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Faculdade de Odontologia

GABRIELA PACHECO DE ALMEIDA BRAGA

Propriedades microbiológicas e indutoras de mineralização de flavonoides isolados ou carreados em hidrogéis termorreversíveis para aplicação como medicação endodôntica

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(Willian Shakespeare)

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RESUMO

Este trabalho foi dividido em dois capítulos que apresentaram como objetivo: 1) avaliar a ação antibiofilme e citotoxicidade de hidrogéis termorreversíveis de poly-n-vinylcaprolactama (PNVCL) carreados com flavonoides e 2) avaliar o efeito de flavonoides isolados ou incorporados em hidrogéis de PNVCL sobre a viabilidade e na indução de marcadores de mineralização em células odontoblastóides. No capítulo 1 foram determinadas as concentrações inibitórias mínimas (CIM) e a concentrações bactericidas mínimas (CBM) dos flavonoides ampelopsina (AMP), isoquercitrina (ISO) e rutina (RUT) sobre *Streptococcus mutans*, *Enterococcus faecallis*, *Actinomyces israelli*, *Lactobacillus casei* e *Fusobacterium nucleatum*. O flavonoide que obteve os menores valores de CIM/CBM foi avaliado quanto a toxicidade em fibroblastos e foi incorporado em hidrogel de PNVCL para análises posteriores. A síntese do hidrogel de PNVCL foi realizada a partir da polimerização radicalar dos monômeros N-vinilcaprolactama e acetato de vinilae sua caracterização por transmitância e ensaios de liberação dos compostos. Como controles positivos foram avaliados hidrogéis de PNVCL contendo clorexidina (CHX) e hidróxido de cálcio (HC) e controle negativo o hidrogel de PNVCL sem antimicrobianos. Os hidrogéis de PNVCL, PNVCL+AMP, PNVCL+CHX, PNVCL+HC foram avaliados quanto ao seu efeito sobre biofilmes multiespéciespor microscopia confocal de varredura a laser (MCVL) e por microscopia eletrônica de varredura (MEV). Os extratos dos hidrogéis foram coletados após 48h e 7 dias em meio de cultura e avaliadosquanto a toxicidade sobre os fibroblastos L-3T3 por meio do ensaio colorimétrico com resazurina. Os resultados do capítulo 1 mostraram que AMP apresentou os menores valores de CIM/CBM contra os microrganismos avaliados e reduziu o metabolismo das células L-3T3 em concentrações acima de 0,125 mg/mL. A análise de MEV mostrou que PNVCL+AMP causou redução bacteriana significativa e desorganização do biofilme multiespécie, similar ao obervado para o PNVCL+CH. Na análise de MCVL, PNVCL+AMP levou à significativa redução das células bacterianas viáveis no interior dos túbulos dentinários, superior aos demais grupos. Baseado nos resultados do capítulo 1, conclui-se que dentre os flavonóides testados, AMP apresentou o melhor efeito antimicrobiano e quando incorporada no hidrogel de PNVCL mostrou ação contra os biofilmes multiespécies avaliados, com efeito superior ou semelhante ao PNVCL+CH.

No capítulo 2, células odontoblastóides (MDPC-23) foram tratadas com flavonoides AMP, ISO e RUT, em três concentrações diferentes (100 µM, 50 µM e 25 µM) e avaliada sua citotoxicidade em 48 h, 8 dias e 14 dias. Após 48h de tratamento e trocas de meio osteogênico por 8 e 14 dias, foram determinadas a atividade de fosfatase alcalina (ALP), produção de proteína total (PT) e deposição de nódulos mineralizados (NM). Os resultados mostraram que AMP, ISO e RUT não afetaram a viabilidade celular em nenhum dos períodos avaliados. A atividade de ALP foi superior ao controle meio osteogênico quando MDPC-23 foram tratadas com AMP a 100 e 50 µM, ISO a 50 µM e RUT a 100 µM. Maior deposição de NM em comparação ao controle foi observada nas concentrações 100 a 25 µM para AMP, 25 µM para ISO e entre 100 e 25 µM para RUT. Após 48 h de exposição, os extratos de 48 h de PNVCL+AMP e de PNVCL+HC, independente da diluição, não afetaram a viabilidade das células, o que não foi observado para os extratos de 7 dias, que causaram citotoxicidade entre as diluições 1/2 e 1/8. Após 8 e 14 dias de exposição, somente os extratos de 7 dias dos hidrogéis causaram redução da viabilidade celular, porém, não diferiram entre si. A atividade de ALP foi superior ao controle para os extratos de 48 h e 7 dias de PNVCL+AMP e PNVCL+HC, independente da diluição. Para a produção de NM, efeito superior de ambos os extratos (48 h e 7 dias) de PNVCL+AMP foi observado em comparação ao PNVCL+HC. A partir dos resultados do capítulo 2, pode-se concluir que a AMP isolada ou incorporada ao hidrogel de PNVCL apresentou citocompatibilidade e capacidade de induzir marcadores de mineralização em células odontoblastóides. Este estudo demonstrou que AMP carreada em hidrogel de PNVCL apresentou efeito antibiofilme e capacidade de estimular marcadores de mineralização dentinária, propriedades interessantes para aplicação como medicação intracanal.

Palavras-chaves: Flavonoides; Antimicrobianos; Biofilmes; Células cultivadas; Hidrogel; Fosfatase Alcalina.

Braga, G.P.A. **Microbiological and mineralization-inducing properties of flavonoids alone or carriedin thermoreversible hydrogels for application as endodontic medication**. 2022. 106 p. Dissertação (Mestrado) em Ciência Odontológica - área Endodontia, Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, 2022.

ABSTRACT

This dissertation was divided into two chapters that aimed to: 1) evaluate the antibiofilm action and cytotoxicity of thermoreversible poly-n-vinylcaprolactam hydrogels (PNVCL) containing flavonoids and 2) evaluate the effect of flavonoids alone or incorporated in PNVCL hydrogels on the viability and induction of mineralization markers in odontoblast-like cells. In chapter 1, the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of the flavonoids ampelopsin (AMP), isoquercitrin (ISO) and rutin (RUT) were determined *on Streptococcus mutans*, *Enterococcus faecallis*, *Actinomyces israelli*, *Lactobacillus casei* and *Fusobacterium nucleatum*. The flavonoid that had the lower MIC/MBC values was evaluated for fibroblast toxicity and was incorporated into PNVCL hydrogel for further analysis. The synthesis of the PNVCL hydrogel was carried out from the radical polymerization of the monomers N-vinylcaprolactam and vinyl acetate and its characterization by transmittance and release assays of the compounds. As positive controls were evaluated PNVCL hydrogels containing chlorhexidine (CHX) or calcium hydroxide (CH) and PNVCL hydrogel without antimicrobials as negative control. PNVCL, PNVCL+AMP, PNVCL+CHX, PNVCL+HC hydrogels were evaluated for their effect on multispecies biofilms by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). The hydrogel extracts were collected after 48h and 7 days in culture medium and evaluated for toxicity on L-3T3 fibroblasts by colorimetric assay with resazurin. The results of chapter 1 showed that AMP presented the lowest MIC/MBC values against the evaluated microorganisms and reduced the metabolism of L-3T3 cells at concentrations above 0.125 mg/mL. SEM analysis showed that PNVCL+AMP caused significant bacterial reduction and disorganization of the multispecies biofilm, similar to what was observed for PNVCL+CH. CLSM analysis demonstrated that PNVCL+AMP led to a significant reduction of viable bacterial cells inside the dentinal tubules, higher than the other groups. Based on the results of chapter 1, it is concluded that among the flavonoids tested, AMP showed antimicrobial activity and effect against multispecies biofilms when incorporated into the PNVCL hydrogel, and this effect was superior or similar to PNVCL+CH. In chapter 2, odontoblastoid cells (MDPC-23) were treated with flavonoids AMP, ISO and RUT at three different concentrations (100 µM, 50 µM and 25 µM) and their cytotoxicity was evaluated at 48 h, 8 days and 14

days. After 48 h of treatment and changes of osteogenic medium for 8 and 14 days, alkaline phosphatase (ALP) activity, total protein production (PT) and mineralized nodule deposition (NM) were determined. The results showed that AMP, ISO and RUT did not affect cell viability in any of the periods evaluated. ALP activity was superior to the osteogenic medium control when MDPC-23 were treated with AMP at 100 and 50 μM, ISO at 50 μM and RUT at 100 μM. Higher NM deposition compared to the osteogenic control medium was observed at concentrations 100 to 25 μM for AMP, 25 μM for ISO and between 100 and 25 μM for RUT. After 48 h of exposure, the 48 h extracts of PNVCL+AMP and PNVCL+HC, regardless of dilution, did not affect the viability of MDPC-23 cells, which was not observed for the 7 days extracts, which caused cytotoxicity between 1/2 and 1/8 dilutions. After 8 and 14 days of exposure, only the 7 days extracts of the hydrogels caused a reduction in cell viability, but they did not differ from each other. The ALP activity was superior to the control for the 48 h and 7 days extracts of PNVCL+AMP and PNVCL+CH, regardless of the dilution. For NM production, a higher effect of both (48 h and 7 days) extracts of PNVCL+AMP compared to PNVCL+CH. From the results of chapter 2, it can be concluded that AMP alone or incorporated into the PNVCL hydrogel showed cytocompatibility and the ability to induce mineralization markers in odontoblastoid cells. In general, this study demonstrated that AMP carried in PNVCL hydrogel presents antibiofilm effect and the ability to stimulate dentinal mineralization markers, interesting properties for application as intracanal medication.

Keywords: Flavonoids; Antimicrobials; Biofilms; Cell culture; Hydrogel; Alkaline Phosphatase.

Lista de Figuras

Capítulo 1

Figure 1. Effect of the flavonoid ampelopsin (AMP) 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 antimicrobials groups and concentrations, according to ANOVA and Tukey test (p<0.05). 40

Figure 2. Variation of G' and G'' with time for PNVCL – L (20% w/v) hydrogel at 25 °C and 37 °C, indicated by the temperature profile with time. 41

Figure 3. In vitro percent drug release vs time profile for PNVCL hydrogels containing ampelosin (AMP), chlorhexidine (CHX) and calcium hydroxide (CH) at pH 7 and pH 10 42

Figure 4. Representative scanning electron microscopy images of 7-days multispecies biofilms under 5000x magnification. Biofilms were treated for 48 hours with A: PNVCL containing ampelopsin (AMP) at 2.5 mg/mL; B – PNVCL containing calcium hydroxide (CH) at 1 mg. C – PNVCL containing chlorhexidine (CHX) at 0.5 mg/mL; D – PNVCL without antimicrobials; E - Control – Bacterial growth without antimicrobial agents. F. Mean (SD) of the bacterial counts detected after 48h of the biofilm treatment with flavonoids and controls. ª Different letters show statistical difference among the antimicrobials groups and concentrations, according to ANOVA and Tukey test (p<0.05). 44

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: PNVCL containing ampelopsin (AMP) at 2.5 mg/mL; B – PNVCL containing calcium hydroxide (CH) at 1 mg/m. C – PNVCL containing chlorhexidine (CHX) at 0.5 mg/mL; D – PNVCL without antimicrobials; E - Control – Bacterial growth without antimicrobial agents. G. Mean (SD) of the percentages of dead cells of E. faecalis biofilm formed in root dentin of bovine teeth, after 48h of treatment with antimicrobial agents and controls. ª Different letters show statistical difference among the antimicrobials groups and concentrations, according to ANOVA and Tukey test (p<0.05). 45

Figure 6. Effect of the 48h (A) and 7 days (B) extracts of PNVCL hydrogels containing ampelopsin (AMP), 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 antimicrobials groups and concentrations, according to ANOVA and Tukey test (p<0.05). 46

Capítulo 2

Figure 1. Experimental design of the study. A. MDPC-23 treatments with ampelopsin (AMP), isoquercitrin (ISO), rutin (RUT) and calcium hydroxide (CH). B. MDPC-23 treatments with 48h and 7 days extracts of the hydrogels: PNVCL, PNVCL +AMP and PNVCL + CH 63

Figure 2. Percentage of MDPC-23 viability after 48h treatment with ampelopsin (AMP), isoquercetrin (ISO), rutin (RUT) and calcium hydroxide (CH) and evaluation at 48h (A), 8 days (B) and 14 days (C) of evaluation. The values are expressed in means/standard deviations 67

Figure 3. ALP activity of MDPC-23 cells after 48h of the treatment with ampelopsin (AMP), isoquercetrin (ISO), rutin (RUT) and calcium hydroxide (CH) and 8 days of evaluation. The values are expressed in means/standard deviations.....68

Figure 4. Mineralized nodules deposition of MDPC-23 cells at 14 days of evaluation after 48h of the treatment with ampelopsin (AMP), isoquercetrin (ISO), rutin (RUT) and calcium hydroxide (CH). The values are expressed in means/standard devations 69

Figure 5. Percentage of MDPC-23 viability after 48 h treatments with 48h (A) and 7 days (B) PNVCL hydrogels extracts containing or not ampelopsin (PNVCL+AMP) and calcium hydroxide (PNVCL+CH). The values are expressed in means/standard deviations.....70

Figure 6. Percentage of MDPC-23 viability after 48h of treatments with 48h (A) and 7 days (B) PNVCL hydrogels extracts containing or not ampelopsin (PNVCL+AMP) and calcium hydroxide (PNVCL+CH) and evaluation at 8 days. The values are expressed in means/standard deviations.....71

Figure 7. Percentage of MDPC-23 viability after 48h of treatments with 48h (A) and 7 days (B) PNVCL hydrogels extracts containing or not ampelopsin (PNVCL+AMP) and calcium hydroxide (PNVCL+CH) and evaluation at 14 days. The values are expressed in means/standard deviations.....72

Figure 8. ALP activity of MDPC-23 cells after 48h treatments with 48h (A) and 7 days (B) hydrogels extracts containing or not ampelopsin (AMP) and calcium hydroxide (CH) and evaluation at 8 days. The values are expressed in means/standard deviations.....74

Figure 9. Mineralized nodules deposition by MDPC-23 cells at 14 days of evaluation after 48h treatments with 48h (A) and 7 days (B) hydrogels extracts containing or not ampelopsin (AMP) and calcium hydroxide (CH). The values are expressed in means/standard deviations.....75

Suppl.1. Images of alizarin red staining obtained by inverted light microscope, indicating the bioactive effect of flavonoids on the MDPC-23 cells relative to mineralized nodules formations. All groups induced high nodules deposition dependent on the concentration. AMP at 10 µM (A), AMP at 50 µM (B), AMP at 25 µM (C), ISO at 100 µM (D), ISO at 50 µM (E), ISO at 25 µM (F), RUT at 100 µM (G), RUT at 50 µM (H), RUT at 25 µM (I) and CH at 100 µM (J), CH at 50 µM (K), CH at 25 µM (L)

Control DMEM (M). 4x magnification 79

Suppl.2. Images of alizarin red staining obtained by inverted light microscope, indicating the bioactive effect of 48 h hydrogels extracts on the MDPC-23 cells relative to mineralized nodules formations. The greatest nodules deposition can be seen in the groups PNVCL+AMP 1/32 and both dilutions of PNVCL+CH (1/16 and 1/32) PNVCL+AMP 1/16 (A), PNVCL+AMP 1/32 (B), PNVCL+CH 1/16 (C), PNVCL+CH 1/32 (D), PNCVL 1/16 (E), PNVCL 1/32 (F) and control DMEM (G). 4x magnification 80

Suppl.3. Images of alizarin red staining obtained by inverted light microscope, indicating the bioactive effect of 7 days hydrogels extracts on the MDPC-23 cells relative to mineralized nodules formations. The greatest nodules deposition can be seen in the groups PNVCL+AMP and PNVCL+CH , both at 1/16 and 1/32 dilutions. PNVCL+AMP 1/16 (A), PNVCL+AMP 1/32 (B), PNVCL+CH 1/16(C), PNVCL+CH 1/32 (D), PNCVL 1/16 (E), PNVCL 1/32 (F) and control DMEM (G). 4x magnification 81

Lista de Tabelas

Table 1. Flavonoids used in this study: codes, synonyms, chemical structure, empirical formula, and molecular weight.....32

Table 2. Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) in mg/mL for the flavonoids and control chlorhexidine against the oral bacteria tested 39

SUMÁRIO

Sumário

Introdução Geral	18
Objetivos	27
Capítulo 1	28
Abstract	29
Introduction	30
Material and Methods	32
Results	39
Discussion	47
References	50
Capítulo 2	57
Abstract	58
Introduction	59
Material and Methods	61
Results	66
Discussion	76
Suplemmentary material	79
References	82
Anexo A	88
Anexo B	93
Anexo C	99
Anexo D	100

Introdução Geral

O tratamento endodôntico de dentes permanentes jovens que sofreram danos pulpares ainda é um desafio clínico, visto que o dente ainda não completou a formação radicular e existe a necessidade de preservar as células remanescentes, principalmente as células da bainha epitelial de Hertwig, responsáveis por guiar a formação das raízes, e também células adjacentes a ela como odontoblastos recém-diferenciados, que sintetizam dentina, o que remete à necessidade do uso de materiais e soluções biocompatíveis e que possam estimular a regeneração tecidual (Rafter et al., 2005; Iglesias-Linares et al., 2013).

