric acid, caffeic acid, ferulic acid and sinapic acid by determining the minimum inhibitory and bactericidal concentration (MIC/MBC) and Fractional Inhibitory Concentration (FIC) on *Enterococcus faecalis*, *Streptococcus mutans*, *Lactobacillus casei*, *Actinomyces israelii* and *Fusobacterium nucleatum*. The phenolic acids were selected and their effect on dual-species and multispecies biofilms with the same standard strains or clinical strains were evaluated by bacterial counts, scanning electron microscopy and confocal microscopy. L929 fibroblast and RAW 264.7 macrophage viability in the presence of these phenolic acids were evaluated by resazurin assays. In addition, mRNA levels of pro-inflammatory markers TNF-α, IL-1β, iNOS and COX-2 were determined by TaqMan quantitative PCR after macrophage exposure to phenolic acids and LPS. Data were statistically analyzed considering $p < 0.05$. Cinnamic acid and caffeic acid presented inhibitory and bactericidal effect against all bacterial species tested, with the lowest MIC and MBC values. No synergistic effect was noted between the compounds (FICI>0.5). Both compounds at 5x the highest MIC were effective in eliminating dual-species biofilms and significantly decreasing multispecies biofilms, especially cinnamic acid. Cinnamic acid caused low toxicity to both cell cultures at MIC concentrations and caffeic acid was not cytotoxic at concentrations below 0.125 mg/mL. Both compounds significantly reduced TNF-α, IL-1β, iNOS and COX-2, in a dose dependent manner. In conclusion, cinnamic acid and caffeic acid showed antibactericidal and antibiofilm effect against bacterial species related to endodontic infections, causing low cytotoxicity. Besides, both compounds demonstrated antiinflamatory effect, inhibiting pro-inflammatory markers expression in LPS-stimulated macrophages.

Key words: cinnamic acid, caffeic acid, antimicrobial activity, biofilms, cytotoxicity, antiinflamamtory activity

1. Introduction

The fundamental principles of endodontic treatment are based on the removal of pulp necrotic tissues, bacteria and their byproducts from infected canal systems and sealing of root canals (Kawashima et al. 2009). Even with the introduction of new heat-treated nickel-titanium instruments and new irrigation protocols, mechanical and chemical debridements are not able to reach microorganisms in inaccessible areas, such as secondary and accessory canals, isthmuses, apical deltas or until extrarradicular lesions and control periapical inflammation (Ordinola-Zapata et al. 2022). Consequently, anaerobes and facultative bacteria are commonly found in post-treatment apical periodontitis especially Gram-positive bacteria such as *Streptococcus*, *Actinomyces*, *Lactobacillus, Propionibacterium, Parvimona* and *Enterococcus* species and some Gram-negative bacteria such as *Fusobacterium nucleatum* and *Prevotella* spp. (Siqueira and Roças 2009, 2021). These microorganisms are frequently organized in multispecies biofilms attached to canal walls and represent a challenge for appropriate infection control (Siqueira and Roças 2021).

Intracanal medicaments are indicated after initial disinfection appointment to eliminate residual bacteria, decrease periapical inflammation and as a barrier against microleakage from the temporary sealing (Vera et al. 2012). Calcium hydroxide is the most recommended intracanal medicament due its antimicrobial activity and ability to dissolve residual tissue (Türkün and Cengiz 1997; Siqueira and Lopes 1999; Nelson-Filho et al. 2002). Despite these advantages, studies have demonstrated the presence of biofilm/bacteria in the root canals even weeks after filling with calcium hydroxide medication, with no significant reduction of lipopolysaccharide (LPS) endotoxin (Gomes et al. 2003; Vianna et al. 2007; Roças and Siqueira 2011). Chlorhexidine is a cationic bisbisguanide with wide-range antibacterial effect against Gram-positive and Gram-negative bacteria, fungi, and virus (Gomes et al. 2003; Vasudeva et al. 2017). It has been demonstrated that chlorhexidine is more effective in eliminating *E. faecalis* from dentin tubules than calcium hydroxide (Gomes et al. 2003; Basrani et al. 2004; Vasudeva et al. 2017). However, chlorhexidine has presented cytotoxicity when in contact to cells even at low concentrations (Lessa et al. 2010).

Phenolic compounds extracted from plants have been largely studied for medical purposes due to their wide range of therapeutic properties, such as antimicrobial, antioxidant, anti-inflammatory, anticarcinogenic, and other effects and by possessing many advantages, such as biocompatibility, bioavailability, and low toxicity (Daglia 2012; Pinho et al. 2014). Depending on their chemical structure, phenolic and polyphenolic compounds can be classified into several classes, namely phenolic acids, flavonoids, stilbenes, coumarins, lignins, and tannins (Shahidi and Yeo 2018). Phenolic acids are found in natural sources such as grains, seeds, legumes, and cereals building the cell wall structures by forming brigdes with cellulose, hemicellulose, and pectin. They are divided in two groups, hydroxybenzoic acids such as *p*-hydroxybenzoic, protocatechuic, vanillic, syringic and gallic acids and hydroxycinnamic acids including *p*-coumaric, caffeic, ferulic and sinapic acids (Shahidi and Yeo 2018).

The antimicrobial action of cinnamic acid derivatives is considered to be concentration dependent. These small lipophilic molecules can cross the cell membrane by passive diffusion in their undissociated form, disrupting or even disrupting the membrane structure, acidifying the cytoplasm, and causing protein denaturation in bacteria (Malheiro et al. 2016; Ribeiro et al. 2018; Qian et al. 2019). Extracts of *Nidus vespae* honeycomb, a natural product rich in phytochemicals, including vanillic acid, p-coumaric acid, ferulic acid, quercetin and kaempferol, showed antimicrobial potential against *S. mutans* and *S. sobrinus* in concentrations ranged from 2 to 8 mg/mL (MIC) (Guan et al. 2012). Hydroxycinnamic acids also showed anti-inflammatory properties *in vitro* and *in vivo* (Itagaki et al. 2009; Kim et al. 2012). Four cinnamic acid derivatives, including two free cinnamic acids, p-coumaric acid and ferulic acids, inhibited NO production and iNOS expression in a dose-dependent manner in macrophages cultures exposed to LPS (Kim et al. 2012). Ferulic acid and derivatives suppressed NF-κB activation and modulating the expression of proinflammatory COX-2, iNOS, VCAM-1 and ICAM-1 in rats (Jung et al. 2009). Caffeic acid reduced the effect of COX-2 and its product PGE_2 as well as the biosynthesis of IL-8 in IL-1β-treated cells (Zielinska et al. 2021).

Natural antimicrobial products have demonstrated to reduce the ability of microorganisms to adhere, communicate and form biofilms, without causing bacterial tolerance or resistance (Borges et al. 2012; Silva et al. 2016). Although the ability of

hydroxicinnamic acids to inhibit microbial growth in planktonic conditions has been frequently reported in the literature, studies evaluating their effect in biofilms are relatively scarce (Silva et al. 2016) especially against multispecies biofilms. Besides, cytotoxic effect and anti-inflammatory potential of cinnamic acid derivatives needs to be better explored before considering their clinical application. The objectives of this study were to evaluate the antimicrobial, antibiofilm and anti-inflammatory properties of hydroxycinnamic acids, as well as their cytotoxicity for endodontic purposes.

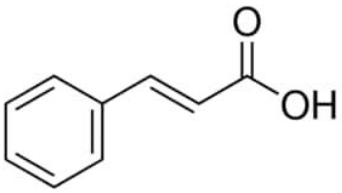
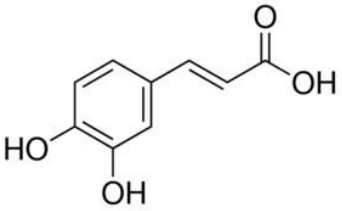
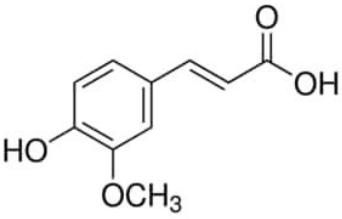
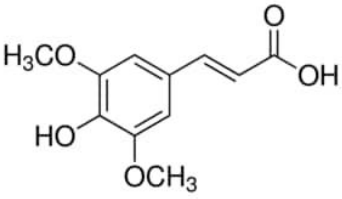
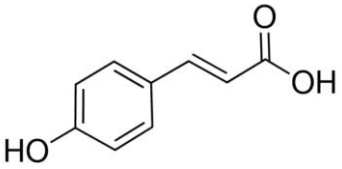
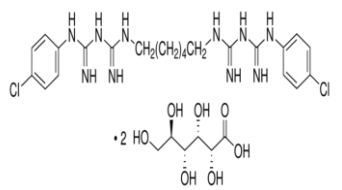
2. Materials and methods

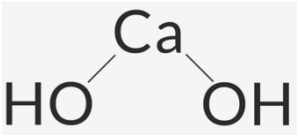
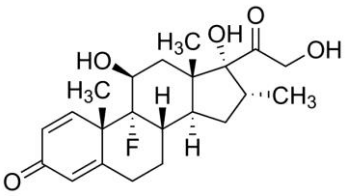
2.1 Phenolic acids and controls

The following phenolic acids were evaluated: cinnamic acid (#C80857), p-coumaric acid (#C9008), caffeic acid (#C0625), ferulic acid (#128708) and sinapic acid (#D7927) which were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of phenolic acids were prepared and frozen in dimethylsulfoxide – DMSO and only work solutions containing up to 5% DMSO were used for the subsequent tests not to influence the results. For microbiological and cytotoxicity assays, positive controls were calcium hydroxide PA (Biodynamics, Ibiporã – PR/BR) dissolved in saline solution (1mg/mL) and chlorhexidine digluconate (Farmácia Manipullis, Araçatuba – SP/BR) diluted in sterile water. For antiinflamatory assays, positive control was dexamethasone (#D4902, Sigma-Aldrich) also diluted in sterile water. All compounds were sterilized by filtration through a 0.22 µm membrane filter. The assays were performed in triplicate in three independent days. **Table 1** shows the phenolic acids and controls used in this study, their codes, synonyms, chemical structure, empirical formula, and molecular weight.

Table 1. Flavonols and controls used in the present study.

Compound Code*	**Synonyms**	**Chemical structure**	**Empirical formula Molecular weight (g/mol)**

Cinnamic acid (CI) #1133933	*trans*-3-Phenylacrylic acid, (2*E*)-3-Phenyl-2-propenoic acid, *trans*-Cinnamic acid, Cinnamic acid		$C_6H_5CH=CHCOOH$ 148.16
Caffeic acid (CA) #C0625	3,4-Dihydroxybenzeneacrylic acid, 3,4-Dihydroxycinnamic acid, 3-(3,4-Dihydroxyphenyl)-2-propenoic acid		$(HO)_2C_6H_3CH=CHCO_2H$ 180.16
Ferulic acid (FE) #PHR1791	*trans*-4-Hydroxy-3-methoxycinnamic acid, Ferulic acid, *trans*-Ferulic acid		$HOC_6H_3(OCH_3)CH=CHCO_2H$ 194.18
Sinapic acid (SI) #D7927	Sinapinic acid, 3,5-Dimethoxy-4-hydroxycinnamic acid, 4-Hydroxy-3,5-dimethoxy-cinnamic acid		$C_{11}H_{12}O_5$ 224.21
p-Coumaric acid (CO) #C9008	*trans*-4-Hydroxycinnamic acid		$HOC_6H_4CH=CHCO_2H$ 164.16
Chlorexidine digluconate (CHX) #C9394	1,6-Bis(N^5-[p-chlorophenyl]-N^1-biguanido)hexane; 1,1'-Hexamethylenebis(5-[p-chlorophenyl]biguanide)		$C_{22}H_{30}Cl_2N_{10}$ 505.44

Calcium hydroxide (CH) #232932	calcium hydrate, lime, hydrated lime, caustic lime, lime hydrate, slaked lime		$Ca(OH)_2$ 74.09
Dexamethasone (DEX) D4902	(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione, 9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione, 9α-Fluoro-16α-methylprednisolone, Prednisolone F		C22,H29,FO5 392.46

*Codes and information taken from the Sigma-Aldrich company website (https://www.sigmaaldrich.com).

Determination of antimicrobial activity

2.2. Standard strains

To evaluate the antimicrobial and antibiofilm activities of the compounds, the following standard strains were used: *Streptococcus mutans* (ATCC 25175), *Lactobacillus casei* (ATCC 393), *Actinomyces israelii* (ATCC 12102), *Enterococcus faecalis* (ATCC 51299) and *Fusobacterium nucleatum* (ATCC 25586). The strains were donated by the Oswaldo Cruz Foundation – FIOCRUZ, Rio de Janeiro. The culture media used for 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*, the medium used was BHIA containing 5mg/L of hemin, 5mg/mL of menadione and 5% of defibrinated sheep blood. The plates were incubated at 37°C under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, UK).

2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC assays were measured by the microdilution method, based on the CLSI standards (Clinical and Laboratory Standards Institute - M7-A9, 2012) with some modifications (dos Santos et al. 2021). Cultures of the bacterial species mentioned in item 2.2 were subcultured in Miller Hinton (MH) broth medium (Difco) for 24h at 37° C. Then cells were separated by centrifugation and resuspended in sodium phosphate buffer – PBS (10 mM, pH 6.8) and the number of cells was adjusted to obtain 1-5x 10^5 cells/mL in MH. Counts were validated by counting on MH agar. A 50 µL inoculum of each bacterium was then incubated in 50 µl of the serial dilution of the phenolic acid (from 1 to 0.0001 mg/mL) and controls dissolved in sterilized water. After incubation for 24 h (and 48h for *F. nucleatum*) at 37°C under the previously atmospheric conditions described in item 2.2, the microtiter plates were stained with rezasurin solution (Sigma-Aldrich) at 5 µg/mL for 3h and analyzed at 595 nm in a spectrophotometer (Biotek, Winooski, VT). Bacterial suspensions were incubated with chlorhexidine (CHX) or without any antimicrobial agent, as positive and negative controls, respectively. The MIC was defined as the lowest concentration of the compound in which there was no detectable growth (blue staining). Media containing the MIC and two higher concentrations were serially diluted and plated on MH agar and incubated at 37 °C for 48 h. Colonies were counted using a binocular stereomicroscope and the number of colony forming units/ml (CFU/mL) was determined. MBC was considered when the compound killed more than 99.9% of the bacteria.

2.4. Determination of Fractional Inhibitory Concentration (FIC)

Only the phenolic acids that showed antimicrobial activity against all species evaluated, determined in the previous tests (item 2.3), were evaluated in combination using the checkerboard microdilution method, according to Tong et al. (2014). The fractional inhibitory concentration (FIC) was derived from the highest dilution of the antimicrobial agent's combination in which did not show detectable growth using resazurin staining. The FIC index was determined by the equation: FIC index = FIC A + FIC B (MIC of antimicrobial A in combination/MIC of A alone) + (MIC of antimicrobial B

in combination/MIC of B alone). Synergy was defined when the FIC index (FICI) is ≤ 0.5, additive 0.5 > FICI < 1, indifferent 1 > FICI < 4 and antagonistic when FICI > 4.

Effect of compounds on dual-species and multispecies biofilms

2.5. Assessment of the viability of dual-species biofilm by bacterial counting

Biofilm assays were performed with all standard bacterial species (*S. mutans*, *L. casei*, *A. israelii* and *F. nucleatum*) combined in dual-species biofilms with *E. faecalis* as proposed by Gao et al. (2016) and modified by dos Santos et al. (2021). Initially, bacterial cultures were centrifuged, washed in sodium phosphate buffer and cell number adjusted to 1-5x10^3 CFU/mL in BHI broth. Sterile 96-well U-bottom polystyrene microplates were pre-treated with 150 µL of artificial saliva (composition: 800mL of deionized water, 1.6 g of yeast extract, 4 g of peptone, 0.28 g of NaCl, 4 g of sucrose, 0.16 g of $CaCl_2$, 0.16 g of KCl and 0.8 g of mucin) for a period of 4 h at 37°C in the 5% CO_2 (coating phase) proposed by Lamfon et al. (2003), and modified by Cavazana et al. (2018). After the incubation period, the saliva was removed and 10 µl of each bacterial culture were inoculated into each of the wells containing 180 µl of BHI broth supplemented with 0.5% sucrose (for *S. mutans* + *E. faecalis*) or 1% glucose (for the other bacterial strains). The plates were incubated at 37°C in an atmosphere of 5% CO_2 or anaerobiosis for *F. nucleatum* + *E. faecalis*. The cultures were incubated for 2 weeks, and the medium was changed every 48 h for cultures and once a week for *F. nucleatum*. After these periods, the culture medium was removed and the wells were washed with sterile saline (0.9% NaCl) for subsequent addition of 100 µL of the compounds - Cinnamic acid (CI) at 5 mg/mL, caffeic acid (CA) at 5 mg/mL (5x the highest MIC), chlorhexidine (CHX) 0.5 mg/mL (approximately 100x the highest MIC) and calcium hydroxide (CH) at 1 mg/mL – concentration currently used for endodontic applications) in each well. Culture medium without antimicrobial agents was considered as negative control. Microplates containing or not antimicrobial agents were incubated for 48h at 37°C in an atmosphere of 5% CO_2 or anaerobic conditions. The treatments were removed and the wells washed with sterile saline. Aliquots from all wells were serially diluted and plated on BHI agar. The plates were incubated for 48h for subsequent determination of CFU/mL using a binocular stereomicroscope.

2.6. Effect of the compounds on multispecies biofilms and scanning electron microscopy

The multispecies biofilm assays were conducted following the same steps described previously for dual-species biofilms, however, all bacteria (*E. faecalis*, *A. israelii*, *S. mutans* and *F. nucleatum*) were mixed in equal aliquots at the same concentration (1-5x10^3 CFU/mL) in BHI broth containing 1% glucose. After 1 week of growth in anaerobic conditions, biofilms were washed twice with sterile saline solution and 100 µl of CI at 5 mg/mL, CA at 5 mg/mL, CH at 1mg/mL and CHX at 0.5 mg/mL were inserted into each well. The plates were incubated for 48h at 37°C in anaerobic conditions. After scraping of biofilms, aliquots from all wells were resuspended, serially diluted, and plated on BHI agar. The plates were incubated for 48h for further counting of CFU/mL. The same experiments were conducted in parallel in coverslips for microscopic analysis. The samples were dehydrated by washing in a series of ethanol (70% for 10min, 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 (Leo, Cambridge, MA, USA) (Jacob et al. 2020).

2.7. Effect of compounds on the multispecies biofilms formed on root dentin and analysis by Confocal Microscopy

2.7.1. Collection of clinical samples of human necrotic root canals

For this part of the study, multispecies biofilms were prepared from the collection of biofilm samples from the necrotic root canals of human teeth, as described by Yang et al. (2016) and dos Santos et al. (2021). Samples were collected from three healthy adult donors aged 25-45 years already under endodontic treatment at the dental clinics of the faculty after approval by Human Research Ethics Committee - FOA/UNESP (CAAE # 22735019.0.0000.5420). The criteria of inclusion were fully erupted first permanent molars with intact pulp chamber walls and sufficient tooth structure for absolute isolation, diagnosed with apical or periradicular periodontitis. Briefly, after rubber dam isolation, operative field and teeth were disinfected with 0.12% CHX and samples collected from all root canals using sterile paper cones (n° 25, Tanari, Manaus, AM, Brazil), before the start of treatment. Biofilm samples were inserted into a tube containing BHI broth and incubated under anaerobic conditions at 37 °C for 72 h. The

cultures were centrifuged, and the number of cells adjusted to $3x10^6$ cells/mL by counting in blood agar BHI medium.

2.7.2. Preparation of dentin specimens and confocal analysis

The biofilm assay for analysis by confocal microscopy was performed according to Ma et al. (2011) modified by dos Santos et al. (2021). A 4 mm bovine root dentin block (n=6/group) was sectioned horizontally from each tooth 1 mm away from the cemento-enamel junction with a 0.6 mm diamond disk (Isomet 5000; Buehler Ltd, LakeBluff, IL) at 1000 rpm, under irrigation with water. The root canal wall of each specimen was ground with a #6 wide bur and the specimen was again sectioned into two semi-cylindrical halves. The smear layer was removed by immersion in a 17% EDTA solution in ultrasound for 3 min and 5 min with deionized H_2O. The specimens were sterilized in an autoclave at 121°C for 15 min. Then, the specimens were washed for 1 minute with distilled water and inserted into a microtube with the canal side up. Then, 500 µL of the bacterial culture from necrotic canals were inoculated and microtubes were centrifuged at 1400xg, 2000 xg, 3600 xg and 5600 xg in this sequence, twice each, for 5 minutes. A fresh aliquot of bacteria was added between each centrifugation and the old one was discarded. All microtubes were incubated at 37 °C in BHI broth for 2 weeks under anaerobic conditions to form multispecies biofilms in dentin tubules. The medium was changed every 72 h. The dentin specimens were removed from the microtube and washed with sterile water for 1 minute. The external surfaces (cementum side) of the specimens were closed with nail polish. The specimens were randomized and divided into groups: negative control – sterile water; CI at 5 mg/mL , CA at 5 mg/mL, CH at 1 mg/mL and CHX at 0.5 mg/mL. Each specimen was immersed in 100 µL of each solution for 48 h. Then, the specimens were washed with sterile water for 1 minute and immersed in a solution of 0.9 mM KH_2PO_4 and 1.5 mM $CaCl_2$ (pH 7.0) at 37 °C to avoid residual effects of the treatments. Then the specimens were fractured into two new halves and stained with the fluorescent stain LIVE/DEAD BacLight Bacterial Viability stain (Molecular Probes, Eugene, OR). Two uninfected specimens were stained with the same protocol and used as an internal control. The fluorescence of the stained cells was evaluated in a Zeiss fluorescence confocal microscope (model LSM 780) and the 2D images acquired by the software Zen using the resolution of

1024x1024 pixels. The proportion of dead cells in the 2D images was determined by counting of dead cells (stained in red) over total cell counts (live – in green + dead – in red) using the Image J 1.48 program (NIH, Bethesda, MA, USA).

Determination of the compounds citotoxicity

2.8. Evaluation of cell viability by resazurin assays

The cytotoxicity of compounds was determined on culture of murine fibroblasts L929 fibroblasts and murine macrophages RAW264.7, according to Duque et al. (2022). Cells were grown in Dulbecco's modified Eagle medium (DMEM, Dulbecco's Modified Eagle's, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine (Gibco) containing 5% CO_2 and 95% air at 37°C until reaching 80% confluence. Then cells were cultured at density of 5x 10^4 cells/well in 96-well plates and incubated for 24 h. The cells were treated with CI, CA, CH and CHX (from 1 to 0.0019 mg/mL) for 24 h. Cells were washed with PBS and resazurin 70µM in DMEM was added for 4h. After that, the plates were analyzed in a spectrophotometer (Biotek, Winooski, VT) at 570 and 600nm. The final values were obtained by the subtractions between the absorbance values at both wavelengths, and they were converted into percentage of cell viability considering the growth in DMEM medium as 100% and the means determined for each group.

Anti-inflammatory activity of the compounds

2.9. Gene expression of pro-inflamamatory markers by quantitative PCR (qPCR)

Murine macrophages RAW264.7 were cultured in 24-wells microplates in DMEM supplemented with fetal bovine serum and antibiotics for 48h. Then, cells were pre-treated with CI, CA, or dexamethasone (DEX) at 0.25 and 0.025 mg/mL for 2h with a further 6h exposure of 100ng/mL lipopolysachharides (LPS) from *Escherichia coli* O111:B4 (#L2630, Sigma-Aldrich). After that, macrophages were washed with PBS at 4°C and total RNA was isolated using the protocol recommended by the manufacturer of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by DNase I treatment (Sigma, Saint Louis, MO, USA). Then, the amount and purity of the genetic material were verified by A260/A280 spectrophotometry (BioTek/Synergy H1). Reverse

transcription in 20 µL of cDNA was performed with 500 ng of RNA, following the recommendations of the manufacturer of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Foster City, CA, USA).

The qPCR reactions were performed using 1 µL of cDNA and standardized assays containing primers and hydrolysis probes (TaqMan Gene Expression Assays; Applied Biosystems, Life Technologies) to amplify the sequences corresponding to the genes TNF-α (ID:Mm00443258_m1), IL-1β (ID:Mm00434228_m1), iNOS (ID:Mm00440502_m1) and COX-2 (ID:Mm03294838_g1) The reactions were processed in the StepOnePlus system (Applied Biosystems, Life Technologies) following thermocycling conditions standardized by the manufacturer of the gene expression assays and the Master Mix (TaqMan Fast Advanced Master Mix; Applied Biosystems, Life Technologies): Uracil-DNA glycosylase incubation at 50 °C for 2 min, DNA polymerase activation at 95 °C for 2 min; 40 cycles of qPCR with denaturation at 50 °C for 1 s and annealing at 60 °C for 20 s. Gene expression was calculated by the comparative quantification cycle method (2-ΔΔCq) using the expression of the β-actin (ACTb) reference gene (ID:Mm02619580_g1), being presented relatively (fold change) to the control.

2.10. Statistical analysis

In virtue of amplitude of CFU/mL counts, microbial data were transformed in Log (CFU+1/mL). The constant +1 was added because some specimens had a zero count. Data from antibiofilm assays, cytocompatibility and anti-inflammatory tests were expressed in means/standard deviation 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

Antimicrobial activity of the cinnamic acid derivatives

3.1. Determination of MIC and MBC values

Table 2 shows the MIC and MBC values obtained for the phenolic acids against the bacterial species tested. Ferulic acid and sinapic acid inhibited only against *A. israelli* and *F. nucleatum* growth, at the maximal concentration tested of 1 mg/mL. Coumaric

acid presented bactericida effect against *A. israelli* and inhibited *S. mutans* and *F. nucleatum* growth. Cinnamic acid (CI) and caffeic acid (CA) presented inibibitory and bactericidal effect against all bacteria tested with MIC values ranged from 0.5 and 1 mg/mL and MBC values from 0.5 to 1 mg/mL. The control chlorhexidine (CHX) showed the lowest MIC and MBC values for all bacterial species tested.

Table 2. Minimal inhibitory concentration - MIC and minimal bactericidal concentration - MBC in mg/mL for the phenolic acids and chlorhexidine against the oral bacteria tested

	E. faecalis ***MIC (MBC)***	***A. israelli*** ***MIC (MBC)***	***S. mutans*** ***MIC (MBC)***	***L. casei*** ***MIC (MBC)***	***F. nucleatum*** ***MIC (MBC)***
CI	0.5(1)	0.5 (1)	0.5 (1)	1(1)	0.25(0.5)
CA	0.5(1)	0.125 (0.25)	0.5 (1)	1(1)	0.25(0.5)
FE	>1(>1)	1 (1)	>1(>1)	1(>1)	1(>1)
SI	>1(>1)	1 (>1)	>1(>1)	>1(>1)	0.5(>1)
CO	>1(>1)	0.5 (1)	1(>1)	1(>1)	1(>1)
CHX	0.004 (0.009)	0.002 (0.002)	0.0006 (0.0006)	0.001 (0.001)	0.009 (0.009)

*CI – cinnamic acid, CA – caffeic acid, FE – ferulic acid, SI – sinapic acid, CO – p-coumaric acid. MIC results were based on resazurin staining and MBC results were based on CFU/mL count in Miller Hinton Agar (MHA) medium. **MBC = >99.9% bacterial reduction. The growth of microorganisms without antimicrobials in MHA was considered 100%.