Para o preparo químico-mecânico tradicional são utilizadas substâncias químicas irrigadoras como o hipoclorito de sódio ou a clorexidina que, nas altas concentrações indicadas, não são consideradas biocompatíveis, podendo inviabilizar as células remanescentes presentes do tecido pulpar ou periapical, interrompendo a proliferação celular e posterior secreção de dentina e continuação da formação da raiz (Ring et al., 2008). Além disso, embora o preparo químico-mecânico reduza significativamente os microrganismos presentes nos canais radiculares, bactérias residuais podem permanecer no interior de túbulos dentinários, canais secundários e istmos devido à dificuldade de limpeza desse complexo sistema de canais radiculares e a resistência microbiana ao tratamento, o que pode gerar infecções persistentes ou secundárias (Siqueira et al., 2007a; Siqueira et al., 2007b).

As bactérias Gram-positivas facultativas têm sido consideradas as mais resistentes ao preparo químico-mecânico, dentre elas, espécies de *Streptococcus*, *Actinomyces* (*A. israelii e A. odontolyticus*), *Propionibacterium*, *Lactobacillus*, e *Enterococcus faecalis*. Entretanto, algumas espécies Gram-negativas como *Fusobacterum* spp, *Prevotella* spp. também podem ser detectadas, mostrando que ainda há certa diversidade microbiana, mesmo após o uso das soluções antimicrobianas de rotina (Sakamoto et al., 2007; Siqueira et al., 2007a; Siqueira & Rôças, 2009). Por este motivo, medicações intracanais são frequentemente utilizadas visando eliminar a microbiota remanescente, sendo que a pasta de hidróxido de cálcio (HC) é a mais indicada, principalmente para dentes permanentes jovens, por suas

propriedades biológicas e físico-químicas (Sheehy & Roberts, 1997; Da Silva et al., 2010).

Os tratamentos endodônticos convencionais preconizados para dentes permanentes jovens frequentemente conduzem à resolução dos sinais e sintomas das patologias pulpares/periapicais (Parirokh et al., 2010), porém, promovem pouco ou nenhum benefício para a continuidade da apicigênese, processo de desenvolvimento radicular (Diogenes et al., 2014). Dessa forma, novos protocolos biológicos vêm sendo estudados buscando combinar materiais que que possam reduzir substancialmente a microbiota residual e permitir a regeneração tecidual natural preservando as células remanescentes e a continuação da formação radicular (Iglesias-Linares et al., 2013).

Um desses protocolos biológicos é baseado no conceito de revascularização pulpar que combina a irrigação passiva com hipoclorito de sódio em baixa concentração (1,5%) e a aplicação de medicação intracanal com uma pasta antibiótica (1-5mg/mL) ou pasta de hidróxido de cálcio (1mg/mL) (AAE, 2018) em uma primeira sessão. Essa técnica propõe a indução de sangramento intracanal, formação de coágulo sanguíneo e aplicação de matriz de colágeno reabsorvível em uma segunda sessão (até um mês depois) para estimular a revascularização pulpar e o reparo periapical (AAE, 2018). O uso de antibióticos apresenta algumas limitações como o desenvolvimento de resistência bacteriana, descoloração da coroa (Kim et al., 2010), além de toxicidade sobre as células indiferenciadas (Althumairy et al., 2014). Além disso, esses medicamentos são mais efetivos quando os microorganismos estão em estado ativo de síntese das paredes celulares, proteínas e DNA, do que em fase estacionária, como ocorre no interior dos espaços radiculares quando bacterias resistem ao preparo químico-mecânico (Saoud et al., 2016). O hidróxido de cálcio também apresenta limitações para eliminar as bactérias intracanais e parte está relacionada aos efeitos inibitórios da dentina e hidroxiapatita sobre a sua atividade antimicrobiana (Haapasalo et al., 2007).

O princípio do revascuralização embora tenha se mostrado favorável em alguns estudos clínicos promovendo o reparo dos tecidos periapicais em dentes permanentes imaturos com periodontite apical (Banchs & Trope, 2004; Thibodeaud

et al., 2007; Jung et al., 2008; Ding et al., 2009; Reynolds et al., 2009; Kim et al., 2010; Taneja et al., 2010; Iwaya et al., 2011), tem sido considerado de baixa previsibilidade desde que o desenvolvimento radicular pode variar de 21% a 100%, e existem algumas controvérsias quanto ao tecido formado no canal radicular que consistiria em um tecido conjuntivo com cemento associado à bactéria residual (Schmalz et al., 2020). Assim, embora a técnica de revascularização pulpar pareça promissora, ainda são necessários novos medicamentos que possam permanecer dentro do canal radicular reduzindo as bactérias residuais aos procedimentos de irrigação passiva e ainda consigam estimular as células remanescentes a continuarem a síntese das paredes dentinárias e completarem a formação radicular (Iglesias-Lineares et al., 2013).

Flavonoides são metabólitos secundários responsáveis pela cor, fragância e sabor de diversas espécies vegetais. Além disso, eles regulam o crescimento celular, protegem as plantas contra espécies bióticas e abióticas, atuam como moléculas de sinalização, e são sequestradores de espécies reativas de oxigênio, entre outras funções (Dias et al., 2021). A ampelopsina (2,3-dihydrochromen-4-one), também conhecida como dihidromiricetina, é um flavanonol extraído das folhas da *Ampelopsis grossendata*. Dentre as suas propriedades biológicas e farmacológicas, foram relatados efeitos anti-inflamatórios, antimicrobianos, antioxidantes, anti-tumores, cardioprotetores, neuroprotetores, dermoprotetores, além de atuar na regulação da insulina e colesterol (Zhang et al., 2016; Ran et al., 2019; Chen et al., 2020; Liang et al., 2020). A ampelopsina também aumenta a atividade da fosfatase alcalina, a deposição mineral e a expressão de genes específicos de osteoblastos, sem apresentar citotoxicidade e efeito negativo sobre a proliferação celular (Zhang et al., 2016; Weng et al., 2017). Outro flavonoide, a isoquercitrina (quercetin 3-O-beta-Dglucofuranoside), é um composto fenólico encontrado em plantas como *Mangifera indica* (Wu et al., 2020) e ervas como a *Camellia sinensis* (Sakakibara et al., 2003) apresentando propriedades terapêuticas, como ação anti-inflamatória, antioxidante, antialérgica, antifúngica e antibacteriana (Yun et al., 2018). A rutina (quercetin-3-O-rutinoside), um biflavonoide presente em algumas hortaliças e frutas cítricas tem sido indicado como um promissor agente antibiofilme (Al-Shabib et al., 2017). Além disso,

possui propriedades antioxidante, anti-inflamatória, anticancerígena, antialérgica, entre outras (Yong et al., 2020). Embora os flavonoides são promissores biomoléculas devido a sua amplitude terapêutica, seu uso clínico é limitado, desde que apresentam baixa solubilidade, baixa estabilidade e degradação em ambientes ácidos o que reduz sua biodisponibilidade ao longo do tempo (Ferreira et al., 2022).

Polímeros naturais ou sintéticos têm sido usado como carreadores de biomoléculas desde que promovem a liberação controlada desses compostos e prolongam seu efeito biológico. No caso da atividade antimicrobiana, por exemplo, esses carreadores reduzem a necessidade de doses excessivas, exposição frequente e probabilidade de resistência bacteriana (Li et al., 2018). Os principais carreadores poliméricos atualmente estão sendo empregados em diferentes formas: *scaffolds*, hidrogéis e nanopartículas ou combinações dessas formas. Hidrogéis são capazes de transitar do estado líquido para o estado gel pela formação de uma rede tridimensional polimérica como resultado de estímulos externos e interações físicas, como calor, pH ou ligações químicas (Li et al., 2018; Chenite et al., 2000).

Os hidrogéis termossensíveis transitam do estado físico em temperatura específica, baseada nas interações hidrofílicas-hidrofóbicas entre as cadeias de polímeros e moléculas de água (Ponce-Vargas et al., 2013). Além de termossensíveis, alguns polímeros podem ser considerados termorreversíveis, como o poly(N-vinylcaprolactam) (PNVCL), pois apresentam valores críticos de temperatura de conversão similares à temperatura fisiológica corporal humana podendo transitar do estado líquido para gel quando injetados dentro do corpo humano e retornar ao estado líquido quando resfriados (Sala et al., 2017; Chenite et al., 2000). Além disso, esses hidrogéis quando sofrem hidrólise não produzem compostos tóxicos e têm apresentado boa compatibilidade celular, quando comparados a outros géis de similar característica química, podendo ser um adequado veículo para o carreamento de medicamentos (Liang et al., 2012). O hidrogel PNVCL também tem mostrado aumentar a solubilidade de alguns fármacos em solução aquosa, facilitando seu carreamento, além de minimizar os efeitos colaterais associados a eles (Paramenswara-Thankam et al., 2018). O presente estudo foi dividido em dois

capítulos e tem por objetivos: 1) avaliar a ação antibiofilme e citotoxicidade de hidrogéis termorreversíveis de poly-n- vinylcaprolactama (PNVCL) contendo flavonoides e 2) avaliar o efeito de flavonoides isolados ou incorporados em hidrogéis de PNVCL sobre a viabilidade e na indução de marcadores de mineralização em células odontoblastóides.

*Referências da Introdução Geral em **Anexo A**

OBJETIVOS

1. Determinar a concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM) dos flavonoides ampelopsina, isoquercitrina e rutina visando determinar o flavonoide com melhor ação antimicrobiana para os testes microbiológicos;
2. Avaliar o efeito de hidrogéis de PNVCL carreados com ampelopsina em comparação comcom hidrogéis de PNVCL contendo hidróxido de cálcio ou clorexidina, sobre biofilmes multiespécies, além de determinar a toxicidade dos extratos desses hidrogéis sobre fibroblastos;
3. Avaliar a toxicidade, efeito sobre a atividade de fosfatase alcalina e sobre a produção de nódulos de mineralização dos flavonoides ampelopsina, isoquercitrina e rutina sobre células odontoblastóides (MDPC-23);
4. Avaliar a citotoxicidade, a atividade de fosfatase alcalina e produção de nódulos de mineralizados de extratos de hidrogéis de PNVCL contendo ampelopsina em comparação com hidrogéis de PNVCL contendo hidróxido de cálcio sobre as células MDPC-23

CAPÍTULO 1

Microbiological properties and cytotoxicity of PNVCL hydrogels containing flavonoids as intracanal medication for endodontic therapy

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***The manuscript is according to the guide for authors of Biofouling (Anexo B).**

Abstract

Objetives: to evaluate the microbiological properties and cytotoxicity of poly (N-vinylcaprolactam) - PNVCL hydrogels containing flavonoids as intracanal medication for endodontic therapy.

Materials and Methods: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the flavonoids ampelopsin (AMP), isoquercitrin (ISO) and rutin (RUT) were determined against *Enterococcus faecalis*, *Streptococcus mutans*, *Lactobacillus casei*, *Actinomyces israelii* and *Fusobacterium nucleatum* by microdilution method after 24h exposure. The flavonoid with the lowest MIC/MBC values were chosen for cytotoxicity analysis and the posterior incorporation in PNVCL hydrogels. PNVCL hydrogels were synthesed and characterized by rheological assays. AMP and controls calcium hydroxide (CH) and chlorhexidine (CHX) were incorporated in PNVCL hydrogels and determined the release profile of these compounds. The effect of PNVCL+AMP, PNVCL+CH, PNVCL+CHX were evaluated on multispecies biofilms and analysed by Scanning Electron Microscopy (SEM) and Confocal LaserScanning Microscopy (CLSM). L-3T3 fibroblast cells were exposed to serial dilutions of AMP and 48 h and 7 days hydrogels PNVCL extracts and cytotoxicity analyzed by the resazurin colorimetry method.

Results: AMP had the lowest MIC/MBC values against all bacteria tested. *S. mutans* and *A. israelli* growth was affected only for AMP and CHX. AMP affected L-3T3 cell viability at concentrations above 0.125 mg/mL. SEM analysis showed that PNVCL+AMP significantly reduced bacterial counts and cause multispecies biofilm disorganization, similar to PNVCL + CH. The CLSM analysis demonstrated that PNVCL + AMP caused a significant reduction of viable cells in dentin tubules, superior to the other groups. 48h and 7 days extracts of PNVCL hydrogels containing AMP showed low toxicity on 3T3 cells, similar to PNVCL + CH.

Conclusion: AMP presented better antimicrobial effects compared to ISO and RUT on bacteria commonly found in infected root canals, in addition caused low toxicity to fibroblast cells. PNVCL hydrogel containing AMP demonstrated potent effect against multiespecies biofilms and compatibility in culture of fibroblasts and could be a potential intracanal medication for endodontic purposes.

Keywords: Flavonoids; Hydrogel; PNVCL; Cytotoxicity; Biofilms.

Introduction

Intracanal bacteria are generally organized in multispecies biofilms embebbed in polymeric matrix and attached to the dentinal root canal walls. Core microbiome of endodontic infections are composed most frequently by up to thirty microbial species belonging to five phyla: Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria and Fusobacteria (Shin et al., 2018; Siqueira & Rôças,2021). In primary endodontic infections, the most abundant species are Gram negative obligate anaerobic species, however, anaerobic facultative bacteria, including *Streptococcus*, *Actinomyces* species, *Lactobacillus* species, *Parvimona micra*, *Pseudoramibacter alactolyticus, Enterococcus faecalis* and others, predominate in post-treatments infections, demonstrating their resistance to conventional chemical-mechanical therapies (Siqueira & Rôças, 2009,2021).

In infected root canals, intracanal medication is needed to eliminate remaining bacteria after canal instrumentation, to reduce inflammation of periapical tissues and pulp remnants, to neutralize tissue debris and as barrier against leakage from the temporary filling (Vera et al., 2012). Calcium hydroxide is considered the recommended intracanal medication due to its favorable antimicrobial action, anti-inflamamatory properties and ability to induce the deposition of mineralized tissue (Mohammadi & Dummer, 2011). Calcium hydroxide has been mixed with different vehicles (aqueous, viscous, and oily) which influences its biological and physico-mechanical properties, including release profile of calcium and hydroxyl ions and time of action (Estrela & Holland, 2003). Despite these advantages, studies have demonstrated the presence of biofilm/bacteria in theroot canals even weeks after filling with calcium hydroxide medication, with and significant reduction of lipopolysaccharide endotoxin (Vianna et al., 2007; Roças & Siqueira, 2011).

In immature permanent teeth with pulp necrosis and apical periodontitis, high bacterial load and the presence of virulent species causes severe inflammation which interferes with tissue healing and root maturation (Schmalz et al., 2020; Fouad, 2020). Current studies have evaluated biological protocols which promote significant level of disinfection and preserve the remaining cells present in the pulp tissue and/or in the Hertwig sheath allowing natural tissue regeneration (Iglesias-Lineares et al., 2013; Fouad, 2020). One of these protocols is based on the concept of pulp revascularization combining passive irrigation with low concentration of sodium hypochlorite with subsequent intracanal medication with a

triantibiotic paste (Glynis et al., 2021) or calcium hydroxide (AAE, 2018). Although this protocol has shown some favorable clinical results with the promotion of periapical tissue repair (Jung et al., 2008; Ding et al., 2009; Taneja et al., 2010, Trope, 2010), it is considered of low predictability. This is because root development can vary from 21% to 100%, and there are some controversies regarding the tissue formed in the root canal that would consist of connective tissue with cementum associated with residual bacteria (Schmalz et al., 2020).

In the search for new biological compounds with multiple pharmacological properties, a variety of flavonoids, compounds derived from fruits, plants, and roots, have been investigated and demonstrated their antimicrobial, antioxidant, osteogenic and antiosteoclastogenic activities (Juccá et al., 2018; Maleki et al. 2019; Liu et al.„ 2021). Ampelopsin (dihydromyricetin) is a flavanonol derived from myricetin present in vine tea with biological properties such as anticancer, antioxidant and antimicrobial effects (Chang et al. 2020; Jiang et al., 2021). In addition, ampelopsin has increased the activity of alkaline phosphatase, mineral deposition, and expression of specific genes of osteoblasts without presenting cytotoxicity and negative effects on cell proliferation (Zhang et al. 2016; Weng et al. 2017). Isoquercitrin has presented several therapeutic properties, including anti-inflammatory, antioxidant, anti-allergic, antifungical and antibacterial activities (Yun et al., 2018). Rutin (quercetin-3-O-rutinoside), abiflavonoid, present in some vegetables and citrus fruits has showed antibiofilm effect (Al-Shabib etal., 2017), antioxidant, anti-inflammatory, anticancer and antiallergic properties (Yong et al. 2020).

Natural or synthetic polymers have been used as drug carriers to promote the controlled release of compounds and extend their biological effect. Advantages of these carriers include reduction of doses or frequent exposure to antimicrobials and the risk of bacterial resistance (Li et al. 2018; Yuan et al., 2011). Poly (N-vinylcaprolactam) (PNVCL) is a thermosensitive hydrogel, which presents critical values of temperature for the conversion to the gel state at the physiological human body temperature andcan be a suitable aqueous vehicle for drug delivery. PNVCL hydrogel can transition from liquid to gel state when injected into the human body and return to liquid state when cooled (thermoreversibility), without additional chemical reactions or environmental treatments (Liang Xet al., 2012; Sala et al. 2017). Furthermore, PNVCL increases the low solubility of some drugs in aqueous solution, facilitating drug delivery, in addition to minimizing associated side effects (Paramenswara

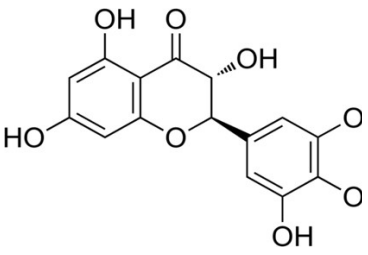
et al., 2019). Considering the wide range of therapeutic effect of flavonoids and the potential of PNVCL hydrogels as carrier of biomolecules, this present study aimed to evaluate the antibiofilm properties and cytotoxicity of PNVCL hydrogel containing flavonoid in comparison to PNVCL containing calcium hydroxide or chlorhexidine.

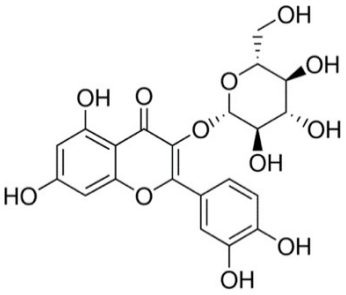
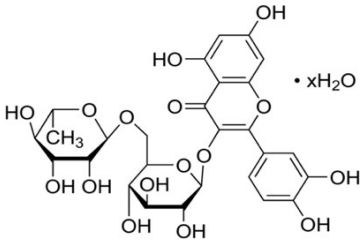
Material and Methods

Preparation of flavonoids and controls

The flavonoids evaluated in the study were: ampelopsin (AMP, #42866), isoquercitrin (ISO, #17793) and rutin (RUT, #R5143), all obtained from Sigma-Aldrich (St. Louis, MO/ USA). **Table 1** shows the flavonoids used in the study, their codes, synonyms, chemical structure, empirical formula, and molecular weight. Flavonoid stock solutions were prepared in dimethylsulfoxic – DMSO at 30 mg/mL. Calcium hydroxide PA (CH, Biodynamics, Ibiporã – PR/BR) at 1 mg/mL and chlorhexidine gluconate (CHX, Manipullis Pharmacy, Araçatuba – SP/BR) at 20 mg/mL were diluted in sterile deionized water as positive controls. All compounds were filtered in a 0.22 µm membrane filter and store at – 20 °C.

Table 1. Flavonoids used in this study: codes, synonyms, chemical structure, empirical formula, and molecular weight.

Compound Code*	Synomyms	Chemical structure	Empirical formula Molecular weight
Ampelopsin (AMP) **42866**	(2R,3R)-3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one, Ampelopsin, DHM, 3,3',4',5,5',7-Hexahydroxyflavanone, Ampeloptin		$C_{15}H_{12}O_8$ 320.25

Isoquercitrin (ISO) #17793	3,3',4',5,7-Pentahydroxyflavone 3- β-glucoside, Isoquercitrin		$C_{21}H_{20}O_{12}$4 64.38
Rutin (RUT)#R5143	Quercetin-3-rutinoside hydrate, Vitamin P hydrate		$C_{27}H_{30}O_{16} \cdot xH_2O$ 610.52

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

Determination of antimicrobial activity

Microbial strains and growth conditions

The following standard strains used: *Enterococcus faecalis* (ATCC 51299), *Streptococcus mutans* (ATCC 25175), *Actinomyces israelii* (ATCC 12102), *Lactobacillus casei* (IAL#523) and *Fusobacterium nucleatum* (NCTC 11326) provided by Oswaldo Cruz Foundation – FIOCRUZ, Rio de Janeiro, Brazil. Microbial suspensions were prepared from cultures previously grown in Mitis Salivarius Agar (Difco Laboratories, Kansas City, MO, USA) with 0.2 U/mL bacitracin (Sigma-Aldrich) for *S. mutans*, MRS Rogosa Agar (Difco) for *L. casei*, Brain Heart Infusion Agar – BHIA (Difco Laboratories) for *A. israelii* and *E. faecalis* and incubated at 37 °C for 24 h in a 5 % CO_2 atmosphere (Incubator Ultra Safe, HF212-UV). *F. nucleatum* was grown in BHI blood agar (Difco Laboratories) containing 5 mg/mL of hemin, 5 mg/mL of menadione, 0.5% yeast extract powder (YE, Difco Laboratories) and 5% defibrinated sheep blood at 37 °C in an anaerobic chamber (AnaeroGen, Oxoid, Thermo Scientific, Waltham, MA, USA). All experiments were performed in triplicate in three independent experiments.

Determination of minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC)

MIC and MBC assays were performed as a screening for the selection of the flavonoid with the

highest antimicrobial effect. These assays followed the criteria described by the Clinical and Laboratory Standard Institute M7-A9 (CLSI, 2012) for microdilution method in bacteria with modifications (Massunari et al., 2017; Caiaffa et al., 2017). Briefly, microbial cultures were grown until reaching the standard optical density - OD (0.5) in specific media as described in the previous item and harvested by centrifugation (Centurion Scientific, K3 Series, Core Life Sciences) for 10 min, at 3000 x g. The supernatant was discarded, and the pellet re-suspended in 2X concentrated Mueller-Hinton broth (Difco Laboratories) and 2X concentrated BHI broth containing hemin, menadione, and YE for *F. nucleatum*. The final concentration of bacterial suspension inside the wells was 1-5x10^5 CFU/mL. Chlorhexidine digluconate was used as positive controls and cultures without antimicrobial agents as negative controls. All antimicrobial agents were serially diluted in sterile deionized water, to obtain concentrations ranging from 1 to 0.0001 mg/mL. The microbial suspensions were inoculated in each well containing the diluted flavonoids (AMP, ISO and RUT) and CHX. The microplates were incubated at 37 °C for 24 h for all microorganisms, except *F. nucleatum*, which was incubated for 48h. Afterwards, 15 µL of 0.01% resazurin (#R7017, Sigma-Aldrich) was applied in each well and incubated for 3 h and analyzed at 570 and 600 nm in a spectrophotometer (Biotek, Winooski, VT). MIC was defined as the lowest concentration of each compound in which there was no detectable growth by the colorimetric method. After incubation, cultures from MIC wells and at least three previous wells were serially diluted and plated on Mueller-Hinton Agar (MHA) for facultative anaerobic bacteria, and in blood agar for *F. nucleatum* for 48 h in the same conditions. Subsequently, viable bacteria were counted and the number of colony forming units/mL was determined (CFU/mL). The minimal bactericidal concentration (MBC) was obtained when the antimicrobial agents killed more than 99% of the tested microbial strains.

Cytotoxicity assays

Cell culture

Fibroblast cells from L3T3-L1 (CL-173^{TM}) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine (Gibco BRL, Gaithersburg, MD) in a humidified incubator with 5% CO_2 and 95% air at 37 °C (Isotemp Fisher

Scientific, Pittsburgh, PA, USA). Cell cultures were subcultured every 48 hours until reaching 80% confluence. Then, cells were treated with trypsin-EDTA (0.25% Trypsin-EDTA 1x, Gibco) for 5 min at 37°C, cells were stained with Trypan Blue (Sigma-Aldrich) and counted in a Neubauer chamber using an Automatic Cell Counter (Bio-rad, Hercules, CA, USA). Next, they were seeded at a cell density of $5x10^4$ cells/well in 96-well plates and incubated for 24 h for subsequent cytotoxicity assay.

Determination of cell viability

The viability rate was evaluated via colorimetry resazurin assay. This method allows determining cellrespiration (mitochondrial), considering the metabolic rate of cells. The cells were treated with AMP, CHX and CH (from 1 to 0.004 mg/mL) prepared in DMEM. After 24 h of treatment, cells were washed with PBS and resazurin 70µM (Sigma-Aldrich) in DMEM was added for 4h. Plates were read at 570 and 600 nm in spectrophotometer (Biotek, Winooski, VT). The final values were obtainedby the subtractions between the absorbance values at both wavelengths, and they were converted into percentage of cell viability considering the growth in DMEM medium without antimicrobials as 100% and the means determined for each group (Borra et al., 2009; Duque et al., 2022).

Synthesis and characterization of PNVCL hydrogels

Thermosensitive PNVCL hydrogels were synthetized and characterized by rheological assays as described previously by Sala et al. (2017). Briefly, 5g of N-vinylcaprolactam (NVLC) monomer were dissolved in 20 g of dimethyl sulfoxide (DMSO) and heated to 70 °C under N_2 atmosphere. Then, 0.112 g of azobisisobutyronitrile (AIBN) was dissolved in 8.33 g of DMSO and added (in drops) to the reaction system. The system was kept under agitation for 4h and the resultant polymer was purified by dialysis against deionized water for 4 days. PNVCL was dryed in an owen at 50 °C and stored at4 °C before use. Rheological analysis was performed to evaluate the hydrogel formation at 37 °C. Solutions with a concentration of 20 wt% of PNVCL were chosen based on the work of Sala et al., who studied the hydrogel formation at different concentrations. The tests were performed on an Anton-Paar (Modular Compact Rheometer, Graz, Austria) model MCR 302, equipped with parallel geometry plate with 25 mm of diameter and gap of 1 .0 mm. The time sweep analysis over 10 min was performed using

strain of 1.0 % and frequency of 1 Hz. For antibiofilm and cytotoxicity assays, PNVCL hydrogel were loaded with AMP at 2.5 mg/mL, CH at 1 mg/mL and CHX at 0.5 mg/mL separately and incubated for 24 h at 5 °C for total solubilization to prepare the polymeric gel.

Evaluation of AMP, CH and CHX release from PNVCL hydrogels

For release assays, AMP solution was prepared by adding 5 mg of AMP in 0.25 mL of DMSO, followed by the addition of 9.5 mL of distilled water, at a concentration of 500 ppm. The samples were prepared by adding 200 mg of polymer to 1 mL of drug solution, this mixture was left for 24 hat 5 °C for total solubilization. 300 µL of sample was incubated for 10 min in the thermostatic bath to form the hydrogel, followed by the addition of 1.8 mL of buffer. A temperature of 37 °C and two different buffers were used to control the release: phosphate buffer saline (pH 7.4) and glycine buffer (pH 10.0). At predetermined times, 1 mL of supernatant was withdrawn and replaced with fresh buffer. AMP aliquots were analyzed in a UV-vis spectrometer at a wavelength of 325 nm. The release of CHX and CH, both at 500 ppm, from PNVCL hydrogels was also evaluated. The CHX aliquots were analyzed in a UV-vis spectrometer at a wavelength of 255 nm. For CH, a colorimetric method was used to allow its quantification (Calcio Liquidform #90, Labtest, Lagoa Santa, MG, Brazil). 1 mL of arsenazo III was added to each aliquot, turning it into a purple solution and determination ofcalcium content was determined at a length of 600 nm in a UV-vis spectrometer.

Analysis of antibiofilm activity

Multispecies biofilms assays and analysis by Scanning Electron Microscopy (SEM)

E. faecalis, *A. israelii*, *S. mutans*, *L. casei* and *F. nucleatum* cultures were mixed in equal aliquots at the same concentration (1-5x 10^3 CFU mL^{-1}) in BHI broth containing 1% glucose and inserted in 24 wells microplates. After 1 week of growth in anaerobic conditions and change of culture media every 48 h, biofilms were washed twice with sterile saline solution, and PNVCL (at 200 mg/mL) without antimicrobials; PNVCL+AMP at 2.5 mg/mL (10x the highest MIC); PNVCL + CH at 1 mg/mL (usual concentration for clinical application); PNVCL+CHX at 0.5 mg/mL (100x the highest MIC) were inserted into each well. The plates were incubated for 48h at 37°C in anaerobic conditions. Aliquots from all wells were plated on BHI agar and the plates incubated for 48 h for further counting of CFU/mL. Concomitantly, the same experiments were

conducted in coverslips for microscopic analysis. The samples were dehydrated by washing in a series of ethanol (70% for 10 min, 95% for 10 min and 100% for 20 min) and air-dried in a desiccator. Afterwards, coverslips were mounted onto aluminum stubs, sputter coated with gold and analyzed in a scanning electron microscope (Leo, Cambridge, MA, USA) (Jacob et al., 2020).

Multispecies Biofilms assays on dentin tubules and analysis by Confocal Laser Scanning Microscopy (CLSM)

This study was approved by the Animal Committee of Araçatuba Dental School, UNESP, Brazil (Protocol: 00444-2020 – Anexo C) and conducted in accordance with Ma et al. (2011). Bovine incisors (n= 6/group) were extracted and stored in a 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. Initially, roots were separated from crowns, using a diamond disc (KG Sorensen D 91, Barueri, SP, Brazil) at 1 mm below the cement-enamel junction. The roots were fixedto acrylic plates, then with two diamond discs of 0.6 mm (Isomet 5000, BuehlerLtd, LakeBluff, IL) at1000 rpm under water irrigation using a precision saw (IsoMet 1000, Buehler, Lake Bluff, IL, USA), a 4 mm cylinder was horizontally sectioned. The root canal wall of each specimen was abraded with a# 6 wide drill. The cylindrical specimen was again sectioned in two cylindrical halves with a 0.6 mm diamond disc. The dentin samples were washed with distilled water and ultrasonically cleaned with 17% EDTA for 3 min and deionized water for 5 min to remove the smear layer. After autoclaving for 15 minutes at 121 °C, they were then inserted into a microtube with the canal side (pulp) up. Any gap between the specimen and the inner wall of the microtube was sealed with composite resin and polymerized for 20 s. Cultures of *E. faecalis*, *A. israelii*, *S. mutans, L. casei* and *F.nucleatum* were mixed in equal aliquots at the same concentration (1-5x 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. A fresh aliquot of bacteria was added between each centrifugation and the old discarded. All microtubes were incubated at 37°C in BHI supplemented with 1% glucose broth for 2 weeks in an atmosphere of 5%CO_2. The culture medium was changed every 48 h.

Dentin disinfection

The dentin samples were removed from the microtube and washed with sterile water for 1 min. These specimens were treated with PNVCL (at 200 mg/mL) without antimicrobials, PNVCL loaded with AMP at 2.5 mg/mL, PNVCL loaded with CH at 1 mg/mL, PNVCL loaded with CHX at 0.5 mg/mL as positive controls and sterile water as negative controls. Each sample was immersed in 350 µl of each solution for 48h, with initial shaking of 1 h and was then washed with sterile water for 1 min at 37 °C to avoid residual effects of the treatments. Subsequently, the samples were cut into two new halves, for observation of the surface longitudinally to the dentinal canals visible by Confocal Microscopy (Ma et al., 2011). The samples were stained with 100 µL of fluorescent LIVE/DEAD BacLight Bacterial Viability stain (L13152, Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide, according to the manufacturer's instructions. The excitation/emission wavelengths were 480/500 nm for SYTO 9 and490/635 nm for propidium iodide. Fluorescence from the stained cells was viewed by CLSM (Leica TCS SP5, Microsystems GmbH), using a 63x oil immersion lens. CLSM images were acquired using software (LAS AF Leica Microsystems) at a resolution by 1024 pixels. Ten-micrometer-deep scans were obtained with the CLSM from two randomly selected places. In order to analyze the Live/Deadcells ratios of the infected dentinal tubules, all scans were reconstructed in a three-dimensional model by the same software, and quantification of the red fluorescence ratio in relation to green- and-red fluorescence was determined by software denominated Image J 1.48 (NIH, Bethesda, MA, USA), indicating the proportion of dead cells for each antimicrobial agent tested (Caiaffa et al., 2017,2021).