3.2. Determination of FIC values and synergistic effect

Only the CI and CA were chosen for this assay because they presented antimicrobial effect against all bacteria tested in the previous assays. Synergy was defined when the FIC index (FICI) is ≤ 0.5, additive 0.5 > FICI < 1, indifferent 1 > FICI < 4 and antagonistic when FICI > 4. For *E. faecalis*, *A. israelii* and *S. mutans* the FIC index was indifferent. *For L. casei* and *F. nucleatum*, FIC index was considered additive. Therefore, there was no synergistic effect between CI and CA.

Table 3. Fractional inhibitory concentration (FIC) in mg/mL and FIC index (FICI) for the phenolic acids against the oral bacteria tested

	E. faecalis	***A. israelii***	***S. mutans***	***L. casei***	***F. nucleatum***
CI + CA	0.06+ 0.5 FICI= 1.12 Indifferent	0.078+0.125 FICI = 1.15 Indifferent	0.06+0.5 FICI = 1.12 Indifferent	0.078+0.5 FICI = 0.57 Additive	0.06 +0.125 FICI = 0.74 Additive

Effect of the cinnamic acid and caffeic acid on biofilms growth

3.3. Effect of the compounds on dual-species biofilms and evaluation by bacterial counts

Figures 1A-D show the effect of CI, CA, calcium hydroxide (CH), and CHX on the total bacterial counts of dual-species biofilms (*E. faecalis + S. mutans, E.faecalis + A. israelii, E. faecalis + L. casei, and E. faecalis + F. nucleatum*) after 2 weeks of growth and 48h of treatment. No detectable bacterial counts were observed for all dual-species biofilms treated with both phenolic acids – CI and CA at 5x the highest MIC. The positive controls CH at 1mg/mL (concentration clinically used) and CHX at 100x the highest MIC significantly reduced total bacterial counts for all dual-species biofilms and CHX eliminated *E. faecalis* and *L. casei* biofilms.

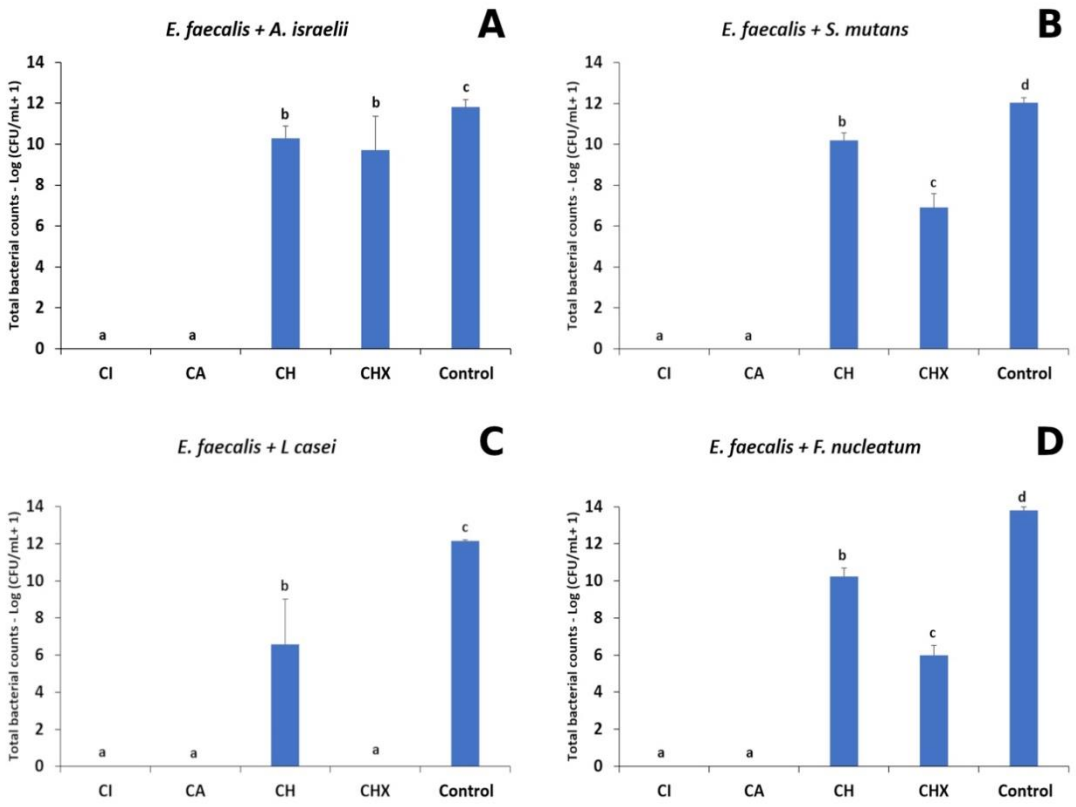


Figure 1. Effect of phenolic acids and controls on 2 weeks dual-species biofilms of oral bacteria combined with *E. faecalis* after 48h of treatment. Graphics represent total bacterial counts in Log CFU/mL+1. Values are presented as means and standard deviations.
Cinnamic acid (CI) - 5 mg/mL (5x the highest MIC value); Caffeic acid (CA) – 5 mg/mL (5x the highest MIC value), Calcium hydroxide (CH) – 1 mg/mL, CHX - 0.5 mg/mL (100x the highest MIC value), and Control – Culture medium without antimicrobial agents.
[a] Different letters show statistical difference between the groups of antimicrobials for each dual-species biofilms tested, according to One-Way ANOVA and Tukey test ($p<0.05$).

3.4. Effect on multispecies biofilms and scanning electron microscopy

Figure 2 (A-F) shows representative images of scanning electron microscopy of multispecies biofilms with *E. faecalis, S. mutans, A. israelli, L. casei and F. nucleatum* after treatment with the phenolic acids and controls. Evident biofilm disorganization and areas of "crack" and a substantial reduction in bacterial presence and in extracellular matrix are observed in **Figures 2A, B, C and D** when multispecies biofilms were treated with CA, CI at 5x MIC and CHX at 100x MIC. **Figure 2E** evidenced the complex and organized structure of multispecies biofilms with no influence of antimicrobial agents. It can be seen bacteria co-aggregated and incorporated in an extracellular polymeric matrix. **Figure 2F** shows the effect of all compounds on total

bacterial counts (in CFU/mL) isolated from the multispecies biofilms, confirming that CI and CA were the most effective treatments, followed by CHX and CH.

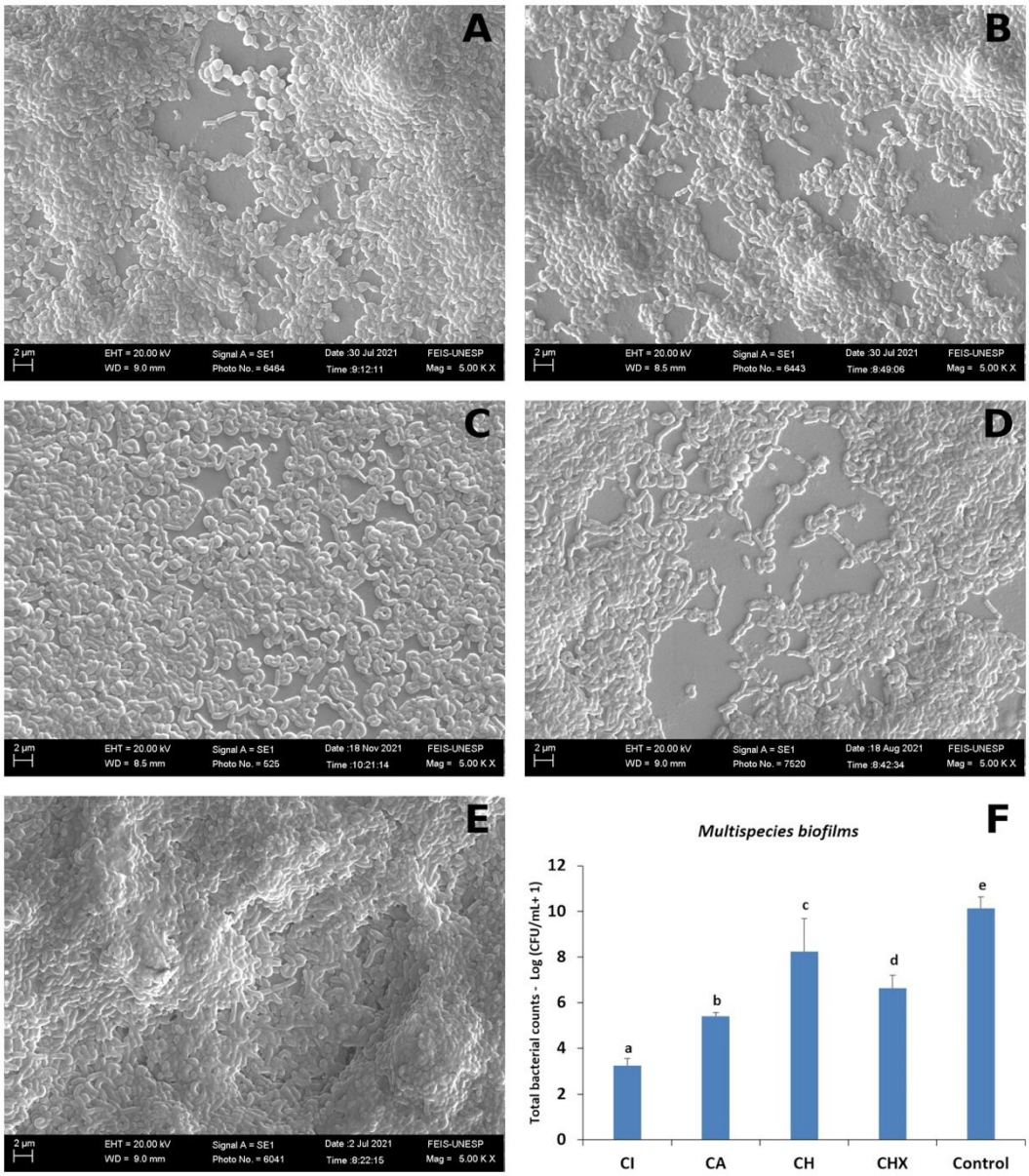


Figure 2. Representative scanning electron microscopy images of 7 days multispecies biofilms under 1000x magnification. Biofilms were treated for 48 hours with A: Cinnamic acid (CI) - 5 mg/mL; B – Caffeic acid (CA) – 5 mg/mL, C - Calcium hydroxide (CH) – 1 mg/mL, D - CHX - 0.5 mg/mL, and E - Control – Culture medium without antimicrobial agents. F. Mean (SD) of the total bacterial counts detected after 48h of the biofilm treatment with the compounds.
[a] Different letters show statistical difference between the antimicrobial groups, according to One-Way ANOVA and Tukey test ($p<0.05$).

3.5. Effect on multispecies biofilms inside root dentin tubules and confocal microscopy

Figure 3 shows the effect of 48h treatment with CI, CA, positive controls CH and CHX on 2-weeks multispecies biofilms formed inside human root canals. Representative confocal images of each group are observed in **Figures 3A-E** showing the dead (red spots) and live (green spots) patterns among the groups. CI led to 79.7% of bacterial reduction, an effect more than 2x higher than that of CHX (34.8%) and HC (35.8%). CA showed similar effect when compared to CH and CHX, reducing 47.1% of bacteria in multispecies biofilms.

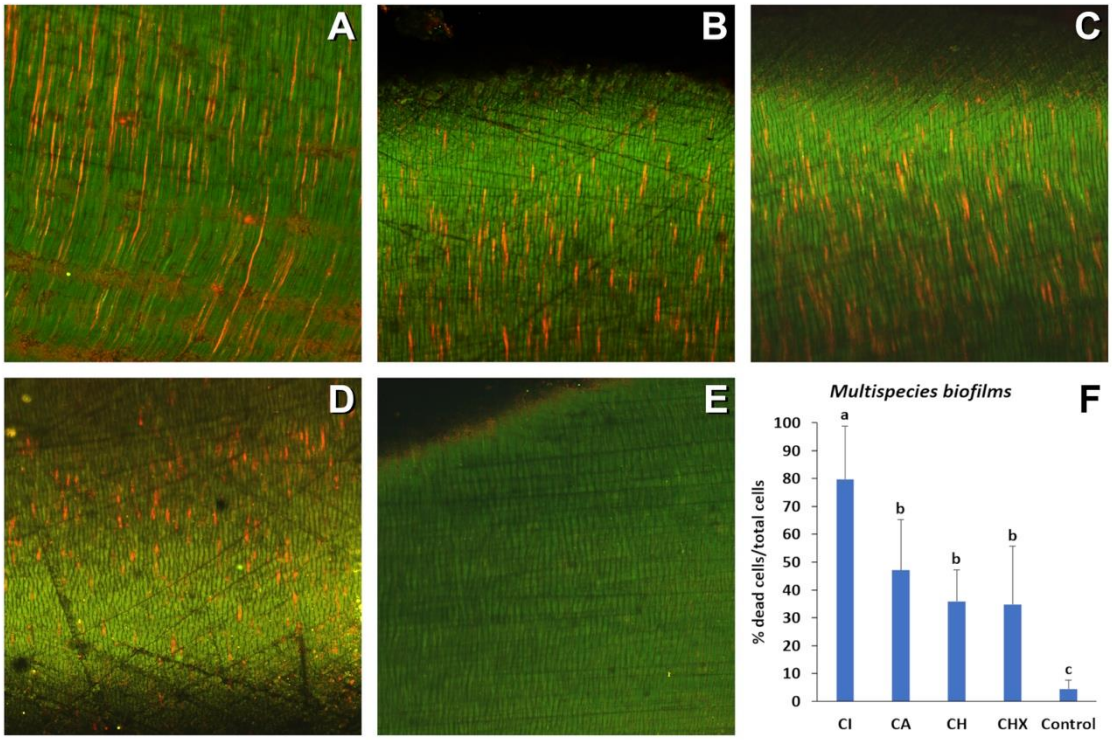


Figure 3. Representative confocal microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms isolated from necrotic human pulps and treated for 48 hours. The groups of the study were: A - Cinnamic acid (CI) - 5 mg/mL; B – Caffeic acid (CA) – 5 mg/mL, C - Calcium hydroxide (CH) – 1 mg/mL, D - CHX - 0.5 mg/mL, and E - Control – Culture medium without antimicrobial agents. F. Mean (SD) of the percentages of dead cells of multispecies biofilms after 48h of treatment with phenolic acids and controls. Bacterial counts were obtained by Image J analysis.