Cytotoxicity assays with hydrogels extracts

PNVCL hydrogel were loaded with AMP at 2.5 mg/mL, CH at 1 mg/mL and CHX at 0.5 mg/mL separately and incubated for 24 h at 5 °C for total solubilization. After 24h, the samples at the liquid state were incubated overnight at 37 °C 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 harvested after 48 h and 7 days for sequential treatment. The cell viability rate was evaluated by colorimetry resazurin assay (Sigma-Aldrich), as previously described. Briefly, fibroblastic L-3T3 cells were seeded into the 96 well plates (1-5x 10^4 cells/well) and incubated for 24 h under standard cell culture conditions. After incubation, DMEM was

removed, and the dilutions of the hydrogel's extracts wereadded to the cells (from 1/2 to 1/64 dilutions). L-3T3 cultured in DMEM without any extract was used as the control. After the 24h treatment, the extracts were removed and 200 µL of resazurin (70µM) were added for incubation at 37° C for 4 h. The plates were analyzed in a spectrophotometer (Biotek, Winooski, VT) at 570 and 600nm.

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 cytocompatibility and microbiological assays were expressed in means/standard deviation and submitted to ANOVA and Tukey tests. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis,considering $p<0.05$.

Results

Antimicrobial activity

Among the microorganisms tested, the flavonoid with superior antibacterial activity was ampelopsin with MIC values ranging 0.125 to 1 mg/mL and MBC between 0.25 and 1 mg/mL. Isoquercitrin and rutin had antibacteridal action only against *F. nucleatum* with MIC at 0.25 mg/mL and 0.5 mg/mL, respectively, and MBC at 1 mg/mL for both flavonoids (**Table 2**).

Table 2. Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) in mg/mL for the flavonoids and control chlorhexidine against the oral bacteria tested.

	E. faecalis	***A. israelii***	***S. mutans***	***L. casei***	***F. nucleatum***
Ampelopsin	1 (1)	0.25 (1)	0.25 (1)	1 (1)	0.125 (0.25)
Isoquercitrin	1 (>1)	1 (>1)	1 (>1)	1 (>1)	0.25 (1)
Rutin	>1(>1)	1 (>1)	1 (>1)	1 (>1)	0.5 (1)
Chlorhexidine	0.005 (0.005)	0.002 (0.002)	0.0002 (0.002)	0.002 (0.004)	0.004 (0.004)

MBC: >99.9% cell reduction. MIC results were based on resazurin staining and MBC results were based on CFU/mL count in Miller Hinton Agar (MHA) medium. The growth of microorganisms without antimicrobials in MHA was considered 100%.

Citotoxicity of ampelopsin and controls

The effect of AMP, CH and CHX on viability of L-3T3 cells can be seen on **Figure 1**. AMP at the concentrations above 0.25 mg/mL of ampelopsin affected cell viability. CH did not affect cell viability at any concentration tested. There was no statistical difference comparing AMP and CH from 0.125 mg/mL. CHX was toxic to cells at a concentration above 0.0078 mg/mL.

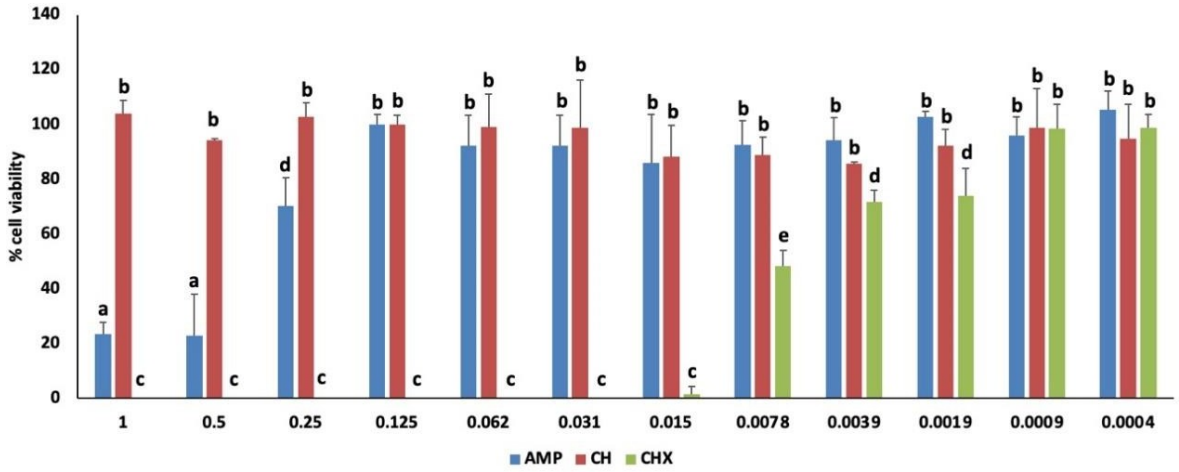


Figure 1. Effect of the flavonoid ampelopsin (AMP) and controls calcium hydroxide (CH) and chlorhexidine (CHX) on the viability of fibroblasts (3T3) after 24h of exposure, using staining with resazurin. Concentrationsin mg/mL.
[a] Different letters show statistical difference among the antimicrobials groups and concentrations, according to ANOVA and Tukey test ($p<0.05$).

Rheological analysis

Figure 2 represents the rheological analysis of PNVCL-L (20% w/v). The formation of a hydrogel is evaluated through the relationship of G' (storage moduli) and G'' (loss moduli) as a function of temperature and time. At 25 °C the loss moduli was greater than the storage moduli (G"> G') characterizing that at this temperature the hydrogel remains in the liquid state. Different from the temperature of 37 °C capable of keeping the hydrogel in a gel state due to G'>G".

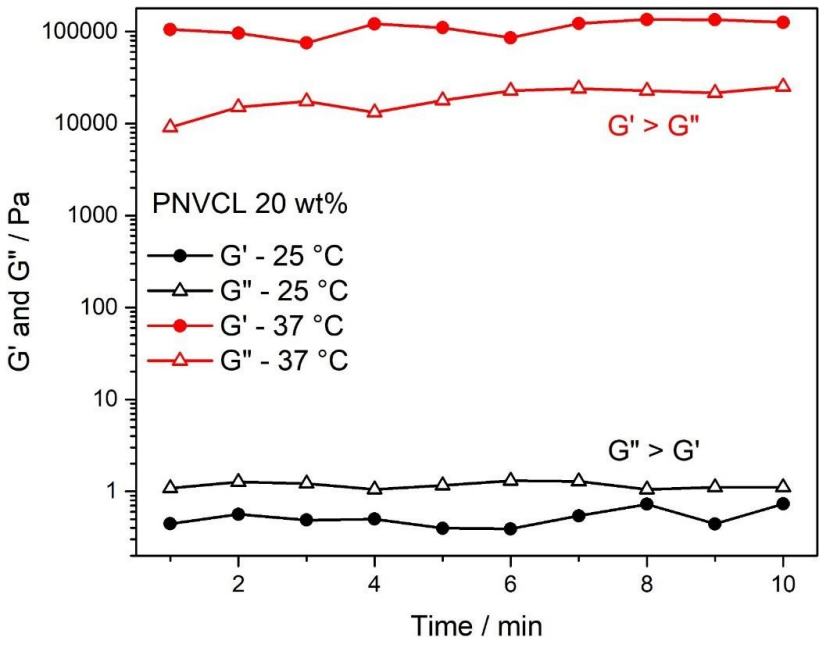


Figure 2. Variation of G' and G'' with time for PNVCL – L (20% w/v) hydrogel at 25 °C and 37 °C, indicated bythe temperature profile with time.

Release Assay

Figure 3 shows the in vitro release of ampelopsin, chlorhexidine and calcium hydroxide from PNVCL at pH 7 and pH 10. PNVCL hydrogel was able to control the output of the three compounds without showing a burst of release in the first hours. This property observed at the beginning of the test was maintained for the entire period of 7 days at neutral pH and at basic pH. The greater release of AMPand CH from PNVCL occurred at basic pH. The release of AMP increased from 13% to 37% at pH 10. For CH the increase occurred from 42% to 67% at the same pH. CHX had similar release patterns (20-25%) independent on the pH, indicating that the change in pH did not affect its release.

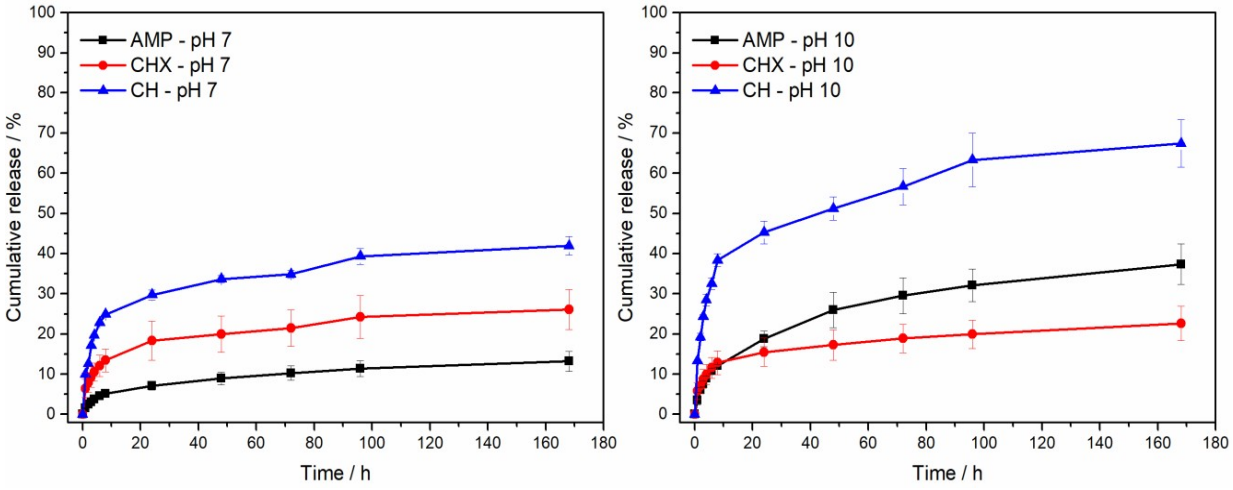


Figure 3. In vitro percent drug release vs time profile for PNVCL hydrogels containing ampelosin (AMP), chlorhexidine (CHX) and calcium hydroxide (CH) at pH 7 and pH 10.

SEM analysis

Figure 4 (A-G) shows representative images of scanning electron microscopy of 7-days multispecies biofilms with *E. faecalis, S. mutans, A. israelli, L. casei and F. nucleatum* after treatment with the PNVCL hydrogels. PNVCL+AMP (**Figure 4A**) caused disorganization on multispeciesbiofilms, with evident reduction of bacterial cells and extracellular matrix, similar to that observed for the PNVCL+CH (**Figure 4B**) and PNVCL+CHX (**Figure 4C**) hydrogels. Pure PNVCL (**Figure 4D**) did not affect biofilm organization, as observed in the control group without any treatment (**Figure 4E**). Quantitative analysis of bacterial counts can be seen in **Figure 4F**. No statistical difference was observed between pure PNVCL and control group. A significant reduction in bacterial counts presented in multispecies biofilms was observed when AMP, CH and CHX were incoporated in the PNVCL hydrogel. No statistical difference between the PNVCL+AMP and PNVCL+CH group was observed, and PNVCL+CHX showed the highest reduction compared to the other groups.

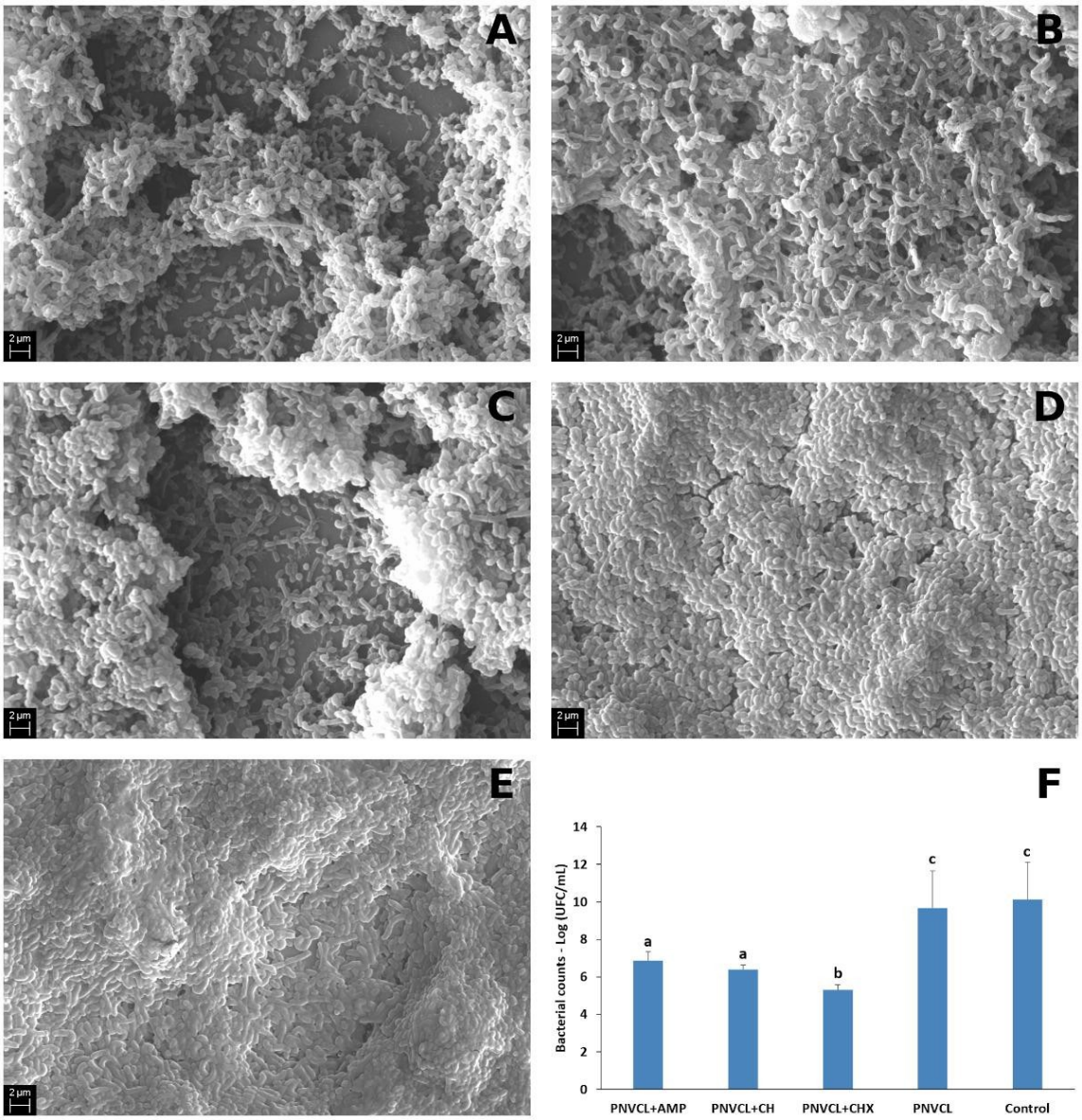


Figure 4. Representative scanning electron microscopy images of 7-days multispecies biofilms under 5000x magnification. Biofilms were treated for 48 h with A: PNVCL containing ampelopsin (AMP) at 2.5 mg/mL; B – PNVCL containing calcium hydroxide (CH) at 1 mg. C – PNVCL containing chlorhexidine (CHX) at 0.5 mg/mL; D – PNVCL without antimicrobials; E - Control – Bacterial growth without antimicrobial agents. F. Mean (SD) of the bacterial counts detected after 48 h of the biofilm treatment with flavonoids and controls.

[a] Different letters show statistical difference among the antimicrobials groups, according to ANOVAand Tukey test ($p<0.05$).

CLSM analysis

Figure 5A-F shows the results observed when 14-days multispecies biofilms (with *E. faecalis, S. mutans, A. israelli*, *L. casei* and *F. nucleatum)* formed inside root canals were exposed to the PNVCL hydrogels containing the antimicrobial agents for 48h. Representative confocal images of PNVCL hydrogels are observed in **Figures 5A-E**. PNVCL+AMP (**Figure 5A)** showed a greater amount of dead cells (red points), followed by PNVCL+CH **(Figure 5B) and** PNVCL+CHX **(Figure 5C)** compared to the control **(Figure 5E)** and PNVCL without antimicrobials **(Figure 5D)** that show more live cells (green points). These results were confirmed in **Figure 5F**. PNVCL associated with ampelopsin reduced 73.8% of bacterial cells inside the tubules and had statistical difference compared to PNVCL+CH and PNVCL+CHX which reduced 58.8% and 50.6 % of bacterial cells. There was no statistical difference between PNVCL+CH and PNVCL+CHX, however, both differed from the control. Pure PNVCL did not differ from the control group.

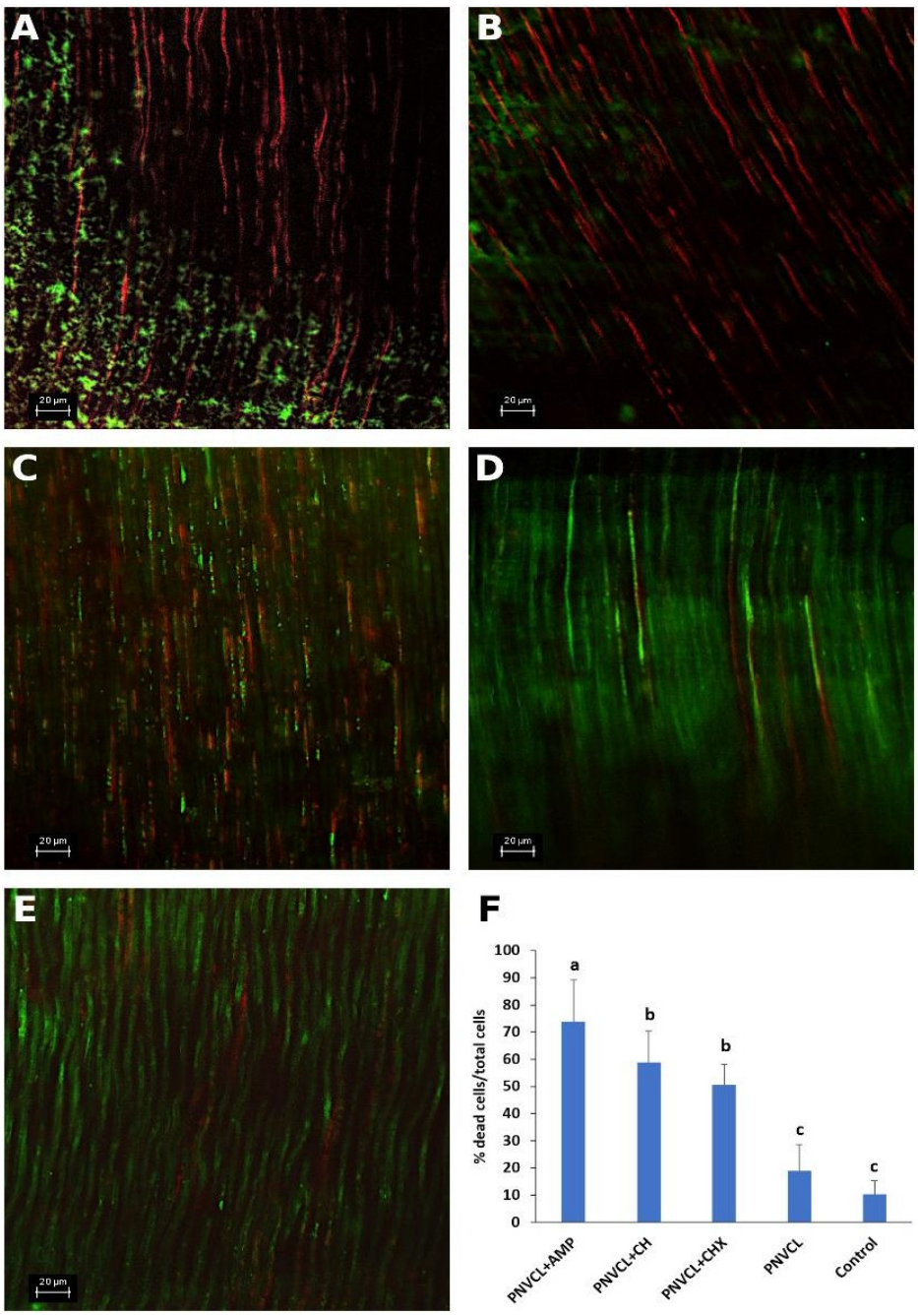


Figure 5. Representative confocal microscopy images of bovine root dentin specimens contaminated for 14 days with multispecies biofilms and treated for 48 h with the following groups with A: PNVCL containing ampelopsin (AMP) at 2.5 mg/mL; B – PNVCL containing calcium hydroxide (CH) at 1 mg/mL. C – PNVCL containing chlorhexidine (CHX) at 0.5 mg/mL; D – PNVCL without antimicrobials; E - Control – Bacterial growth without antimicrobial agents. F. Mean (SD) of the percentages of dead cells of E. faecalis biofilm formed in root dentin of bovine teeth, after 48 h of treatment with antimicrobial agents and controls.

[a] Different letters show statistical difference between the antimicrobials groups, according to ANOVAand Tukey test ($p<0.05$).

Citotoxicity tests with hydrogels extracts

Figure 6A-B shows the effect of the 48 h and 7 days extracts of PNVCL hydrogels containing AMP, CH and CHX, or without antimicrobials agents on fibroblast cells after 24 h of exposure. In **Figure 6A**, viability was superior to 70% when cells were exposed to 48 h PNVCL extracts and PNVCL containing AMP or CH with no statistical difference among them, at any dilution tested. Similar results were observed to PNVCL+CHX from dilutions below 1/8. In **Figure 6B**, 7-days extracts of PNVCL hydrogels containing AMP, CH and CHX did not statiscally differ from each at any concentration and all of them were similar to control from dilution below 1/4. PNCVL+CHX showed no statistical difference with pure PNVCL at any concentration.

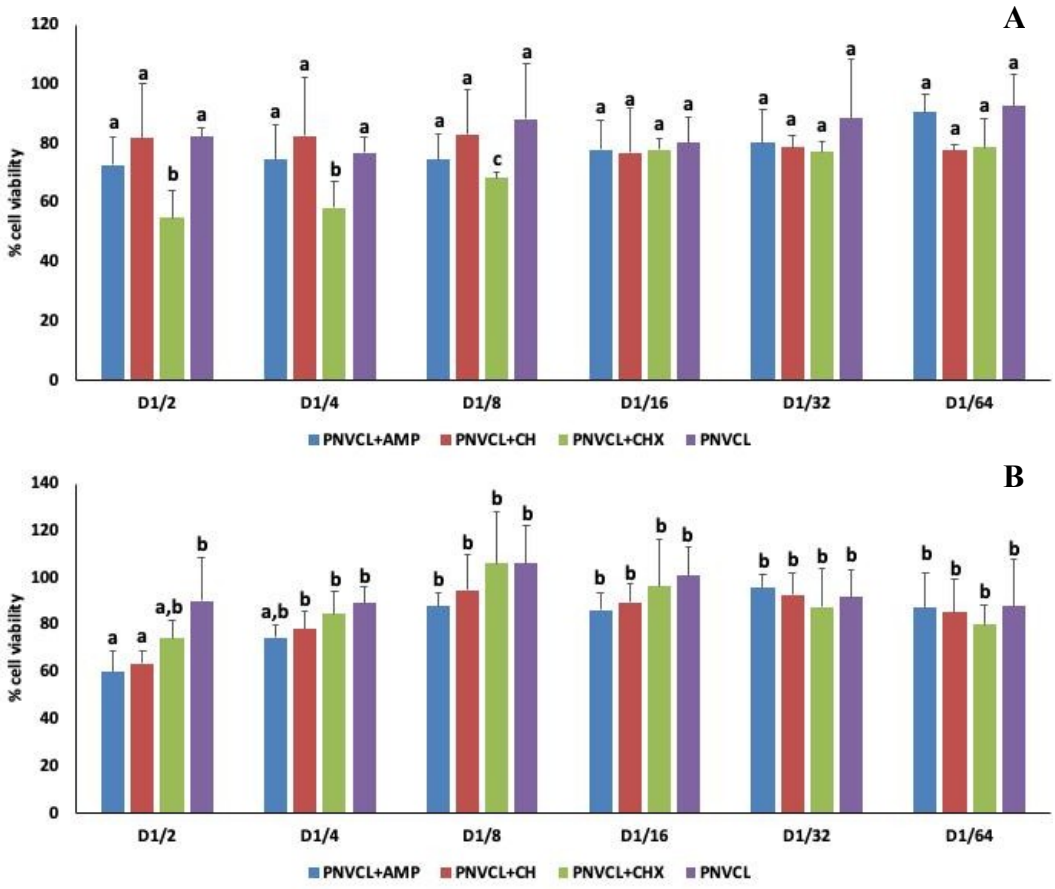


Figure 6. Effect of the 48 h (A) and 7 days (B) extracts of PNVCL hydrogels containing ampelopsin (AMP), calcium hydroxide (CH) and chlorhexidine (CHX) on the viability of fibroblasts (3T3) after 24hof exposure, using staining with resazurin. Concentrations in mg/mL. [a] Different letters show statistical difference among the antimicrobials groups and concentrations, according to ANOVA and Tukey test ($p<0.05$).

Discussion

Considering the challenge of developing a medication capable of substantially reducing residual bacteria in passive irrigation procedures and allowing the apex closure in immature permanent teeth, this study evaluated the antimicrobial activity of the flavonoids ampelopsin (AMP), isoquercitrin (ISO) and rutin (RUT). Ampelopsin had a greater bactericidal effect against the bacteria tested, specially against *A. israelli*, *S. mutans*, *F. nucleatum* with MIC/MBC ranging 0.125 to 1 mg/mL. Our study is according to previous study which observed that AMP (also known as dihydromyricetin) was effective against *Staphylococcus aureus*, *Bacillus subtilis* (Gram-positive bacteria), *Escherichia coli*, *Salmonella paratyphi* and *Pseudomonas aeruginosa* (Gram-negative bacteria) with MIC/MBC ranging 0.312 to 2.5 mg/mL (Xiao et al., 2019). Another study confirmed the antibacterial effect of AMP on *S. aureus* with MIC and MBC of 0.125 and 0.25 mg/mL, respectively (Wu et al., 2018). The same study demonstrated a significant reduction of biofilm biomass of *S. aureus* and a decrease in the metabolic activity of the biofilm cells. No study was found evaluating the effect of AMP on the same bacteria tested in the present study. Some investigations have confirmed a direct relationship between the hydroxylation at position 5 and 7 and the antibacterial activity of flavonoids and the hydroxylation on the B and C rings can also improve this effect (Farhadi et al., 2018; Shevelev et al., 2020).

In the present study, isoquercitrin (ISO) and rutin (RUT) had bactericidal effect only against *F. nucleatum*. Although hydroxylation at position 5 and 7 is presented in all flavonoids tested - AMP, ISO and RUT, AMP presents more hydroxylations on the B ring compared to the other ones. Considering other mechanisms of action, AMP was capable of preventing cell division and proliferation, destroying cell integrity and permeability, in addition to interacting with bacterial DNA of *S. aureus* (Liang et al., 2020). Contrary to our study, some investigations demonstrated antimicrobial effect of RUT on *A. naeslundii*, *A. viscosus, C. albicans* (Gutiérrez-Venegas et al., 2019), *E. coli* and methicillin-resistant *S. aureus* with MIC value ranging 0.4 to 1.6 mg/mL (Al-Shabib et al.,2017) and effect of ISO against *E. coli* and *C. albicans* with MIC value of 2.5 mg/mL (Yun J et al., 2015). Differences in results may be due to different methodologies and tested microorganisms. Based on the results obtained in this study, AMP was selected for the cytotoxicity assays.

In addition to the antimicrobial action, the compound used for endodontic purposes, should present compatibility with the remaining periapical cells, including fibroblasts, that will favor the

tissue repair and allow the development of the root apex in case of immature teeth. In the present study, AMP did not affect fibroblast viability in the concentrations below 0.25 mg/mL. Studies have reported low cytotoxicity of AMP on fibroblasts and keratinocytes cells (Sklenarova et al., 2021). In an in vivo wound-healing study, AMP promoted proliferation of fibroblasts and fibrocytes, reduced the signs of inflammation, manifesting immunostimulating and remodeling properties capable of accelerating wound repair when antimicrobial activities are no longer needed (Shevelev et al.,2020).

Despite the low toxicity and wide range of therapeutic application, flavonoids have some limitations like low solubility in water, easy degradation in highly acidic medium and low bioavailability (Ferreira et al., 2022). To overcome these drawbacks, several new forms of delivery systems have been developed for the encapsulation of these compounds reducing theirlimitations and improving their therapeutic effect (Ferreira et al., 2022). This is the first study which evaluate poly (n-vinylcaprolactam) – PNVCL - as thermosensitive polymer for the delivery of ampelopsin. As demonstrated in a previous study demonstrated, when PNVCL reachs a temperature above its lower critical values of temperature (LCST), it changes from a liquid to a gel state (Sala et al., 2017). PNVCL has demonstrated its ability to carry antibiotics, such as ciprofloxacin,and promoted a time-controlled release (Paramenswara-Thankam et al., 2018).

The ability of the hydrogel to release drugs is directly dependent on characteristics such as temperature, the portionof hydrophilic groups and hydrophobic groups in the copolymer and hydrophilicity/hydrophobicityof the drug (Liu et al., 2016). Vibrational spectroscopy analysis in the infrared region showed that release rate in hydrogels is higher for hydrophilic than hydrophobic drugs (Liu et al., 2016). The low water solubility of ampelopsin may be an advantage, leading to a slower release of the drug release in PNVCL system, making longer the time of the drug action and reducing medication reintake (Solanki et al., 2012). The influence of pH on drug release from PNVCL hydrogel systems was previously studied by Fallon et al. (2019). They observed that PNVCL exhibited a burst of drug releaseat pH 6.8, compared to lower release in an acidic environment at pH 2.2. In the present study, greater drug release was promoted by the pH increase. Calcium hydroxide (CH) presented the highest percentage of release from PNVCL when compared to AMP and CHX. This result can be explained by the alkalinity promoted by CH when it is dissolved (pH 12.6 in water solution), different from chlorhexidine, which is an acidic solution, with pH around 5.0. On the other hand, AMP in solution, has a pH ranging between 5.5 and 6.5, which promoted lower

release at neutral pH (from 5 to 10%) and greater release at basic pH (from 13 to 37%). Previous study showed that nanocapsules containing ampelopsin showed two release phases, one called burst phase when 44%of the drug were release up to 6h, followed by a sustained release phase, where the remaining 56%of the drug were released progressively over time (Dalcin et al., 2017). Cyprofloxacin incorporated in guar-*graft*-poly(N-vinylprolactam) hydrogels showed a initial burst release, followed by a controlled release pattern of sustained release (Paramenswaran-Thankan et al., 2018).

The effect of new antimicrobial agents on biofilms have been considered in the medical studies, since biofilms are implicated in the pathogenesis and chronicity of several bacterial infections, including endodontic infections, that are difficult to successfully eradiate with conventional antimicrobial therapies (Costerton et al., 1999; Hall and Mah, 2017). In this study, the antibiofilm effect of PNVCL associated with AMP, CH and CHX was evaluated by bacterial counts and analysed by electron scanning microscopy (SEM) and confocal laser scanning microscopy (CLSM). The concentration of compounds incorporated in PNVCL hydrogels was chosen based on previous studies which have shown that lower concentrations of antimicrobials (lesser than 10x MIC) have no significant effect on polymicrobial biofilms (Oliveira et al., 2017; O'Brien et al., 2022), since biofilm cells are hundreds oftimes more resistant to antibacterial agents than planktonic cells (up to 1,000-fold increase) (Roy etal., 2018; Nab et al., 2019). In this study, bacterial counts and SEM images showed evident reductionof bacterial cells and extracellular matrix when multispecies biofilms were treated with PNVCL+AMP, PNVCL+CH and PNVCL+CHX. The same was observed for CLSM results, intracanal multispecies biofilms were also reduced by the three formulations of PNVCL, with superior effect for PNVCL+AMP. This is a relevant finding since one of the major limitations of intracanal medication is to reach residual bacteria in dentinal tubules, secondary canals and istmus after chemical-mechanical treatment. Our results are similar to those found to ampelopsin-loaded (dihydromyricetin) nanocapsules at 1 mg/mL reduced 67% of the *P. aeruginosa* biofilm population in 96 h of treatment compared to 41.2% for free ampelopsin and 44.8% for blank nanocapsules, showing sustained release of the drug and an additional antimicrobial effect of nanocapsules (Dalcin et al., 2017). Other formulations of PNVCL hydrogels with pectin (poly(acrylamidoglycolic acid-co-vinylcaprolactam) and silver nanoparticles presented inhibitory effect against *B. subtilis* and *E. coli* (Reddy et al., 2014)and copolymerization of N-

vinylcaprolactam onto caboxymethylcellulose also showed high antibacterial ativity against *S. aureus*, *Proteus vulgaris* and *S. typhi* (Kumar et al., 2018).