[a] Different letters show statistical difference between the antimicrobial groups, according to One-Way ANOVA and Tukey test ($p<0.05$).

Cytoxicity effect of cinnamic and caffeic acids

3.6. Effect on fibroblast and macrophage viability

The effect of the compounds CI, CA, CH and CHX on fibroblasts and macrophages viability can be seen in **Figure 4A** and **Figure 4B**, respectively. Compounds were tested at serial concentrations between 1 mg/mL and 0.00019 mg/mL, considering MIC range evaluated in the present study. For both cells, CI and CA can be considered cytocompatible (around 80% of cell viability) at 0.5 mg/mL and 0.125 mg/mL, respectively. CH was not cytotoxic for both cells at the concentrations tested. In the presence of CHX, cell viability was superior to 80% only at the concentrations below 0.0078 mg/mL and 0.0019 mg/mL for fibroblasts and macrophages, respectively.

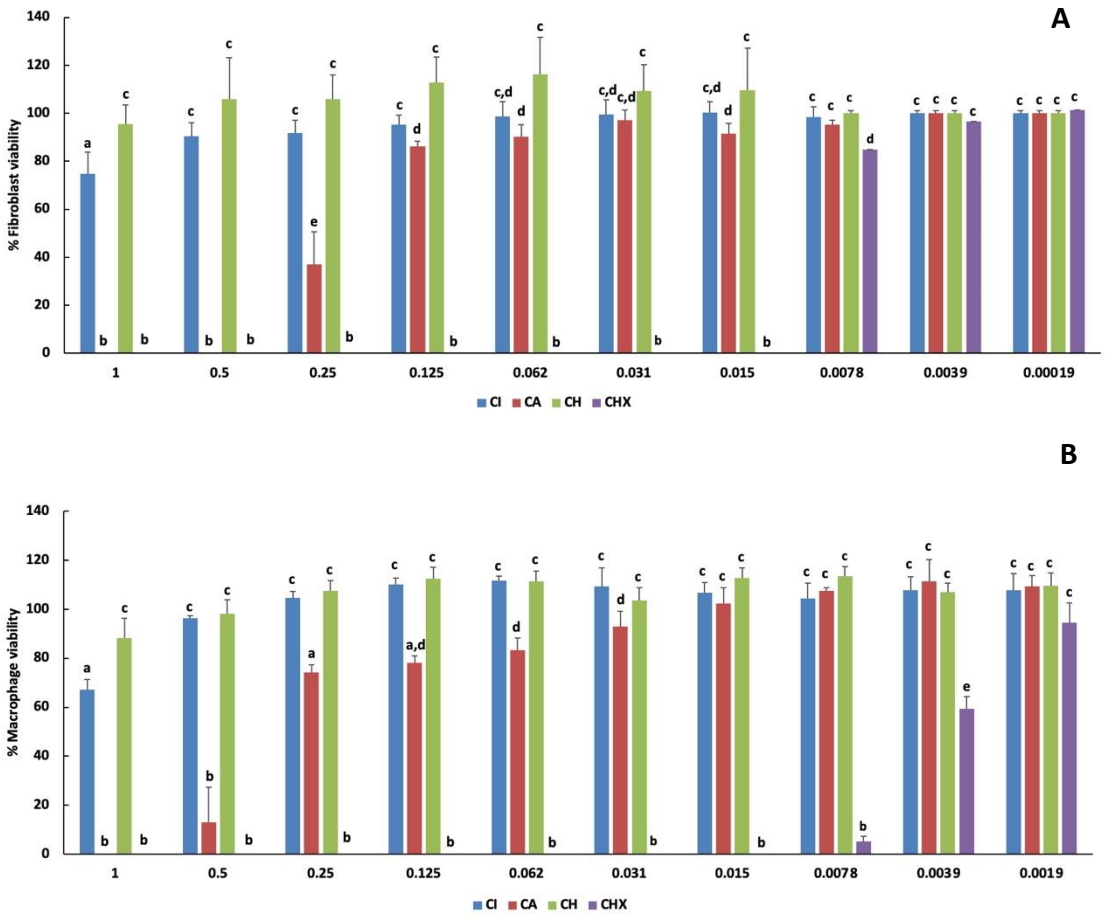


Figure 4. Effect of the cinnamic acid (CI) and caffeic acid (CA) and controls calcium hydroxide (CH) and chlorhexidine (CHX) on the viability of fibroblasts (L929) **(A)** and macrophages **(B)** after 24h of exposure, using staining with resazurin. Concentrations in mg/mL.
[a] Different letters show statistical difference among the groups and concentrations, according to Two-Way ANOVA and Tukey test ($p<0.05$).

Anti-inflammatory effect of cinnamic acid and caffeic acid

3.6. Effect of the compounds on pro-inflammatory markers in macraphages

The effect of CI, CA and dexamethasone control (DEX) on the expression of pro-inflammatory cytokines in macrophages (RAW 264.7) was evaluated after 8 h of exposure to LPS (**Figure 5**). In the presence of LPS, both CI and CA significantly reduced the expression of TNF-α, IL-1β, iNOS and COX-2, in a dose dependent manner, when compared to DMEM+LPS. The mRNA levels of TNF-α were similar between DEX and both CI and CA compounds at 0.025 mg/mL and lower than DEX at 0.25 mg/mL. For IL-1β gene expression, CI at 0.25 mg/mL did not differ from DEX. CI and CA at 0.025 mg/mL had superior effect compared to DEX, drastically reducing the levels of IL-1β transcripts. No detectable mRNA levels of IL-1β were observed when cells were treated with CA at 0.25 mg/mL. Considering the gene expression of iNOS, DEX did not differ form the control, CI at both concentrations and CA at 0.025 mg/mL significantly reduced iNOS levels when compared to DMEM+LPS. No detectable mRNA levels of iNOS were observed when cells were treated with CA at 0.25 mg/mL. The mRNA levels of COX-2 were similar when cells were treated with DEX and CI and CA at 0.25 mg/mL. The lowest levels of COX-2 were observed for cells treated with CI and CA at 0.025 mg/mL.

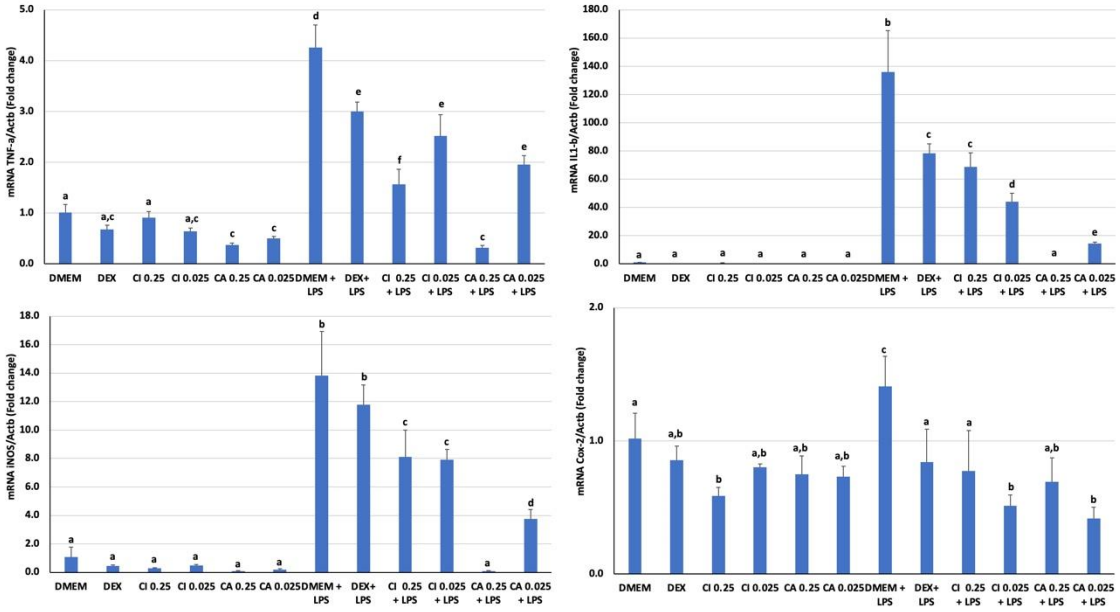


Figure 5. Effect of cinnamic acid (CI) and caffeic acid (CA) and control dexamethasone (DEX) on the expression of pro-inflammatory cytokines in macrophages (RAW 264.7) after 8 h of exposure or not to LPS. Concentrations in mg/mL.

[a] Different letters show statistical difference among the groups and concentrations, according to One-Way ANOVA and Tukey test ($p<0.05$).

4. Discussion

In the present study, only cinnamic acid and caffeic acid showed inhibitory and bactericidal effect against all bacterial species tested, demonstrating their effect against bacteria related to endodontic infections. In a previous study, cinnamic acid showed bactericidal activity against *S. mutans* in planktonic conditions at 0.741 mg/mL (MBC) (Ribeiro et al. 2018), similar to our results (MBC = 1 mg/mL). Al-Ani et al. (2018) investigated the chemical composition and antibacterial effect of ethanol extract (EEP) and water extract (WEP) of propolis. They reported that the most abundant compounds found in the extract composition were aromatic alcohols, aromatic acids, cinnamic acid and its esters, fatty acids, and chrysin. All propolis samples showed moderate antibacterial effect against Gram-positive microorganisms with MIC ranging from 0.08 mg/mL to 2.5 mg/mL.

In this study, the association of cinnamic acid with caffeic acid with had no synergistic effect against the bacterial strains tested. Studies evaluating the synergism between different phenolic acids or flavonoids are not communly found in the literature, although it is known that these compounds are detected simultaneouly in the composition of plant extracts (Al-Ani et al. 2018; Huang et al. 2021). Synergistic effect of cinnamaldehyde and cinnamic acid were found previously with FIC values 0.17x MIC of cinnamaldehyde and 0.33x MIC of cinnamic acid (Huang et al. 2021). Study showed synergism between caffeic acid and antibiotics (erythromycin, clindamycin, cefoxitin and vancomycin) against different strains of *Staphylococcus aureus* (Kepa et al. 2018). The association of caffeic acid with norflaxacin reduced antibiotic MIC from 156.3 mg/mL to 15.5 mg/mL against *Staphylococcus aureus*. For *Escherichia coli*, synergistic effect was observed between caffeic acid and imipenem, reducing antibiotic MIC from 2500 mg/mL to 1574 mg/mL. In *Pseudomonas aeruginosa*, caffeic acid showed a synergistic effect with gentamicin, reducing the antibiotic MIC from 625 mg/mL to 24.61 mg/mL and with imipenem, reducing antibiotic MIC from 1250 mg/mL to 78.13 mg/mL (Lima et al. 2016).

Biofilms are defined as a highly organized structure containing bacterial communities embebed in an extracellular polymeric matrix attached on a surface (Marsh, 2004). Bacteria living within the biofilm can be about 1000-fold more resistant to antimicrobial agents when compared to bacteria in planktonic conditions (Donlan

and Costerton 2002; Patel 2005; Borges et al. 2012). The complexity of root canals anatomy and multispecies biofilms increases the difficulty in eliminating the microorganisms by conventional chemical-mechanical procedures (Susin et al. 2010; Alves et al. 2011). Studies evaluating the antibiofilm action of flavonoids on microorganisms related to endodontic infections is still very scarce. In the present study, cinnamic and caffeic acids at 5x the highest MIC were effective in eliminating dual-species biofilms and significantly decreasing multispecies biofilms, especially cinnamic acid. Previous studies also observed that the phenolic acids, such as caffeic acid, are effective agents in preventing biofilm formation and decreasing biomass and viability of biofilms (Silva et al. 2016; Mandal et al. 2017). In another study, caffeic acid had a similar effect compared to fluconazole against biofilms in formation and mature biofilms of *Candida albicans* (De Vita et al. 2014). The antibacterial effect of the caffeic acid derivative, phenethyl ester caffeic acid, was evaluated on *Streptococcus mutans* biofilms. This derivative at a concentration of 0.08 mg/mL inhibited biofilm formation by more than 90%, demonstrating a good inhibitory effect on *S. mutans* biofilm. Caffeic acid also showed favorable results against *Staphylococcus aureus* and *Staphylococcus epidermidis* mature biofilms, including more virulent antibiotic-resistant strains isolated from patients (Niu et al. 2020). Cinnamic acid effectively inhibited both the production of quorum sensing virulence factors and biofilm formation in *P. aeruginosa*. This phytocompound interfered with the initial binding of planktonic cells to the substrate, causing a reduction in biofilm development (Rajkumari et al. 2018).

Chemically, phenolic acids can contain two distinct carbon structures, the hydroxycinnamic and hydroxybenzoic structures. Although the basic structure remains the same, the numbers and positions of the hydroxyl groups on the phenol ring cause important changes in the biological properties of phenolic compounds (Stalikas 2007; Borges et al. 2012). Generally, the higher number of hydroxylations results in higher antimicrobial effect (Polaquini et al. 2016; Samy and Gopalakrishnakone 2010). However, the effect of the number of hydroxyl groups on the antimicrobial effect of hydroxycinnamic acids was relatively minor (less than 4x change in activity) than hydroxybenzoic acids. Caffeic acid was only about half as active as cinnamic acid (Sánchez-Maldonado et al. 2011). The antimicrobial activity of hydroxycinnamic acids was comparable or higher than that of hydroxybenzoics with same number of hydroxyl

groups (Sánchez-Maldonado et al. 2011). Besides, phenolic toxicity to microorganisms can be related to enzyme inhibition by the oxidized compounds, possibly through reaction with sulfydryl groups or through more non-specific interactions with proteins (Ho et al. 2001).

In addition to the antibacterial activity of phenolic acids, knowledge of their cytotoxic effect is also very important for clinical use. In the present study, cinnamic acid caused minimal toxicity to L-929 fibroblasts and RAW 264.7 macrophages cultures at MIC concentrations and caffeic acid was not cytotoxic at 0.125 mg/mL. Slight decrease in metabolic activity was observed when fibroblasts (also L-929) were exposed to eugenol (89% of cell viability), citronellol (87%), cinnamic acid (more than 90%) and trans-cinnamaldehyde (72%) at 0.741 mg/mL (CBM obtained in the study) for 24 h (Ribeiro et al. 2018). In another study, caffeic acid had no significant influence on fibroblasts growth at concentrations between 0.06 and 1.26 mg/mL (Pinho et al. 2014). In vitro cytotoxic effect of phenolic compounds found in olive oil, including cinnamic acid and caffeic acid was evaluated on GN61 gingival fibroblasts for 24h and caffeic acid reduced fibroblast viability at concentrations greater than 1.5 mM (0.27 mg/mL) and for cinnamic acid this reduction was observed only at 6.75 mM (1 mg/mL). These results were similar to those observed in our study since cinnamic acid presented lower cytotoxicity compared to caffeic acid.

The persistence of endotoxins, especially LPS, in the root canal system after endodontic treatment can lead to injuries to the periapical tissues (Colic et al. 2009). LPS stimulates the production of enzymes that induce oxidative stress, such as iNOS (inducible nitric oxide synthase), which in macrophages modulates the production of nitric oxide (NO). In addition, these endotoxins increase the phagocytic activity of polymorphonuclear cells, and the secretion of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukins (IL-1β, IL-6), prostaglandins (PGE2) (Garlet 2010, Braz-Silva et al. 2018). It is known that the inflammatory response is an ally of the repair process, however, the uncontrolled secretion of these cytokines induces the uncontrolled expression of NF-κβ receptor ligand (RANKL) and bone resorption (Walsh et al. 2006). In the present study, both CI and CA significantly reduced the gene expression of TNF-α, IL-1β, iNOS and COX-2, in a dose dependent-manner. Cinnamic acid also reduced the secretion of pro-inflammatory markers such as IL-1β and TNF-α

and NO production in model of acute myocardial ischemia in rats (Song et al. 2013). Furthermore, cinnamic acid derivatives and hybrids have been demonstrated to reduce nitric oxide (NO) production and the expression of iNOS and COX-2 via the NF-κB signaling pathway (Gabriele et al. 2017; Ruan et al. 2017). Study evidenced that caffeic acid (50-10 μM) targets COX-2 and its product PGE 2 in human colonic myofibroblasts (Zielińska et al. 2021). Wan et al. (2021) evaluated caffeic acid treatment and observed a significant decrease in mRNA expression of pro-inflammatory cytokines IL-1β , IL-6 and TNF-α. An *in vivo* study showed the up-regulation of pro-inflammatory cytokines (IL-6 and TNF-α) in mice treated with cinnamic acid for 14 days (Zhuo et al. 2022). Four hydroxycinnamic acid derivatives (HADs), including two free cinnamic acids, *p*-coumaric acid (CA) and ferulic acid (FA), and their conjugate phenolic amides, *p*-dicoumaroyl-putrescine (DCP) and diferuloylputrescine (DFP) significantly inhibited NO production and iNOS expression in a dose-dependent manner. Among the four HADs tested, DFP showed the most potent inhibition on NO production and iNOS mRNA and protein expression (Kim et al. 2012).

Although cinnamic and cafeic acids presented relevant antibiofilm and antiiflammatory effect in the present study, their application is limited due to problems related to their stability and water solubility, as well as their low bioacessibility and bioavaliability (Ozkan et al. 2020). Further studies evaluating drug-delivery systems capable of encapsulating phenolic acids and controlling their release are necessary before considering these molecules as medications for endodontic purposes.

5. Conclusion

In conclusion, cinnamic acid and caffeic acid showed antibactericidal and antibiofilm effects against bacterial species related to endodontic infections, causing low cytotoxicity in the lowest concentrations. Besides, both compounds demonstrated antiinflamatory effect, inhibiting pro-inflammatory markers expression in LPS-stimulated macrophages.

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Conflict interest

There is not conflict of interest.

6. References

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Capítulo 2

Physicochemical and biological characterization of chitosan-poloxamer thermosensitive hydrogel containing phenolic acids for endodontic applications

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***O manuscrito está de acordo com as normas do periódico Materials Science & Engineering C**

The authors declare that have no conflicts of interest

Abstract

This study carried out a synthesis and physicochemical characterization of chitosan-poloxamer hydrogels (CPH) containing phenolic acids and evaluated their effects on multispecies biofilm and on the cell viability of macrophages and fibroblasts. CPH were characterized by flow and oscillatory rheometry, gelation temperature, texture profile bioadhesion analysis in dentin specimens and scanning microscopic analysis. Cinammic acid (CI), caffeic acid (CA) and controls (calcium hydroxide – CH and chlorhexidine – CHX) were incorporated in CPH and evaluated antibiofilm effect on multispecies biofilms formed with *Streptococcus mutans*, *Enterococcus faecalis*, *Actinomyces israelii*, *Lactobacillus casei* and *Fusobacterium nucleatum* in radicular dentin samples using confocal microscopy. Toxicity of CPH containing or not the compounds was determined on fibroblasts and macrophages cultures by resazurin assays. Data were statistically analyzed considering $p < 0.05$. CPH were characterized as thermoreversible and with adequate mechanical and bioadhesive properties. The antibiofilm effect of CPH+CA (77.8%) and CPH+CI (73.2%) hydrogels were superior to CPH+CH (53.6%) and CPH+CHX (39.9%). In general, CPH + CI caused lower cytotoxicity when compared to CPH + CA, in both cell lines. In conclusion, chitosan-poloxamer 407 hydrogels was characterized as thermoreversible and with adequate physicochemical properties, and when combined with cinnamic acid reduced drastically multispecies biofilms formed in dentin tubules, causing minimal toxicity to fibroblast and macrophages.

Key words: thermosensitive hydrogel, chitosan, poloxamer, cinnamic acid, caffeic acid, biofilms, cytotoxicity

1. Introduction

Conventional endodontic treatment significantly reduces the microbiota present inside the infected root canals (Siqueira et al., 2007), however, the permanence of microorganisms due to the anatomical complexity of the root canal system and their resistance to chemical-mechanical treatment can cause persistent or secondary infections (Siqueira and Rôças 2009). The maintenance or development of periapical lesions after endodontic treatment has been attributed to factors related to the presence and virulence of resistant microorganisms, mainly gram-positive anaerobic bacteria, including *Streptococcus spp*., *Actinomyces spp*., *Lactobacilli spp*., *Enterococcus faecalis*, and gram-negative anaerobic bacteria, such as *Fusobacterium nucleatum*, in the root canal system and/or periapical tissues (Siqueira and Roças, 2009, 2021) .

Intracanal medicaments have been recommended as root canal dressing between appointments to eliminate residual bacteria, reduce inflammation and postoperative pain, help eliminate apical exudate, control inflammatory root resorption, and prevent contamination from temporary sealing (Farhad and Mohammadi, 2005). Calcium hydroxide (CH) is the most recommended intracanal medicament because of its antimicrobial and mineralization activies mainly in long-term outcome of treatment such as dental trauma and treatment of immature teeth with open apices (Mohammadi and Dummer, 2011). However, CH 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 (Haapasalo et al., 2000, 2007; Upadya et al., 2011). Chlorhexidine (CHX) is a cationic bisbiguanide with wide-range antimicrobial activity especially between pH 5 and 7 (Athanassiasis et al., 2007). When used as an intracanal medicament, CHX was more effective than CH in eliminating *E. faecalis* from inside dentin tubules (Gomes et al., 2006; Athanassiasis et al., 2007). However, CHX has presented cytotoxicity when in contact to cells even at low concentrations (Lessa et al., 2010).

Phytochemicals are attractive compounds for therapeutic use since they are considered eco-friendly, relatively inexpensive, with broad-spectrum antimicrobial effect and relative low cytotoxicity, as well as can prevent the emergency of resistant

bacteria, by their multiple antimicrobial mechanism of action (Dahiya et al., 2012; Upadhyay et al., 2014). Natural phenolic compounds detected in plants can form simple molecules, such as phenolic acids (benzoic and cinnamic acids) to highly polymerized molecules such as lignins or flavonoids. Hydroxycinnamic acids or cinnamic acids derivatives have been studied for their antioxidant and antimicrobial properties, confered by the presence of one or more hydroxyl groupings in the molecule (Sánchez-Maldonado et al., 2011). Although studies evaluating their effect on biofilm are relatively scarce (Jagani et al., 2009; Silva et al., 2015; Silva et al., 2016).

The application of phenolic acid is limited due to problems related to their stability and water solubility, as well as their low bioavaliability (Ozkan et al., 2020). Then, natural or synthetic polymers have been used as drug carriers to increase solubility, control release, and extend biological effect of phytomedicines. Hydrogels are polymeric materials with three-dimensional networks that retain large amounts of water in their structures and are of great interest in biomedical and environmental applications (Shariatinia and Jalali, 2018). They have found applications as biomedical materials, particularly drug delivery devices and tissue engineering due to their structural similarity to the extracellular matrix in addition to their excellent chemical, physical, and biological characteristics (Bhattacharyya et al., 2014; Guilherme et al., 2015; Zhang et al., 2017). Many hydrogels have been developed using both natural polymers, including chitosan and synthetic polymers, such as poloxamer-407. Chitosan, a polysaccharide of animal origin obtained from the chitin of the exoskeleton of crustaceans, has the ability to form a hydrogel under suitable conditions, but chitosan hydrogels have little mechanical strength and poor absorption in water solutions (Chenite et al., 2000; Mu et al., 2019). Poloxamer 407 (ethylene oxide and propylene oxide blocks) have been largely used in various drug delivery systems including with chitosan due to their advantages such as thermoreversibility, low toxicity, biocompatibility, easy gel preparation methods, good compatibility with a wide range of biomolecules, including polyphenols, enhancing their solubilization and prolonging their release profile for many types of applications (Dumortier et al., 2006; Jones et al., 2009; Yu et al., 2018).

Recent investigations have demonstrated that the association of cinnamic acids with chitosan (conjugated or in nanogels) not only beneficially alters the

structural and physicochemical properties of chitosan, but also notably increases its antioxidant, antimicrobial, anti-inflammatory properties (Wang et al., 2019, de Carvalho et al., 2021, Yue et al., 2021; Nagy et al., 2022). This study aimed to synthesize and to characterize the physicochemical properties of chitosan-poloxamer hydrogels containing phenolic acids as well as evaluate their effects on multispecies biofilm and cytotoxicity for endodontic application.

2. Materials and methods

2.1. Phenolic acids and controls

The following phenolic acids were evaluated: cinnamic acid (CI, #C80857) and caffeic acid (CA, #C0625) which were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of phenolic acids were prepared and frozen in dimethylsulfoxide – DMSO. For microbiological and cytotoxicity assays, positive controls were calcium hydroxide PA (CH, Biodynamics, Ibiporã – PR/BR) dissolved in saline solution (1mg/mL) and chlorhexidine digluconate (CHX, Farmácia Manipullis, Araçatuba – SP/BR) diluted in sterile water. All compounds were sterilized by filtration through a 0.22 µm membrane filter. The assays were performed in triplicate in three independent days.

2.2. Chitosan-poloxamer hydrogel (CPH) preparation

Hydrogels were prepared according to the cold method first described by Schmolka et al. (1972). First, chitosan low molecular weight (50-190 Da; 75–85% deacetylated) (Sigma Aldrich®) (1% wt/vol) was dissolved in a solution of acetic acid (1% wt/vol) (Synth®, Brasil) using mechanical stirrer (100 rpm) for about 3h. Following complete dissolution, the chitosan dispersion was then refrigerated (4^{o} C) and used as a solvent for the Poloxamer 407 (HP-407) (Sigma Aldrich®, USA) dispersion. After that, HP-407 (18% wt/vol) was added to the chitosan dispersion and stored at 4^{o} C for about 24h. For antibiofilm and cytotoxity assays, the hydrogels were prepared 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 based on previous microbiological assays conducted by this research group. CPH hydrogels were kept under agitation for 1 h and after that they were incubated for 24h at 5 °C for total solubilization before use.

2.3. Physicochemical characterization of CPH

2.3.1. Flow Rheometry

Rheometry was analyzed 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 CPH was performed at 37 ±0.1 °C. The formulations were carefully placed to the inferior plate, and allowed to equilibrate for at least 1 min prior to analysis (Calixto et al., 2016a). Flow curves were measured over shear rates ranged from 0 to 100 s−1 (increased over a period of 120 s, held at the upper limit for 10 s, and then decreased over a period of 120 s). The consistency index and the flow index were determined using eq. 1 (JONES et al., 2009) 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 is the rate of shear (s−1), and n is the flow behavior index (dimensionless).