In general, 48 h- and 7 days- PNVCL hydrogels extracts containing the antimicrobials AMP, CH and CHX caused low toxicity to fibroblasts evaluated in the present study. PNVCL hydrogels have been considerated cytocompatible when exposed to Caco-2 and pulmonary Calu-3 cell lines. It is anadvantage when compared to other hydrogels, such as poly(N-isopropylacrylaminde) - PNIPAM, which hydrolyzes producing toxic compounds (Vihola et al., 2005). In vitro tests showed that cell viability was greater than 90% when PNVCL when exposed to human mesenchymal stem cells and bovine chondrocytes for 24h (Sala et al., 2017). In addition, the cytotoxicity of poly(vinylcaprolactam) – PVCL-based microgels loaded with doxycycline on HK-2 cells were investigated after 48 h incubation and the hydrogel showed no cytotoxicity at the concentration range of 0.1-50 □g/mL and about 90% of cells were viable at the high concentration of 100□g/mL, which is considered biocompatibility and little cytotoxicity of PVCL (Wang et al., 2013). In the present study, 48h extract of PNVCL+CHX caused cytotoxicity in dilution above 1/8. Chlorhexidine has presented cytotoxicity when directly exposed to cells even at low concentrations (Lessa et al., 2010).

In traditional drug administration, high doses or repeated administration are generally used to reach the therapeutic effect, however, as a consequence, there is an increase in the systemic andlocal toxicity, in addition to, a decrease in the general efficacy of the drug along the time. Hydrogels are interesting because they provide a controlled release system and contribute to the continuous and unsaturated release, increasing the time of action against microorganisms (Gonzalez-Urias et al., 2022). Considering the limitations of the intracanal medications used in revascularization protocols, the potent antibiofilm effect of PNVCL containing ampelopsin showed that it could be used as medication in Endodontics. In vivo clinical studies should be conducted to confirm the safety and efficacy of this medication.

Conclusion

PNVCL containing ampelopsin presented potent antibiofilm activity and low cytotoxicity, similar to PNVCL loaded with calcium-hydroxide and both could be useful for further application as intracanal medication in Endodontics.

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Capítulo 2

Cytotoxicity and biomineralization potential of flavonoids alone or incorporated into PNVCL hydrogels

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***The manuscript is according to the guide for authors of Archives of Oral Biology (Anexo D).**

Abstract

Objectives: The aim of the study was to evaluate the effects of ampelopsin (AMP) in comparison with other flavonoids and AMP-loaded PNVCL hydrogel on cell viability and mineralization markersin odontoblast-like cells.

Methods: MDPC-23 odontoblast-like cells were exposed to AMP, isoquercitrin (ISO) and rutin (RUT)at the concentrations 100 µM, 50 µM and 25 µM, and serial dilutions of 48 h and 7 days extracts ofthe hydrogel poly(*N*-vinylcaprolactam) - PNVCL, AMP-loaded PNVCL and calcium-hydroxide (CH)- loaded PNVCL for 48h. Cell viability was evaluated by resazurin colorimetry assays. Total protein (TP) production, alkaline phosphatase (ALP) activity and mineralized nodule deposition assays were carried out for 8 and 14 days in osteogenic medium and analyzed by Folin-Ciocalteu,thymolphthalein monophosphate and alizarin red staining assays, respectively. Data were statistically analyzed using ANOVA/Tukey test, considering $p<0.05$.

Results: Cell viability was above 70% when MDPC-23 were treated with AMP, ISO and RUT at 100 µM, 50 µM and 25 µM at the different time-points (48 h, 8 days and 14 days). In general, among theflavonoids tested, AMP showed the greater ALP activity and mineralized nodules deposition, differing from the control at the highest concentrations. 48h and 7 days extracts of pure PNVCL hydrogel affected cell viability at the dilutions 1/2 and 1/4. Only 7 days extracts of PNVCL+AMP and PNVCL+CH at the dilutions 1/2 and 1/4 affected MDPC-23. Both PNVCL+AMP and PNVCL+CH (at the dilutions 1/16 and 1/32) stimulated ALP activity and mineralized nodules deposition, statistically higher than the control of osteogenic medium.

Conclusion: AMP was cytocompatible and induced the highest levels of mineralized markers in MDCP-23. Low concentrations of AMP-loaded PNVCL extracts were cytocompatible and were able toinduce ALP activity and mineralized nodules deposition.

Keywords: Flavonoids; Hydrogels; Odontoblasts; Cytotoxicity; Alkaline Phosphatase; Dentin mineralization.

Introduction

The physiological process of apexogenesis, which consists of normal root development and closure of the apex mainly through the deposition of dentin by odontoblasts, can be interrupted in young permanent teeth by the occurrence of trauma or pulp necrosis. The conventional apexification procedure that induces the formation of a mineralized barrier in the apical region from frequent exchanges of calcium hydroxide (CH) induces root weakening and increase the susceptibility to root fracture (Andreasen et al. 2002; Kahler et al., 2018), besides to provide little or no benefit to the continuity of apicigenesis (Diogenes et al., 2014). The apical plug created inthe most apical portion of the immature root with mineral trioxide aggregate (MTA) searching for sealing the communication between the root canal and the extra-radicular region, has a greater advantage over frequent exchanges of CH. However, this technique depends on the crown/root ratios, and it does not promote complete root development and presents risk of further root fracture (Torabinejad et al., 2017)

The regenerative endodontic procedure (REP) indicated by the American Association of Endodontics (AAE) consists of careful disinfection the root canal with sodium hypochlorite at low concentrations, avoiding rigorous mechanical instrumentation. This is followed by insertion of an antimicrobial medication, such as antibiotic pastes or calcium hydroxide pastes for the control of remaining microorganisms for posterior blood clot induction and application of MTA as capping material (Diogenes & Ruparel, 2017). REP has demonstrated satisfactory clinical and radiographic results, with no statistical difference compared to treatment with MTA plug, with 79% of the cases with complete root development (Schmalz G et al., 2020). However, some controversies have been raised regarding the tissue formed in the immature root canal which consists of a connective tissue with cementum associated with residual bacteria. The presence of bacteria after REP procedure has shown to prevent root development in 21% to 100 % of cases reducing the predictability of the treatment (Schmalz et al., 2020). In addition, intracanal medication with antibiotic pastes could leadto bacterial resistance, allergies, and tooth discoloration (Reynolds et al. 2009; Khaler et al. 2014). Thereby, a medication that can remain inside the root canal for longer periods, eliminating the residual microorganisms and stimulating remaining cells or progenitor cells of the apical papilla to continue root development is still a promise (Iglesias-Lineares et al., 2013).

Flavonoids are polyphenolic compounds widely found in various fruits, vegetables,

barks, stems, tea plants and derivatives. They are largely used in various sectors ranging from nutritional, pharmaceutical to medicinal and cosmetic applications (Ramesh et al., 2021). Flavonols and derivates, such as dihydroflavonols and glycosylated flavonols, are groups of flavonoids known by their strong antimicrobial and antioxidant activity with multiple benefits to human health (Weidmann, 2012; Xiao et al., 2021; Ramesh et al., 2021; Zhu et al., 2022). Ampelopsin, also called dihydromyricetin, is a flavonoid extracted mainly from the leaves of *Ampelopsis grossendata*. Some studies have shown its biological and pharmacological properties, such as anti-inflammatory, antioxidant, anti-tumor, hepatoprotection, cardioprotection, neuroprotection, dermoprotection, insulin and cholesterol regulation, and antimicrobial activities (Zhang et al., 2018; Chen et al., 2015; Ran et al., 2019, Liang et al., 2020). Isoquercitrin (quercetin-3-O-glucoside) is a flavonoid glucoside found in plants, fruits and vegetables that has antioxidant, anti-inflammatory (Fu et al., 2020), anti-cancer (Chen et al., 2016) and antiviral (Kim et al., 2016) properties. Furthermore, it has demonstrated to enhance the mineralization capacity of osteoblastic cells and promoted alkaline phosphatase activity (Li et al. 2019; Wang et al. 2014). Rutin is another glycosylated flavonol found in teas, fruits such as apples and tomatoes and legumes such as onions, among others (Hertog et al., 1993). Rutin has also demonstrated antioxidant, anti-inflammatory, and anticancer activities in some recent studies (Al-Shabib et al., 2017; Yong et al., 2020). Studies observed the effect of rutin on cell proliferation, enhancing the osteogenic differentiation and mineralization as well as reducing the oxidative stress (Zhao et al., 2020; Liu et al., 2021).

To improve their solubility, bioavailability and biological properties in a controlled-release manner aiming to control residual bacteria, inflammation and the reparative process, flavonoids have been incorporated into drug- delivery vehicles, such as hydrogels (Lišková et al., 2015; Soares et al., 2020). The poly(n- vinylcaprolactam) PNVCL hydrogel is a thermoreversible hydrogel capable of transitioning from the liquid state to the gel when subjected to a similar physiological human body temperature, becoming a gel at approximately 34°C, returning to its liquid form at lower temperatures (Sala et al., 2017). It has showed a great potential in the field of biomedicine as controlled drug delivery systems, for being easily injectable, biocompatible (Ribeiro et al., 2021) as well as, for not producing toxic compounds (Vihola et al. 2007) and increasing the availability of drugs (Paramenswara et al., 2019). The present study aimed to evaluate the cytotoxicity and biomineralization potential of

odontoblast-like cells stimulated by flavonol derivatives (ampelopsin, isoquercitrin and rutin) and by extracts of ampelopsin-loaded PNVCL hydrogel, as a potential injectable delivery system for endodontic purposes.

Material and Methods

Flavonoids and controls

Ampelopsin (AMP, 42866*), isoquercitrin (ISO, #17793) and rutin (RUT, #R5143), all obtained from Sigma-Aldrich (St. Louis, MO/ USA), were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at 30mg/mL and stored at -70 °C, and calcium hydroxide was dissolved in water at 1mg/mL and stored at 4°C. Cell culture medium was used as control in this study. All experiments were performed in triplicate in three independent days.

Synthesis and characterization of PNVCL hydrogels

Thermosensitive PNVCL hydrogels were synthetized and previously characterized by rheological assays according to Sala et al. (2017). Briefly, 5 g of N-vinylcaprolactam (NVLC) monomerwere dissolved in 20 g of dimethyl sulfoxide (DMSO) and heated to 70 °C under N_2 atmosphere. Then, 0.112g of azobisisobutyronitrile (AIBN) was dissolved in 8.33g of DMSO and added (in drops) to thereaction system. The system was kept under agitation for 4h and the resultant polymer was purifiedby dialysis against deionized water for 4 days. PNVCL was dried in an owen at 50^{o} C and stored at4 °C before use. PNVCL molecular structure was analyzed by ^{1}H NMR (DMX 360 MHz; Bruker, Billerica, MA) and their viscoelastic properties confirmed by rheological analysis on AR 200 rheometer (TA Instruments), couped with a Peltier control device (Sala et al., 2017). For subsequentassays, PNVCL hydrogel were loaded with AMP at 2.5 mg/mL (7.8 mM, PNVCL + AMP) and CH at 1 mg/mL (13.5 mM, PNVCL + CH) separately and incubated for 24 h at 5°C for total solubilization to prepare the polymeric gel.

MDPC-23 cell culture

The odontoblast-like cells (MDPC-23 - mouse dental papilla cell line) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; high low glucose, L-glutamine and sodium pyruvate; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and containing 100IU/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamicin

and 0.25 µg/mL amphotericin (Gibco) in a humidified incubator with 5% CO2 and 95% air at 37ºC (Isotemp Fisher Scientific, Pittsburgh, PA, USA). Cells were sub-cultured every 2 days until 80% of confluence was reached.

Study design

For the first part of this study (**Figure 1A**), the following experimental groups were determined: control group (DMEM – no treatment), ampelopsin (AMP), isoquercitrin (ISO), rutin (RUT), and calcium hydroxide (CH, gold standard as medication in endodontics) at concentrations of 100 µM, 50 µM and 25 µM. MDPC-23 were seeded (5 X 10^3 cells/well) onto sterile 96-well plates (T0), which were maintained at 37°C for 48 h. Then, cells were treated with the compounds (T1) for 24 h (T2) and 48 h (T3) and determined cell viability (Leite et al., 2017; Massunari et al., 2021). For alkaline phosphatase (ALP) and mineralized nodules (MN) deposition assay cells were seeded in 48 well plates (3x 10^2 cells/well) and treated for 48 h with the compounds (T3). After that, DMEM was replaced by osteogenic DMEM (DMEM with FBS, antibiotics and supplemented with 50 µg/mL ascorbic acid, 10nmol/L sodium β-glycerophosphate and 1.8 nmol/L KH_2PO_4, which was refreshed every 48h until completion of the experimental period for ALP (8 days – T4) and MN deposition (14days – T5) assays (**Figure 1A**). For the second part of this study (**Figure 1B**), 1mL of PNVCL, PNVCL+AMP and PNVCL + CH at the liquid state were inserted in 24-well plates and incubated overnight at 37 °C to acquire the gel state. Then, 1 mL of DMEM was added over the hydrogels (T0) and incubated for 48 h (T1) and 7 days (T3). Cells were seeded (T2) and evaluated for cytotoxicity at 48 h (T4), 8 days(T5) and 14 days (T6), ALP at 8 days (T5) and MN deposition at 14 days (T6) (**Figure 1B**). All the assays were performed in triplicate in three independent days (Leite et al., 2017; Massunari et al., 2021).

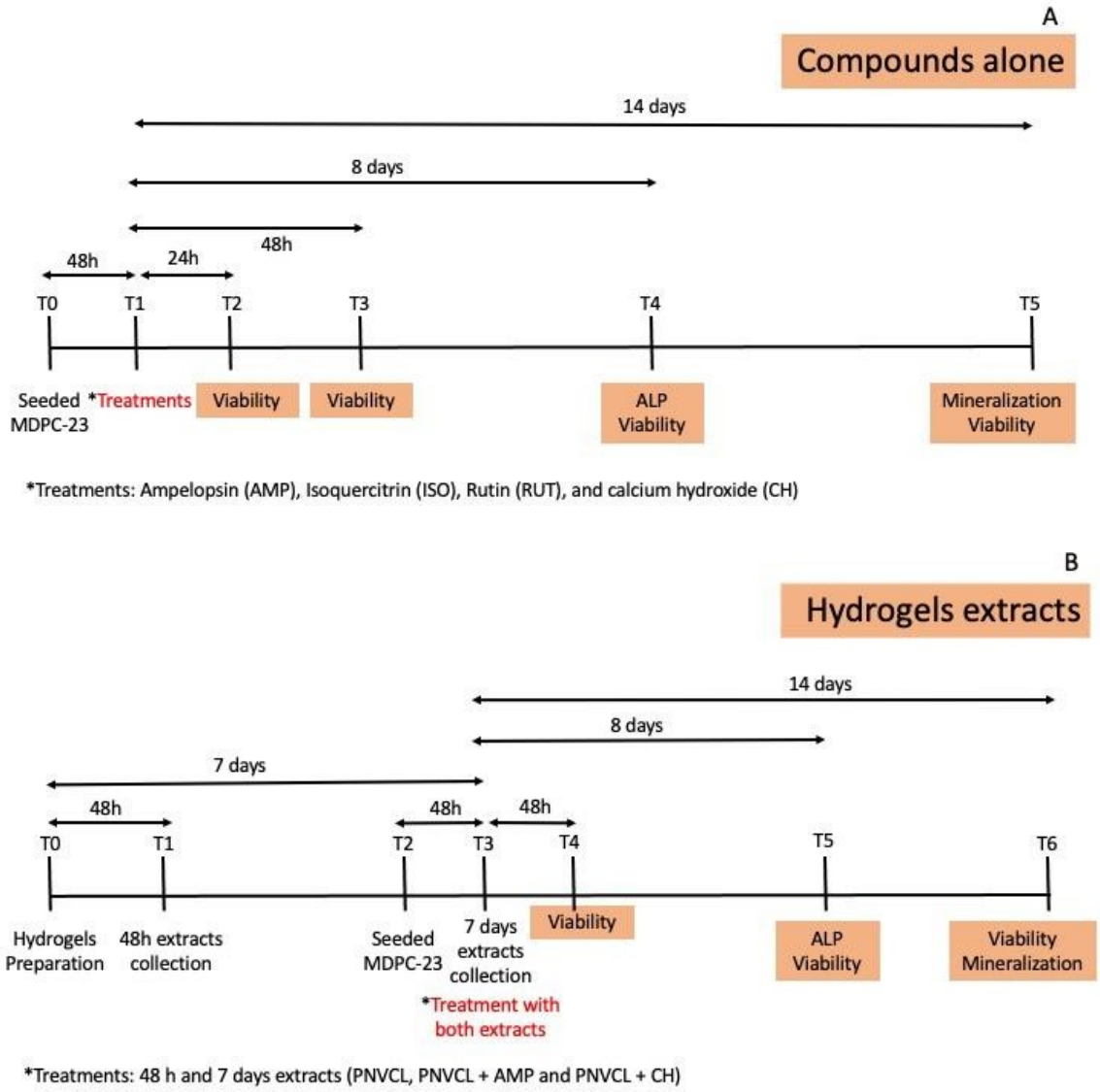

Figure 1. Experimental design of the study. A. MDPC-23 treatments with ampelopsin (AMP), isoquercitrin (ISO), rutin (RUT) and calcium hydroxide (CH). B. MDPC-23 treatments with 48h and 7days extracts of the hydrogels: PNVCL, PNVCL +AMP and PNVCL + CH.