2.3.2. 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 (Calixto et al., 2016a). CPH was 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 at 25 and 37 ± 0.1 °C.

2.3.3. Sol–gel transition temperature

The determination of sol-gel transition temperature (Tsol-gel) of CPH was performed in oscillatory mode with temperature ramp, using cone-plate as described above. The temperature sweep analysis was performed over the temperature range of 18-50°C at a defined frequency (1.0 Hz) and rate of heating 3 °C/min using a controlled stress.

2.3.4. Texture profile analysis

Texture profile analysis (TPA) was performed by using a TA-XT plus texture analyzer (Stable Micro Systems, Surrey, UK) as described by Calixto et al., (2016a). The hydrogel was weighed (7 g), placed in 50 mL centrifuge tube, and centrifuged to remove the air bubbles present. Then, the hydrogel was allowed to stand for 12 hours until analysis. Then, tubes were placed below the 10 mm analytical probe, which was lowered at a constant speed of 1 mm·s−1 until it reached the hydrogel surface. Contact was detected by a triggering force of 2 mN, and then the probe continued down to 10 mm depth in the hydrogel. Then, the probe returned to the surface (0.5 mm·s−1), and after 5 seconds, a second compression was initiated. The test results were used to plot a force–time curve, from which the mechanical parameters were calculated such as hardness, compressibility, adhesiveness, and cohesion. This process was repeated seven times at 37° C± 0.5°C.

2.3.5. Bioadhesion

A TA-XT plus texture analyzer (Stable Micro Systems) was used to measure the tensile strength according to the methodology described by Calixto et al., (2016). The teeth model was fastened to the cylindrical probe (diameter, 10 mm). A 50 mL centrifuge tube containing CPH was placed below the probe. The probe was lowered at a constant speed of 1 mm·s−1 until the teeth model and hydrogels surface were made to be in contact with each other, as detected by a triggering force of 2 mN. The teeth model and hydrogels were kept in contact for 60 seconds, and no external force was applied during this time interval. After 60 seconds, the teeth model was drawn upward (0.5 mm·s−1) until the contact between the surfaces was broken. During this experiment, a force–time curve was plotted, and the work of adhesion and peak adhesion were calculated from this plot. Seven replicates were analyzed at 37 ± 0.5 °C.

2.3.6. Scanning electron microscopy

CPH was applied in coverslips for microscopic analysis. The hydrogel was dehydrated by washing in a series of ethanol (70% for 10min, 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 (Leo, Cambridge, MA, USA) (Jacob et al., 2020).

2.4. Evaluation of CPH on multispecies biofilms in root dentin and analysis by Confocal Microscopy

This study was approved by the Animal Committee of the Faculty of Dentistry of Araçatuba, UNESP, Brazil (Protocol: 00709-2019) and conducted in accordance with Ma et al. 2011 and dos Santos et al. (2021). Briefly, 4 mm cylindrical specimens were obtained from the horizontally sectioned bovine dental roots with diamond discs and their root canals were enlarged with a #6 bur. The cylindrical specimens were again sectioned into two cylindrical halves and the dentin specimens (n=6/group) were washed with distilled water and ultrasonically cleaned with 17% EDTA for 3 min and deionized water for 5 min. After autoclaving for 15 minutes at 121°C, they were then inserted into a microtube with the canal (pulp) side up and fixed with composite resin. Cultures of *E. faecalis, A. israelii, S. mutans, L. casei and F. nucleatum* were pooled in equal aliquots at the same concentration (5 x 10^3 CFU/mL), centrifuged and resuspended in BHI broth at the same initial volume. In the microtubes with the dentin specimens, 0.5 mL of the bacterial culture were inoculated and centrifuged at 1400 x g, 2000 x g, 3600 x g and 5600 x g, twice each, for 5 minutes to promote dentin infection, replacing the culture medium every centrifugation. All microtubes were incubated at 37°C in BHI supplemented with 1% glucose broth for 2 weeks in a 5% CO_2 atmosphere, with culture medium replacement every 72 h. After this period, the dentin specimens were washed with sterile water and treated with 1mL CPH containing phenolic acids (CA and CI at 5 mg/mL) and controls (CH at 1 mg/mL and CHX at 0.5 mg/mL) and sterile water for 48h.Then they were washed with sterile water for 1 min at 37°C, cut into two new halves and stained with 100 µL of LIVE/DEAD BacLight BacLight Bacterial Viability fluorescent stain (L13152, Molecular Probes, Eugene, OR) for observation of the surface longitudinally to the dentinal canals visible by Confocal Scanning Laser Microscopy - CLSM. CLSM images were acquired using software (LAS AF Leica Microsystems) with a resolution of 1024 pixels. Ten-micrometer-deep scans were obtained with the CLSM of two randomly selected locations for each dentin specimen. The quantification of the ratio of red fluorescence in relation to green and red fluorescence was determined by the software called Image J 1.48 (NIH, Bethesda, MA, USA), indicating the proportion of dead cells for each material tested.

2.5. Cytotoxicity of CPH

The cytotoxicity of hydrogels was determined in L3T3-L1 fibroblast culture (CL-173TM) and in RAW 264.7 macrophage culture. After preparation, CPH loaded with CA, CI, CH and CHX were kept at 5°C for 24h. Then, 1mL of liquid hydrogels were incubated in 24-well microplates at 37°C overnight to acquire the gel state. Then, the DMEM culture medium was added over the hydrogel and incubated for 48 h and 7 days. The supernatant was collected after 48 h and 7 days for sequential treatment. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Dulbecco's modified Eagle, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 IU/mL penicillin, 100 µg/mL of streptomycin and 2 mmol/L glutamine (Gibco) in a humidified oven containing 5% CO_2 and 95% air at 37°C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). Cultures were seeded at a cell density of 1 x 10^4 cells/well in 96-well plates and incubated for 24 h. After incubation, the DMEM was removed and dilutions of the hydrogel extracts were added to the cells (from 50% to 6.25%). 3T3 and RAW 264.7 grown in DMEM without any extract were used as controls. After the 24 h treatment, the extracts were removed and 200 µL of resazurin (70µM) were added for incubation at 37 ^oC for 4 h. At each time point, 100 µL of the medium was transferred to another 96-well plate for absorbance reading at 570 and 600 nm on a spectrophotometer (Biotek, Winooski, VT) (Massunari et al., 2021).

2.6. Statistical analysis

Data from cytocompatibility and microbiological assays were expressed in means/standard deviation 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

Physicochemical characterization of hydrogels

3.1. Flow rheometry

Figure 1 demonstrates the hydrogel flow curve. According to the analysis performed, the CPH showed non-Newtonian behavior of the pseudoplastic type since the relationship between the shear rate (Pa) and the shear stress (1/s) is not linear.

Furthermore, according to the descending curve that overlapped the ascending curve, the CPH showed a thixotropic flow behavior characterized by the decrease in viscosity during the increase in the shear rate, and when this rate is decreased, the viscosity increases again. Shear thinning refers to the reduction in viscosity when the shear rate increases, which is a desirable characteristic of pharmaceutical formulations, as the reduction in viscosity facilitates administration of the formulation (Carvalho et al., 2013; Calixto et al., 2016)

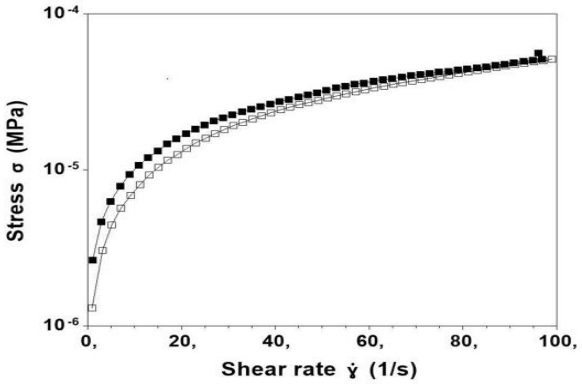

Figure 1. Continuous rheological behavior of chitosan-poloxamer hydrogels. Empty symbols indicate an upward curve, and full symbols, a downward curve.

3.2. Oscillatory Rheometry

The storage module and the loss module were analyzed from the frequency sweep analysis. The storage modulus represents the strain energy stored during the shearing process (characteristic of a solid material). The loss modulus represents the energy dissipated during the shearing process (characteristic of a liquid material) (Laun et al., 2014). **Figure 2** demonstrated the results of CPH oscillatory rheometry. According to the analysis performed, the hydrogel exhibited viscoelastic characteristics at 25 ^{o}C where storage module > loss module. In addition, frequency dependence is observed. At this temperature, the loss modulus is predominant, revealing that the poloxamer micelles are not neatly packed and that they have a less structured system. However, at 37°C, the rapid increase in storage modulus reflects the formation of a strong gel network with storage modulus > loss modulus, independent of oscillatory frequency.

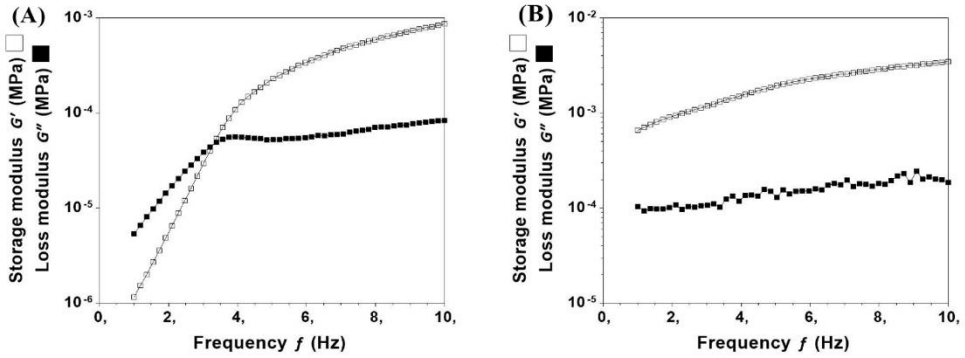

Figure 2. Frequency sweep profile of storage modulus G' (opened symbols) and loss modulus G'' (closed symbols) of chitosan-poloxamer hydrogels. (A) Analyzes performed at 25℃, (B) Analyzes performed at 37 ℃.

3.3. Sol–gel transition temperature

The determination of the sol-gel transition temperature (Tsol-gel) of the hydrogels was performed in oscillatory mode with temperature ramp, using a cone plate as described above. The temperature scan analysis was performed in the temperature range of 18-50°C at a defined frequency (1.0 Hz) and heating rate of 3°C/min using a controlled voltage. According to **Figure 3** can be observed that the Tsol-gel occurs in three phases. First, before the gelation process, the loss module presents values lower than the storage modulus demonstrating viscoelastic characteristics, as shown in oscillatory rheology. The second phase corresponds to the beginning of the gelation process, which starts approximately 24 °C, temperature at which the storage modulus exceeds the loss modulus. In addition, the phase transition can be observed by abrupt change in Tan d is observed with values of <1 indicating a greater storage modulus compared with loss modulus. Rheological analyzes confirmed that CPH was thermosensitive and had thermoreversible properties.

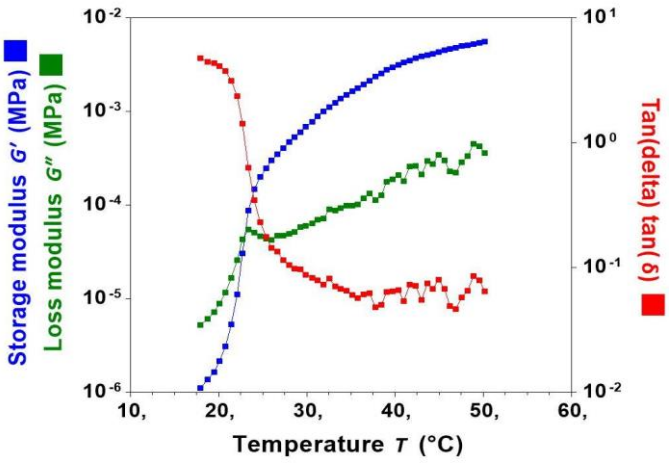

Figure 3. Temperature sweep analyses demonstrating of storage modulus G' (blue symbol) and loss modulus G'' (green symbol) and loss tangent (Tan d) (red symbol) of chitosan-poloxamer hydrogels.

3.4. Texture profile and bioadhesion analysis

Texture profile results are divided in hardness (measured by the peak of force applied by the probe on the hydrogels), compression (the work required to deform the product during compression), adhesiveness (the work required to overcome the attractive forces between the surfaces of the hydrogels and probe) and cohesiveness (the force that binds molecules) (Friedman et al., 1963; Jones et al., 2009). The data presented in **Table 1** obtained by Equation (1) confirm that the hydrogel showed shear thinning behavior (n<1) being characterized as pseudoplastic fluids. Formulations that present pseudoplastic characteristics demonstrate the ability to restructure when in contact with the physiological environment, thus explaining the increase in the consistency index (K) (**Table 1**).

Table 1. Flow behavior (n) and consistency index (K), mechanical properties (hardness, compressibility, adhesiveness, and cohesiveness) and biioadhesion in vitro of chitosan-poloxamer hydrogels

Tests	**Results**	
Flow behavior (n) and consistency index (K)	n = 0.9853	K = 0.5377
Hardness	11.96 mN	
Compressibility	96.47 $mN^{-1}s$	
Adhesivity	90.10 $mN^{-1}s$	
Cohesiveness (dimensionless)	0.89	
Bioadhesion	Peak of adhesion= 0.036 N	Area of adhesion = 0.109 N.s

3.5. Scanning electron microscopy analysis

Figure 4 shows porous network structure of the CP hydrogel and crosslinking between the polymers chitosan and poloxamer 407 at three magnifications.