Cell viability assays

The cell viability rate was evaluated via resazurin colorimetric assays. These assays were performed at 48 h, 8 days and 14 days after treatments. Firstly, cells were seeded into 96-well plates ($5x10^3$ cells/well) and incubated for 48h. For flavonoids screening, DMEM was aspirated and the flavonoids AMP, ISO and RUT at 100 µM, 50 µM and 25 µM were applied on cells and incubated for 48 h under standard cell culture conditions. After incubation, treatments were aspirated, and cells were maintained in osteogenic DMEM (changed every 48h) until completing 8 and 14 days. For hydrogels evaluation, 48 h and 7 days extracts (diluted from 1/2 to 1/64) of PNVCL, PNVCL+AMP and PNVCL+CH were applied to the cells and incubated for 48 h. For the subsequent assays, cells were treated with the extracts (diluted at 1/16 and 1/32) for 48 h and osteogenic DMEM until completing 8 and 14 days. After all these periods, DMEM was removed, and wells washed with sodium phosphate buffer – PBS (10 mM, pH 6.8) and stained with resazurin at 700 µM (#7017, SigmaAldrich) in DMEM for 4 h. At each time point, 100 µL of the medium was transferred to another 96-well plate for the absorbance reading at 570 and 600 nm in a spectrophotometer (Biotek, Winooski,VT). The final values were obtained by the subtractions of the absorbance values at 570 from 600 nm, and they were converted into percentage of cell viability considering the growth in DMEM mediumas 100% and the means determined for each group (Borra et al., 2009; Massunari et al., 2021).

Total protein production and Alkaline phosphatase assays

The quantification of total protein was performed according to Leite et al. 2017, with some modifications. Firstly, MDPC-23 at $3x10^2$ cells/well in 48-wells plates were treated with flavonoids (from 25 to 100 µM) or the PNVCL extracts (diluted at 1/16 and 1/32) for 48 h with subsequent osteogenic DMEM changing until completing 8 days. After that, culture medium was removed, washed with PBS and 200 µL of 0.1% sodium lauryl sulfate (Sigma-Aldrich) previously dissolved in deionized water was added to each well to lyse the cells. After the solution was left to rest 10 min at room temperature, it was homogenized and 100 µL was separated to perform the ALP activity assay. Next, Lowry reagent (Sigma-Aldrich) was added to the lysed cells, and incubated for 20 min at room temperature, followed by adding Folin-Ciocalteu's phenol reagent (Sigma-Aldrich), dilutedin deionized water at a ratio of 1:3, for 30 min. After this period, all the samples were read in a spectrophotometer (Synergy H1) to

determine absorbance values at 655 nm. A standard curve of bovine serum albumin (BSA, Sigma-Aldrich) was determined to measure the total protein of each sample (Leite at al., 2017). The alkaline phosphatase (ALP) assay was performed following the instructions of the ALP kit manufacturer (Labtest Diagnóstico S.A., Lagoa Santa, MG, Brazil). Cell lysate was mixed with 50 µL of thymolphthalein monophosphate at 22 mmol/L and 500 µL of buffersolution at 300 mmol/L (pH 10.1) and incubated for 15 min at 37 ^{o}C. After that, 2 mL of color reagentwas placed in each sample and homogenized. Absorbance was measured at 590 nm with a spectrophotometer (Synergy H1). ALP activity was calculated using a standard curve using known enzyme concentrations. The final ALP data (% in relation to the osteogenic DMEM control) were divided by the values of total protein (% in relation to the osteogenic DMEM control) to normalize the ALP results (Duque et al., 2022)

Mineralized nodule deposition

Mineralized nodule deposition was determined by alizarin red staining method. Briefly, MDPC-23 at $2x10^3$ cells/well were treated with the flavonoids or PNVCL hydrogels for 48h and osteogenic DMEMwas changed until completing 14 days. After that, cells were washed with PBS and fixed in 70% coldethanol for 2 h. Then 40 mmol/L alizarin red S (Sigma-Aldrich) was prepared in distilled water, adjusted to a pH of 4.2 with ammonium hydroxide and applied to the cells for 20 min at room temperature with gentle agitation. The cells were then washed twice with distilled water and allowed to dry. Images of the cultures stained with alizarin red S were obtained using an inverted microscope (Olympus BX51; Olympus, Miami, USA). After capturing the images, 10% of cetylpyridinium chloride solution was added to the wells and left under agitation for 15 min. 100µL of each well were transferred to a microplate reader and the absorbance values obtained at 562 nm a spectrophotometer. The results in absolute values were converted into % considering the osteogenic DMEM control as 100%. The final mineralization nodule deposition data (% in relation to the osteogenic DMEM control) were divided by the values of cell viability obtained by the resazurin method (% in relation to the osteogenic DMEM control to normalize the results (Massunari et al., 2021; Duque et al., 2022).

Statistical analysis

Data from cytotoxicity assays, ALP activity and mineralized nodules deposition were expressed

in means/standard deviation and submitted to ANOVA and Tukey tests. SPSS 19.0 software (SPSS Inc.,Chicago, IL, USA) was used to run the statistical analysis, considering $p<0.05$.

Results

Flavonoids treatments

Analysis of the cell viability

Figure 2 shows the percentage of cell viability obtained by resazurin colorimetric assays after exposure of MDPC- 23 odontoblastic-like cells to AMP, ISO and RUT at three different times 48 h, 8 days and 14 days, normalized by the positive control (DMEM 100% cell growth). Independent on the timepoint, cell viability was above 70% when cells were treated with AMP, ISO and RUT at all concentrations tested (**Figure 2A**). At 48 h, cell viability was dose-dependent only for RUT groups. On the eighth day, AMP and ISO at 100 µM induced cell growth in 114 and 118%, respectively, but only ISO at 100 µM differed from the other concentrations (**Figure 2B**). In 14 days, there was no difference among the concentrations, considering each flavonoid separately (**Figure 2C**).

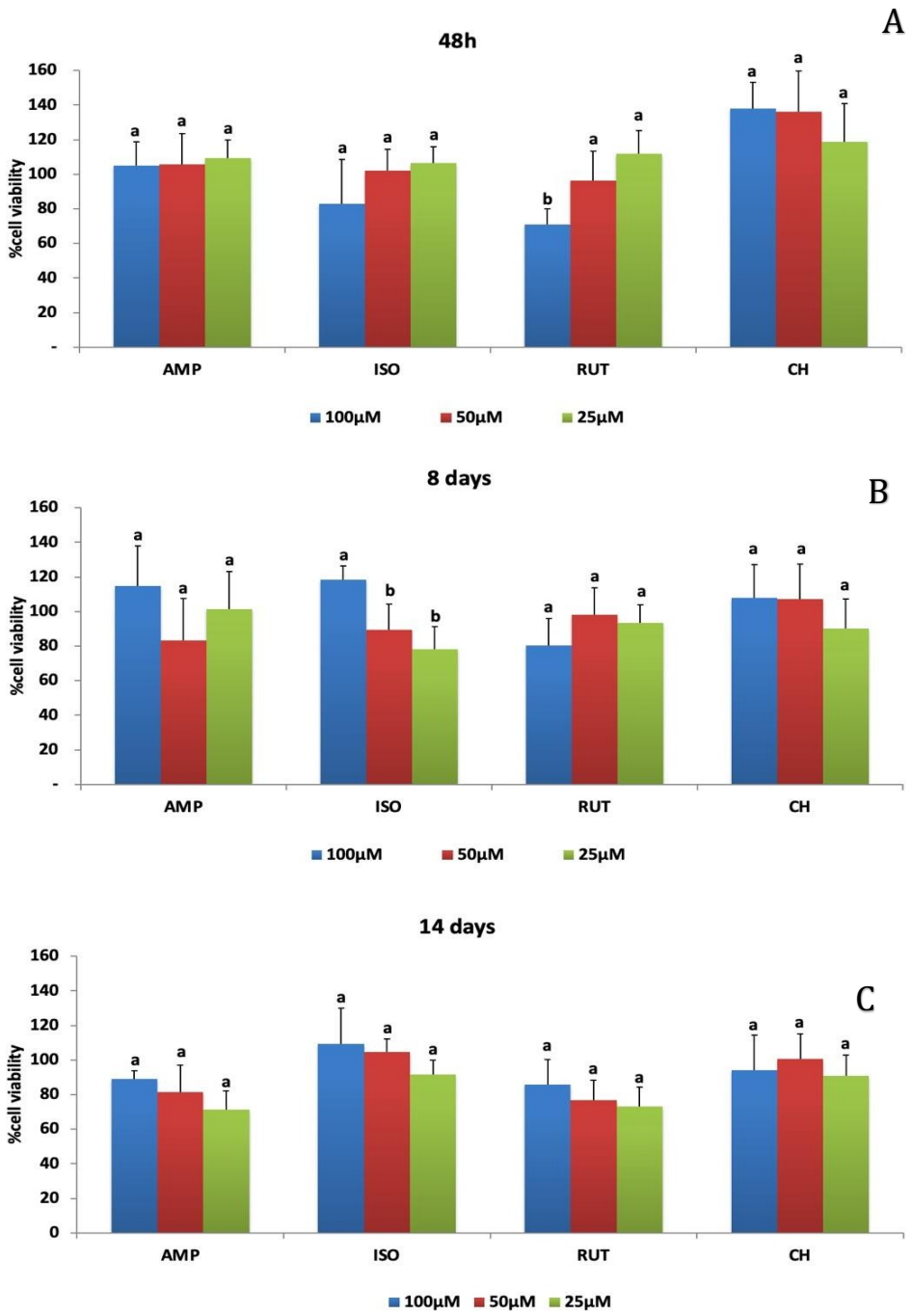

Figure 2. Percentage of MDPC-23 viability after 48h treatment with ampelopsin (AMP), isoquercetrin (ISO), rutin (RUT) and calcium hydroxide (CH) and evaluation at 48h (A), 8 days (B) and14 days (C) of evaluation. The values are expressed in means/standard deviations. [a]Different letters show statistical difference among the concentrations, considering each compoundseparately, according to ANOVA and Tukey post hoc test ($p<0.05$

Alkaline Phosphatase Activity

In **Figure 3** shows means/standard deviations of ALP activity when MDPC-23 were exposed to flavonoids for 48 h and evaluated at 8 days. Among the flavonoids tested, AMP at 100 µM stimulated the greatest ALP activity, 1.8 folds superior to control, different from AMP at 50 µM and 25 µM (1.37 and 1.25 folds, respectively). ISO at 50 µM and RUT at 100 µM increased 1.36- and 1.25-folds ALP activity in MDCP-23 cells, compared to control. CH increased ALP activity between 2.28 and 1.54 times superior to control, showing the highest results in the study.

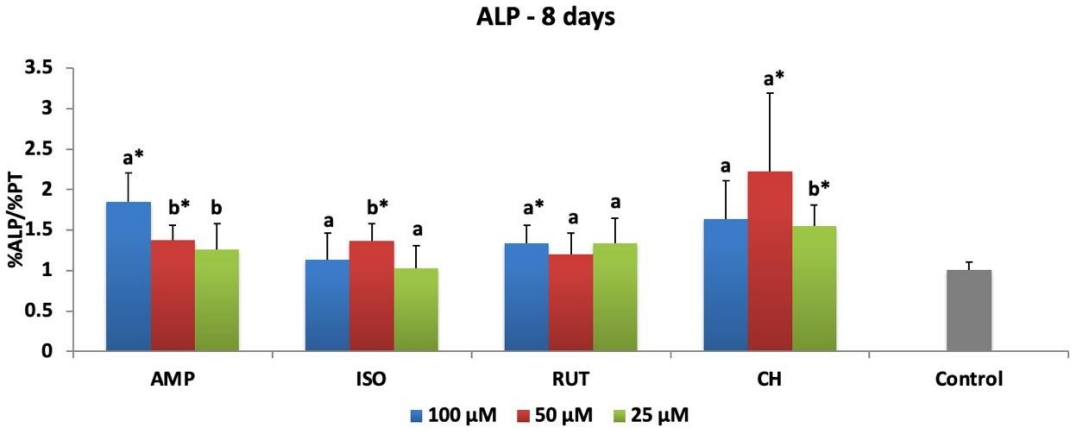

Figure 3. ALP activity of MDPC-23 cells after 48h of the treatment with ampelopsin (AMP), isoquercetrin (ISO), rutin (RUT) and calcium hydroxide (CH) and 8 days of evaluation. The values areexpressed in means/standard deviations.

[a]Different letters show statistical difference among the concentrations, considering each compoundseparately, according to ANOVA and Tukey post hoc test ($p<0.05$).

* Statistical difference from each experimental group and the control group, according to Dunnett test ($p<0.05$).

Mineralized nodule deposition

Figure 4 presents the means/standard deviations of mineralized nodules (NM) deposition after 48 h of MDPC-23 exposure to flavonoids and evaluation at 14 days. Statistical difference from the controlwas observed for all concentrations of AMP, RUT and CH, which increased 1.42-2.16 folds, 1.58-1.78folds, 1.57-1.88 folds the NM deposition after treatments. ISO at 25 µM differed from the control, increasing 1.67 folds the NM deposition.

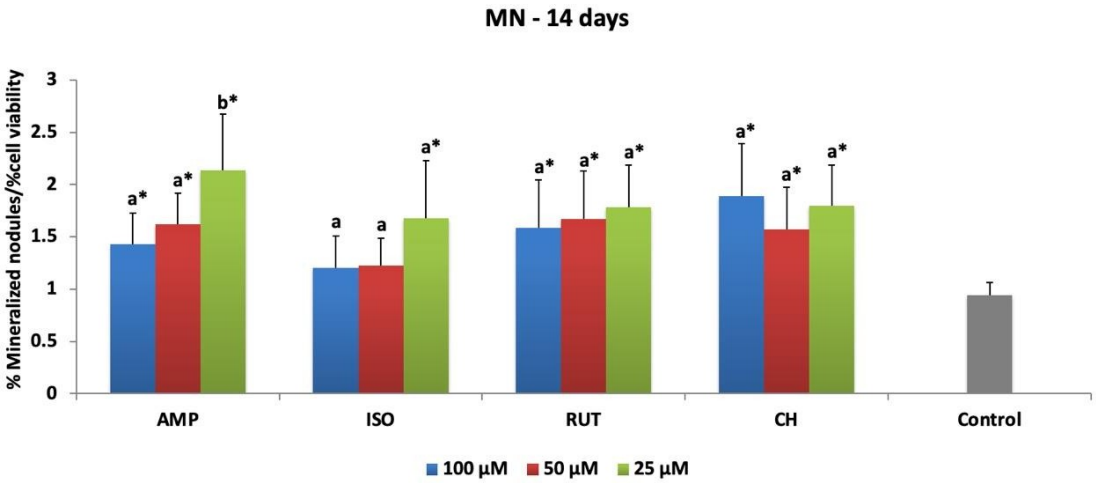

Figure 4. Mineralized nodules deposition of MDPC-23 cells at 14 days of evaluation after 48h of the treatment with ampelopsin (AMP), isoquercetrin (ISO), rutin (RUT) and calcium hydroxide (CH). Thevalues are expressed in means/standard devations.

[a]Different letters show statistical difference among the concentrations, considering each compound/combination separately, according to ANOVA/Tukey post hoc test ($p<0.05$).

* Statistical difference from each experimental group and the control group, according to Dunnett test ($p<0.05$).

Hydrogels extracts treatments

In general, among the flavonoids tested, AMP showed superior ALP activity and mineralized nodules deposition, differing from the control at their highest concentrations. For these reasons, AMP was chosen to be incorporated in PNVCL and evaluated cytotoxicity and its effects on mineralization markers in comparison to PNVCL containing calcium hydroxide.

Cell viability evaluation

Figures 5, 6 and 7 shows the percentage of cell viability after MDPC-23 exposure to 48 h and 7 days hydrogels extracts, and evaluation at 48 h, 8 days and 14 days. 48 h extract of PNVCL+AMP and PNVCL+CH hydrogels did not affect cell viability. PNVCL was cytocompatible (more than 80% cell viability) under 1/8 dilution (**Figure 5A**). **Figure 5B** shows the results of the 7-day extracts, PNVCL, PNVCL+AMP and PNVCL+CH was not cytotoxic from the 1/8 dilution. After 8 days, cell viability remained above 90% for all 48 h extracts (**Figure 6A**) and 7-

day extracts at concentrations of 1/16 and 1/32 (**Figure 6B**). **Figure 7** represents cell viability in 14 days, and it can be seen that 48 h extracts was not cytotoxic, since cell viability was superior to 70-80% for all hydrogels extracts tested (**Figure 7A**). At 7 days, there was no difference among the groups of PNVCL extracts, independent on the dilution tested, however, the percentages of cell viability ranging from % (**Figure 7B**).