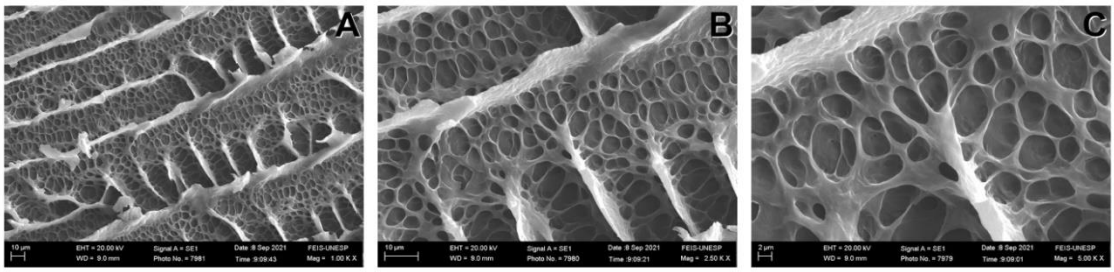

Figure 4. Scanning electron microscopy representative images of chitosan-poloxamer hydrogels. A – 1000x; B – 2.500x and C – 5000x.

Biological properties of hydrogels containing phenolic acids and controls

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

Figure 5A-F shows the results observed when 14-day multispecies biofilms (with *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 48h. Representative confocal images of CPH hydrogels are seen in **Figures 5A-F** comparing dead (red spots) and live (green spots) patterns between groups. The antibiofilm effect of CPH+CA (77.8%) and CPH+CI (73.2%) hydrogels were superior to CPH+CH (53.6%) and CPH+CHX (39.9%). No statistical difference was observed between CPH+CH and CPH+CHX. The CPH without antimicrobials had little antibiofilm effect. These results were confirmed in **Figure 5G**.

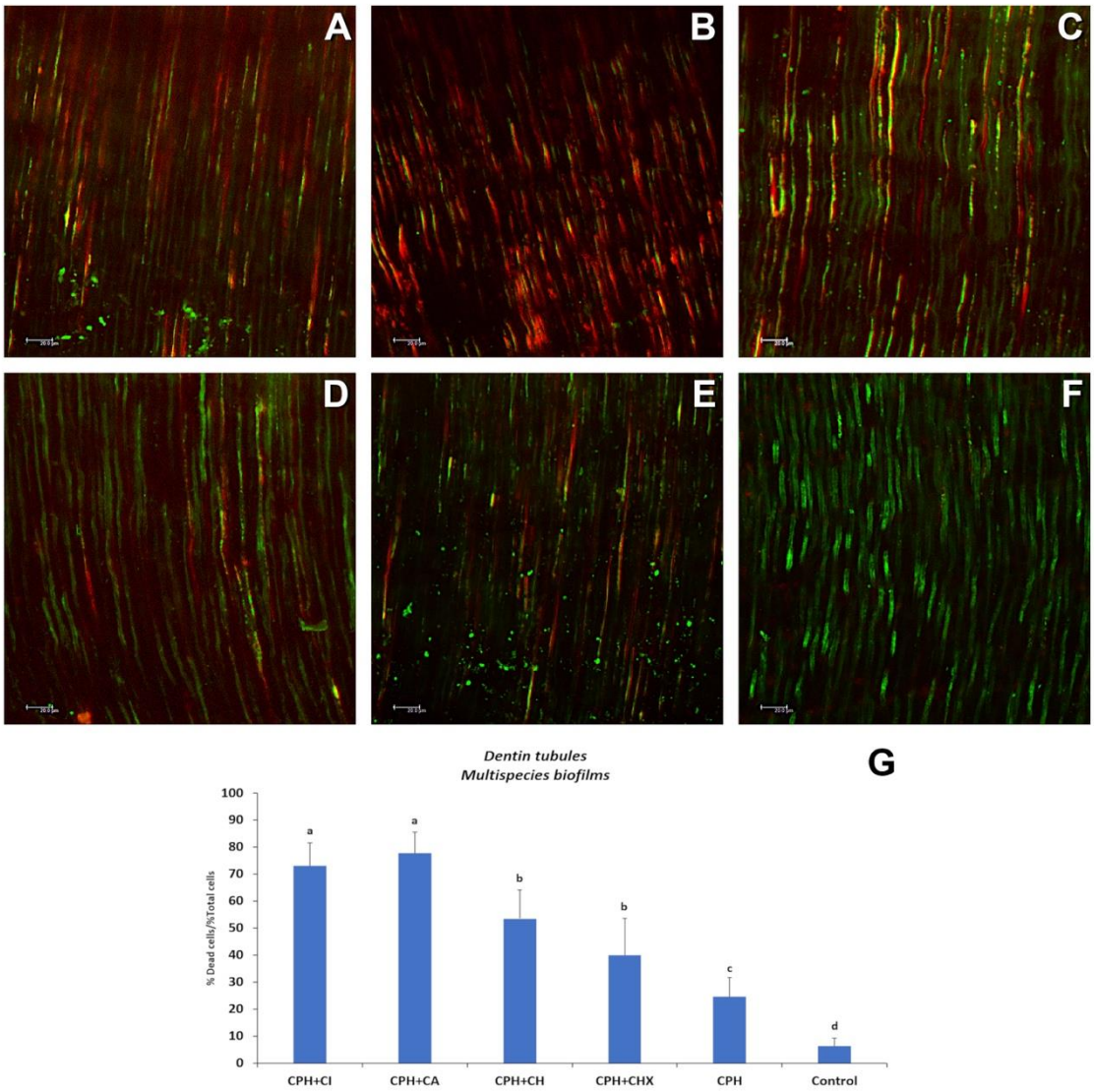

Figure 5. Representative confocal microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms and treated for 48 hours with the following groups with A: Hydrogel (CPH) with cinnamic acid (CI) at 5 mg/mL, B – CPH with caffeic acid (CA) at 5 mg/mL, C – CPH with 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 48h of treatment with hydrogels and controls. Bacterial counts were obtained by Image J analysis.[a] Different letters show statistical difference between the antimicrobial groups, according to One-Way ANOVA and Tukey test ($p<0.05$).

3.7. Cytotoxicity of CPH hydrogels

Figure 6A-B shows the effect of 48 h and 7days extracts of CPH hydrogels containing CI, CA, CH and CHX, or without antimicrobial agents on fibroblast cells after

24h exposure. All CPH hydrogels (with or without antimicrobials) diluted to 1/2 presented cytotoxic effect, reducing cell viability below 80%, both 48h and 7days extracts, with the exception of 7days extracts of CPH+CH. CPH + CI had less cytotoxicity when compared to CPH + CA, especially for 48h hydrogels extracts and CPH + CHX was highly toxic to fibroblast cells, independent on the dilution.

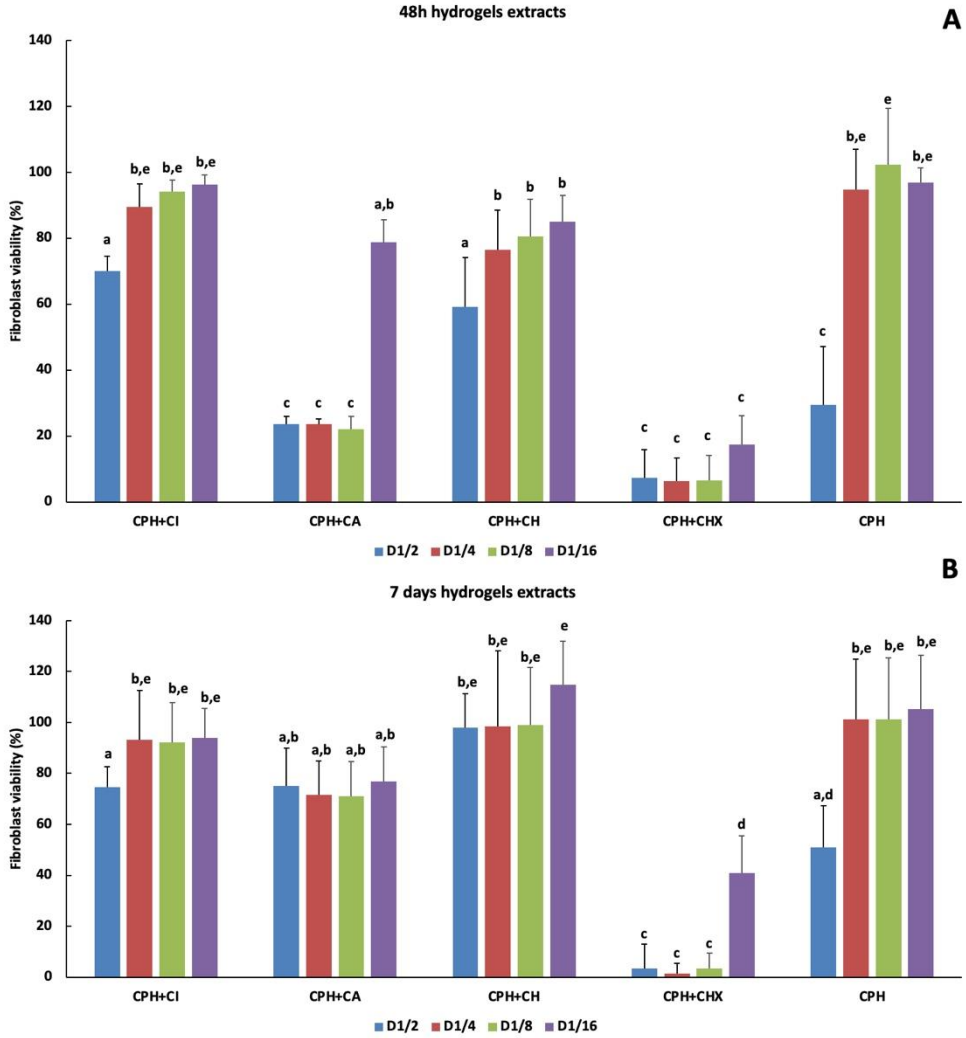

Figure 6. Effect of the 48h **(A)** and 7 days **(B)** extracts of chitosan-poloxamer hydrogels (CPH) containing cinnamic acid (CI) or caffeic acid (CA) and controls calcium hydroxide (CH) and chlorhexidine (CHX) on the viability of fibroblasts (3T3) after 24h of exposure, using staining with resazurin. Concentrations in mg/mL.

[a] Different letters show statistical difference among the groups and concentrations, according to Two-Way ANOVA and Tukey test ($p<0.05$).

Figure 7A-B shows the effect of 48h and 7days extracts of CPH hydrogels containing CI, CA, CH and CHX, or without antimicrobial agents on macrophages after 24h exposure. Cell viability significantly increase with the dilutions, independent on

the CPH hydrogel considering both 48h and 7days extracts. CPH + CI and CPH + CH were cytocompatible at dilution 1/8 and CPH + CA at dilution 1/16, observing more than 70% of cell viability. No cell viability was detected when macrophages were treated with CPH + CHX, independent on the dilution.

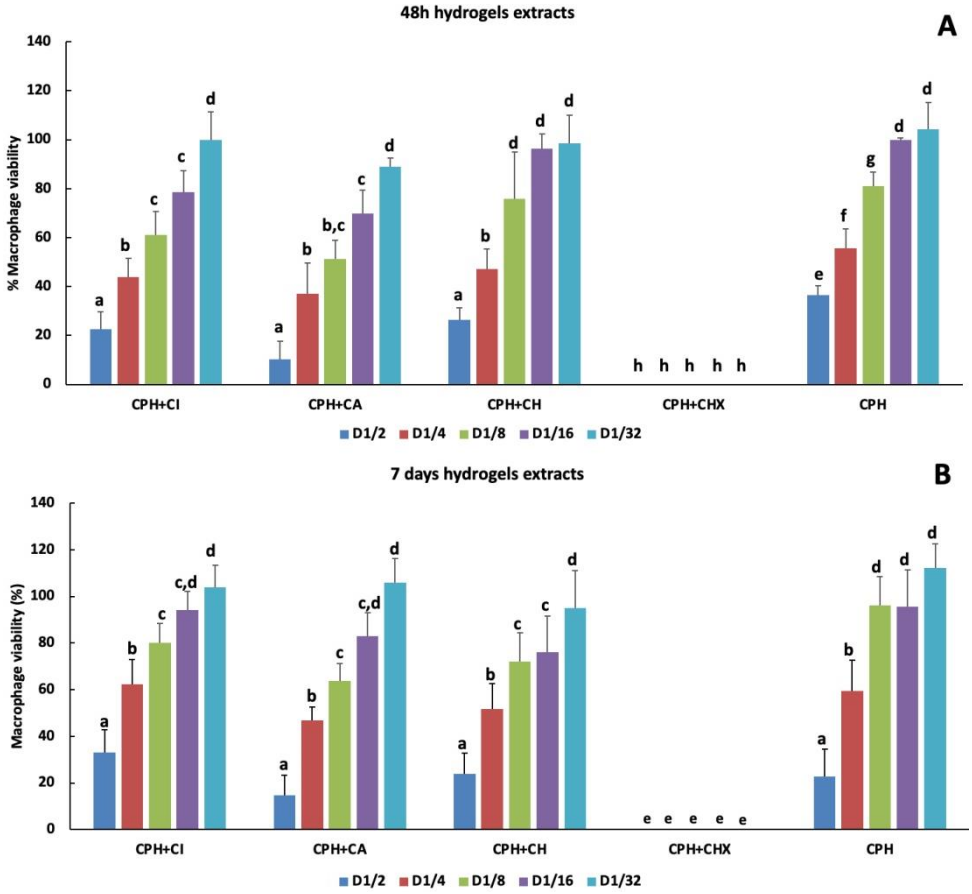

Figure 7. Effect of the 48h **(A)** and 7 days **(B)** extracts of chitosan-poloxamer hydrogels (CPH) containing cinnamic acid (CI) or caffeic acid (CA) and controls calcium hydroxide (CH) and chlorhexidine (CHX) on the viability of macrophage (RAW 264.7) after 24h of exposure, using staining with resazurin. Concentrations in mg/mL.

[a]Different letters show statistical difference among the concentrations for each group and among the groups considering each concentration separately, according to Two-Way ANOVA and Tukey test ($p<0.05$).

4. Discussion

In this present study, chitosan-poloxamer hydrogels was characterized as pseudoplastic fluid with the ability to form a strong gel network with storage modulus superior to 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. Texture profile and bioadhesion analysis revealed adequate hardness, resistance to compression, adhesiveness, and cohesiveness, besides the ability to adhere in dentin, important properties for clinical applications. For endodontic purposes, for example, they could be useful as intracanal medicament for delivery of antimicrobial agents reducing the microbial load and allowing tissue recovery and regeneration.

Thermosensitive hydrogels can generate sol-gel phase transition when they receive temperature stimuli. In recent years, a variety of therapeutic approaches such as chemotherapy and regenerative procedures can be achieved by combining synthetic or natural compounds with chitosan-based hydrogels (Mu et al., 2019). Chitosan has been widely used for medical applications due to its low toxicity, biocompatibility, biodegradability, mucosal adhesion properties and antimicrobial activities (Chenite et al., 2000). By means of physical and chemical modifications, chitosan formulations can be prepared to form hydrogels capable of releasing drugs (Kong et al., 2018; Chen et al., 2021). Gel formation is the result of non-covalent interactions, such as electrostatic, hydrophobic, or hydrogen bonds (Jiang et al., 2016) and drug-releasing capacity is dependent on the pH, temperature, light or ionic strength (Zhao et al., 2017).Poloxamer-407 is a copolymer composed of a polypropylene oxide chain and polyethylene 2-oxides. Structurally, the hydrophobic core consists of a 1-polypropylene oxide chain and a hydrophilic crown constructed with 2-polyethylene oxides (Cabana et al., 1997; Rey-Rico et al., 2015; Ahmad et al. 2020). At a temperature of 2.0 to 15.0 °C, polyethylene oxides are soluble and micelle formation occurs at a temperature > 15.0 °C and then gel formation due to the dehydration reaction (Bonacucine et al., 2008). Studies have already shown that this copolymer is safe in nature for use as an in-situ gelling agent in formulations for intranasal and ocular delivery of many drugs (Gratieri et al., 2010; Fakhari et al., 2017; Salatin et al., 2017).

In this current study, the incorporation of phenolic acids in CPH increased the effect against multispecies biofilms without difference between cinnamic and caffeic acids formulations. There was no found studies evaluating chitosan thermosensitive hydrogels containing cinnamic acid or caffeic acid for antimicrobial purposes. However, cinnamic acid conjugated with chitosan or incorporated in nanogels have been developed to increase mainly antimicrobial activity, confirming that this combination is

promissing (Lee et al., 2014; Badawy et al., 2004; de Carvalho et al., 2021, Navy et al., 2022). Chitosan conjugated with cinnamic acid showed greater antifungal activity against *Pyricularia oryzae* and *Botrytis cinerea* than chitosan. Furthermore, one of the chitosan conjugated with cinnamic acid exhibited a 12-fold increase in activity against *Botrytis cinerea*, compared to chitosan alone, resulting in one of the most active derivatives in the study (Badawy et al., 2004). Studied evaluated the antibacterial properties of chitosan conjugated with caffeic acid against 15 clinical isolates, two standard strains of methicillin-resistant *Staphylococcus aureus*, five foodborne pathogens and three standard strains of *Staphylococcus aureus* and there was an increase in the antimicrobial activity of these conjugates compared to natural chitosan (Lee et al., 2014). Encapsulation of essential oil using cinnamic acid grafted chitosan nanogel increased antifungal activity against *Microsporum canis* and completetly inhibited the mycelial growth (de Carvalho et al., 2021). Navy et al. (2022) also synthetized chitosan-hydroxycinnamic acid conjugates and observed bactericidal effect against *S. aureus* and *Escherichia coli* and increased antioxidant acitivty when compared to natural chitosan. One caffeic acid conjugate was 4000 times more active than chitosan and more active than free caffeic acid (Navy et al., 2022). Phenolic acids (gallic and trans-cinnamic acids) were embedded in a gelatin/chitosan matrix and exhibited higher antioxidant activities compared to the trans-cinnamic acid films and decreases *E. coli* growth (Benbettaieb et al., 2020).

Considering the toxicity of chitosan hydrogels in the present study, hydrogels containing cinnamic acid were more cytocompatible when compared to caffeic acid hydrogels. Cell viability was dependent on the cell type and hydrogel dilutions. Previous studies have demonstrated that chitosan-based hydrogels presented low cytotoxicity in different cell lines (Li et al., 2009; Wang et al., 2011; Deka et al., 2016; Rodriguez-Rodriguez et al., 2020). Water-soluble chitosan-ethylene glycol acrylate methacrylate (CS-EGAMA) and polyethylene glycol diamethacrylate (PEGDMA) were sensitive to pH and have no cytotoxic effect on L929 and SW1353 cells lines (Li et al., 2009). Water soluble phosphonium chitosan derivatives also showed low cytotoxicity to L929 cells (Wang et al., 2011). Curcumin-loaded chitosan phosphate nanoparticle were cytocompatible when tested on culture of peripherical blood mononuclear cells and murine monocyte-macrophage cell lines (Deka et al., 2016).

Gelatin/chitosan/plyvinyl acohol hydrogels were synthetized at different polymer ratios using the freeze-drying and sterilized by steam sterilization. The sterilized hydrogels showed low cytotoxicity (cell viability > 70%) to the HT29-MTX-E12 cell line (Rodriguez-Rodriguez et al., 2020). The only study found evaluating a phenolic acid incorporated in an injectable thermosensitive chitosan/gelatin/ β-glycerol phosphate (C/G/GP) was developed by Dong et al. (2015) for the controlled release of the ferulic acid as antioxidant agent for neurological application. This system was effective in inhibiting the neurological oxidative stress and presented excellent compatibility with the Neuro-2a cells (Dong et al. 2015).

Other researchers have developed chitosan system for endodontic application, mainly for bone repair (Dhivya et al., 2015; Abdel-Fattah et al., 2017), however, this is the first study that evaluate the antibiofilm properties and cytotoxicity of chitosan-poloxamer hydrogels containing cinnamic acid and caffeic acid compared to calcium hydroxide and chlorhexidine, antimicrobial agents currently used as intracanal medications. The outcomes of this study suggested that chitosan-poloxamer especially loaded with cinnamic acid could be a promising drug delivery system for endodontic purposes.

5. Conclusion

Chitosan-poloxamer 407 hydrogels was characterized as thermoreversible and with adequate mechanical and bioadhesive properties, and when combined with cinnamic acid reduced drastically multispecies biofilms formed in dentin tubules, causing minimal toxicity to fibroblast and macrophages.

Acknowledgments

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Conflict interest

There is not conflict of interest.

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Conclusão Geral

CONCLUSÃO GERAL

1. O ácido cinâmico e o ácido caféico apresentaram efeito bactericida e antibiofilme contra espécies microbianas relacionadas a infecções endodônticas, causando baixa citotoxicidade. Além disso, ambos os compostos demonstraram efeito anti-inflamatório, inibindo a expressão de marcadores pró-inflamatórios em macrófagos estimulados por LPS.
2. Os hidrogéis de quitosana-poloxamer 407 foram caracterizados como termorreversíveis e com propriedades químicas e físico-mecânicas adequadas, e quando combinados com ácido cinâmico reduziram drasticamente os biofilmes multiespécies formados nos túbulos dentinários, causando mínima toxicidade aos fibroblastos e macrófagos.

Anexos

Anexo A

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Anexo B

Aprovação do Comitê de Ética em Humanos

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito antimicrobiano e anti-inflamatório de ácidos fenólicos isolados, combinados e incorporados em hidrogéis de quitosana para aplicação endodôntica

Pesquisador: VANESSA RODRIGUES DOS SANTOS
Área Temática:
Versão: 1
CAAE: 22735019.0.0000.5420
Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.649.197

Apresentação do Projeto:
Estudo do biofilme dos canais radiculares, para estudar uma
proposta de incorporação dos compostos com melhor efeito terapêutico em hidrogéis termossensíveis de quitosana como medicação endodôntica
para dentes permanentes jovens.

Objetivo da Pesquisa:
Avaliar in vitro os efeitos antimicrobiano e anti-inflamatório de ácidos fenólicos derivados do ácido cinâmico, isolados e combinados e estudar uma
proposta de incorporação dos compostos com melhor efeito terapêutico em hidrogéis termossensíveis de quitosana como medicação endodôntica
para dentes permanentes jovens

Avaliação dos Riscos e Benefícios:
Riscos:
Riscos:O projeto oferecerá riscos mínimos aos pacientes, uma vez que esta pesquisa visa somente a coleta de biofilme do canal radicular com
auxílio de cone de papel absorvente estéril de pacientes com indicação para tratamento

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Continuação do Parecer: 3.649.197

endodôntico, devido necrose pulpar. As coletas serão
realizadas por equipe especializada, e os pacientes estarão sob tratamento odontológico nas clínicas específicas da FOA. Assim, a participação
nesta pesquisa ocasionará mínimo desconforto e a doação será completamente voluntária. Os procedimentos adotados nesta pesquisa obedecem
aos Critérios da Ética em Pesquisa com Seres Humanos conforme Resolução nº. 466/12 do Conselho Nacional de Saúde. Nenhum dos
procedimentos usados oferece riscos à sua dignidade.

Benefícios:

Benefícios: ao participar desta pesquisa o paciente não terá nenhum benefício direto. Entretanto, pretendemos testar os compostos sobre os
microrganismos que isolaremos do biofilme do canal radicular de dentes com necrose pulpar que foram coletados com o objetivo de verificar se
esses compostos tem capacidade antibacteriana e assim se poderiam ser uma futura medicação para tratamento endodôntico. A pesquisadora se
compromete a divulgar os resultados obtidos, respeitando-se o sigilo das informações coletadas, conforme previsto no item anterior.

Comentários e Considerações sobre a Pesquisa:

Projeto científico dentro das normas da CONEP.

A metodologia proposta é capaz de atender os objetivos do estudo

Considerações sobre os Termos de apresentação obrigatória:

Quanto aos termos de apresentação obrigatória, todos foram apresentados de modo adequado.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Não havendo pendências, recomenda-se a aprovação do projeto.

Considerações Finais a critério do CEP:

Não havendo pendências, o CEP propõe a aprovação do projeto de pesquisa salientando que, de acordo com a Resolução 466 CNS de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI,

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Continuação do Parecer: 3.649.197

endodôntico, devido necrose pulpar. As coletas serão
realizadas por equipe especializada, e os pacientes estarão sob tratamento odontológico nas clínicas específicas da FOA. Assim, a participação
nesta pesquisa ocasionará mínimo desconforto e a doação será completamente voluntária. Os procedimentos adotados nesta pesquisa obedecem
aos Critérios da Ética em Pesquisa com Seres Humanos conforme Resolução nº. 466/12 do Conselho Nacional de Saúde. Nenhum dos
procedimentos usados oferece riscos à sua dignidade.
Benefícios:
Benefícios: ao participar desta pesquisa o paciente não terá nenhum benefício direto. Entretanto, pretendemos testar os compostos sobre os
microrganismos que isolaremos do biofilme do canal radicular de dentes com necrose pulpar que foram coletados com o objetivo de verificar se
esses compostos tem capacidade antibacteriana e assim se poderiam ser uma futura medicação para tratamento endodôntico. A pesquisadora se
compromete a divulgar os resultados obtidos, respeitando-se o sigilo das informações coletadas, conforme previsto no item anterior.

Comentários e Considerações sobre a Pesquisa:
Projeto científico dentro das normas da CONEP.
A metodologia proposta é capaz de atender os objetivos do estudo

Considerações sobre os Termos de apresentação obrigatória:
Quanto aos termos de apresentação obrigatória, todos foram apresentados de modo adequado.

Recomendações:
Não há.

Conclusões ou Pendências e Lista de Inadequações:
Não havendo pendências, recomenda-se a aprovação do projeto.

Considerações Finais a critério do CEP:
Não havendo pendências, o CEP propõe a aprovação do projeto de pesquisa salientando que, de acordo com a Resolução 466 CNS de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI,

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Anexo C

Aprovação do Comitê de Ética em Animais

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Efeito antimicrobiano e anti-inflamatório de ácidos fenólicos isolados, combinados e incorporados em hidrogéis de quitosana para aplicação endodôntica"**, Processo FOA nº 00709-2019, sob responsabilidade de Cristiane Duque apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 12 de Novembro de 2019.

VALIDADE DESTE CERTIFICADO: 12 de Maio de 2022.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 12 de Junho de 2022.

CERTIFICATE

We certify that the study entitled **"Antimicrobial and anti-inflamatory effect of isolated phenolic acids combined and incorporated chitosan hydrogels for endodontic application"**, Protocol FOA nº 00709-2019, under the supervision of Cristiane Duque presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on November 12, 2019.

VALIDITY OF THIS CERTIFICATE: May 12, 2022.
DATE OF SUBMISSION OF THE FINAL REPORT: June 12, 2022.

Profa. Associada Maria Cristina Rosifini Alves Rezende
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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