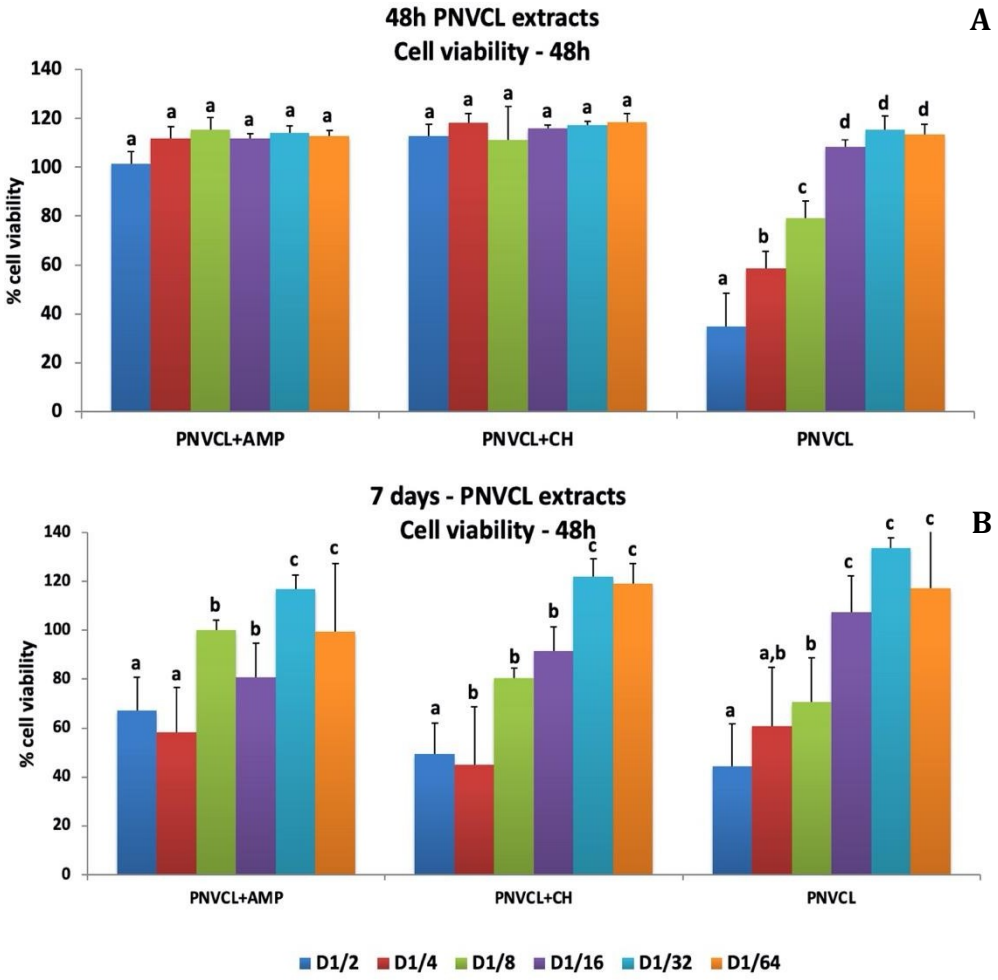

Figure 5. Percentage of MDPC-23 viability after 48 h treatments with 48h (A) and 7 days (B) PNVCL hydrogels extracts containing or not ampelopsin (PNVCL+AMP) and calcium hydroxide (PNVCL+CH).The values are expressed in means/standard deviations.

[a] Different letters show statistical difference among the concentrations, considering each PNVCL hydrogel separately, according to ANOVA and Tukey post hoc test ($p<0.05$).

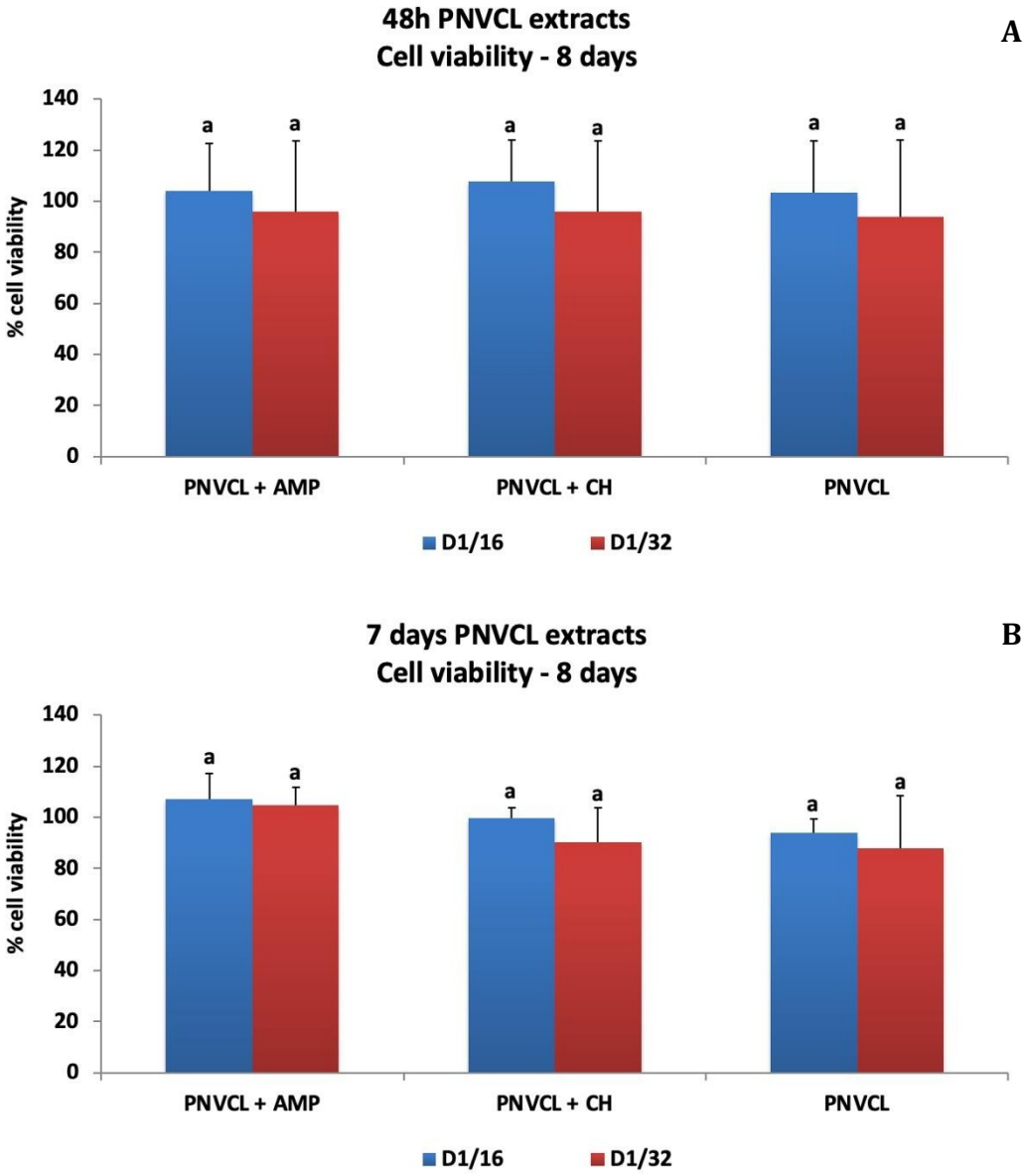

Figure 6. Percentage of MDPC-23 viability after 48h of treatments with 48h (A) and 7 days (B) PNVCL hydrogels extracts containing or not ampelopsin (PNVCL+AMP) and calcium hydroxide (PNVCL+CH)and evaluation at 8 days. The values are expressed in means/standard deviations. [a] Different letters show statistical difference among the groups, according to ANOVA and Tukey posthoc test ($p<0.05$).

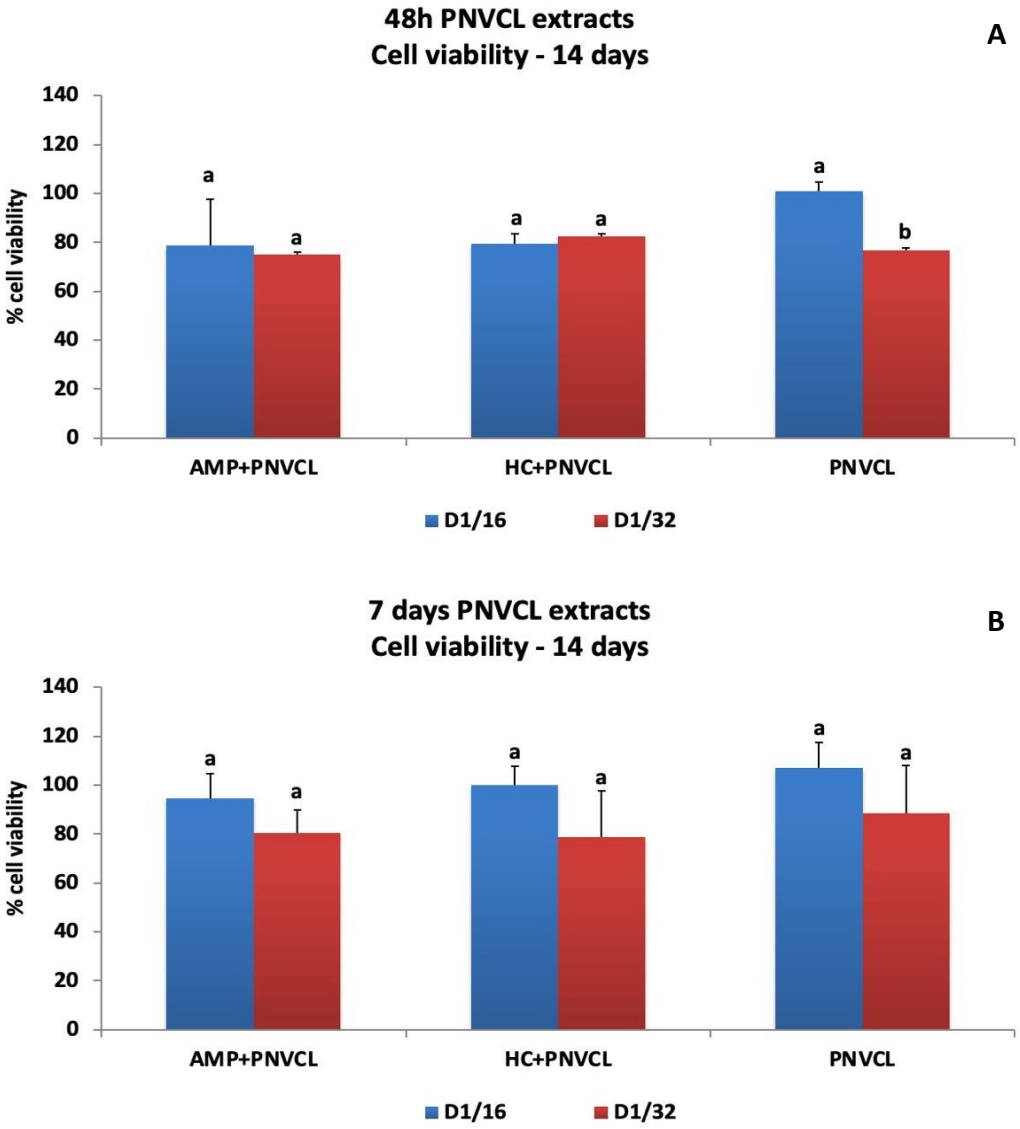

Figure 7. Percentage of MDPC-23 viability after 48h of treatments with 48h (A) and 7 days (B) PNVCL hydrogels extracts containing or not ampelopsin (PNVCL+AMP) and calcium hydroxide (PNVCL+CH) and evaluation at 14 days. The values are expressed in means/standard deviations. [a] Different letters show statistical difference among the groups, according to ANOVA and Tukey posthoc test ($p<0.05$).

Alkaline Phosphatase Activity of hydrogel extracts

The alkaline phosphatase activity of MDPC-23 exposed to 48 h extracts and 7 days extracts of the hydrogels can be seen in **Figure 8.** For 48 h hydrogels extracts, there was an increase on ALP activityfor both groups – PNVCL + AMP (1.30-1.34 folds) and PNVCL + CH (1.27-1.43 folds) without difference between the dilutions (1/16 and 1/32), when compared to the control group (**Figure 8A**). For 7 days hydrogels extracts, both dilutions of PNVCL+AMP group promoted an increase on ALP activity (1.35-1.39 folds), without difference between them, statistically superior to control. The highest ALP activity was observed for PNVCL+CH at the dilution 1/32 (2.1 folds) and different fromthe dilution 1/32 (1.37 folds) and all other groups of the study (**Figure 8B**). It was observed that PNVCL alone did not affected ALP activity, independent on the type of hydrogel extract applied on cells.

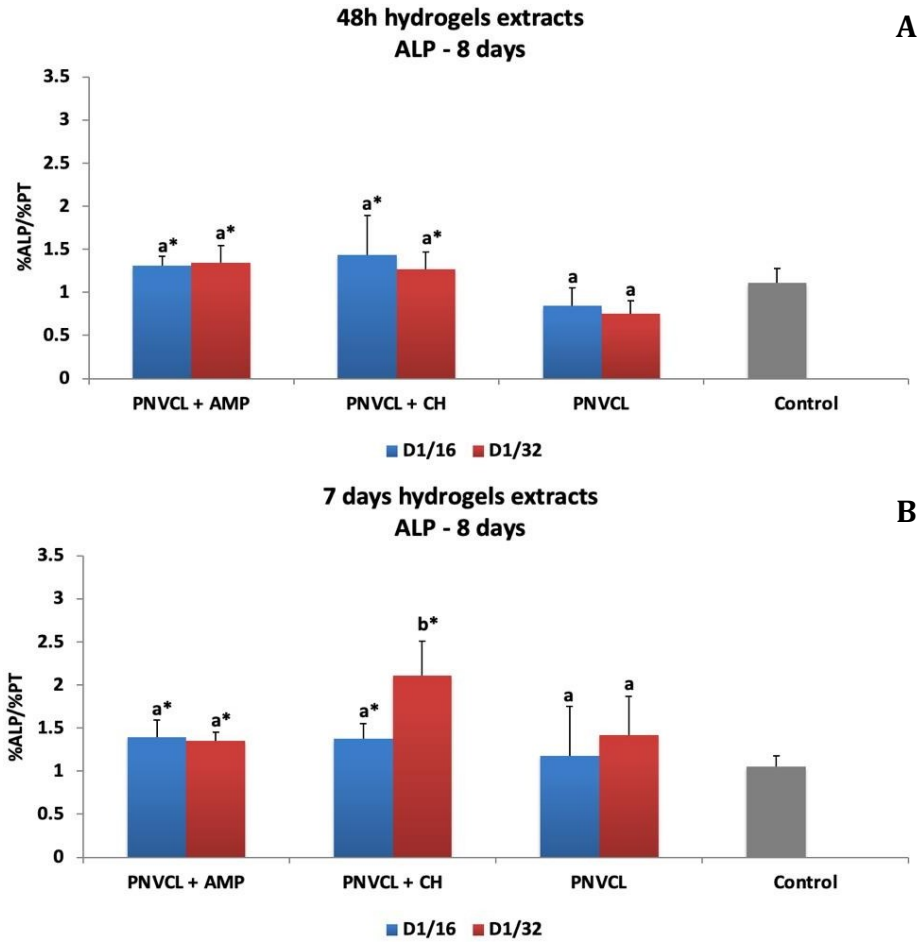

Figure 8. ALP activity of MDPC-23 cells after 48 h treatments with 48 h (A) and 7 days (B) hydrogels extracts containing or not ampelopsin (AMP) and calcium hydroxide (CH) and evaluation at 8 days. The values are expressed in means/standard deviations.

[a]Different letters show statistical difference among the concentrations, considering each PNVCL hydrogel separately, according to t Student test ($p<0.05$).

* Statistical difference from each experimental group and the control group, according to Dunnett test ($p<0.05$).

Mineralized nodule deposition of extracts

Figure 9 shows the effect of hydrogel extracts on mineralized nodules (MN) deposition by MDPC-23 cells. For 48 h hydrogels extracts, PNVCL+AMP at 1/32 dilution (2.6 folds) induced the highest MN deposition, differing from all other groups. PNVCL+CH, at both dilutions, significatively increased NM deposition (1.97-2.23 folds) (**Figure 10A**). For 7 days hydrogels extracts, PNVCL+AMP at 1/32 (2.9 folds) presented the highest values of NM deposition, without difference from the dilution 1/16 (2.24 folds), but both differed statistically from all

other groups. PNVCL+CH at 1/32 (1.78 folds) and at 1/16 (1.47) had similar results and differed statistically from the control (**Figure 10B**). Pure PNVCL hydrogel did not influence NM deposition by cell

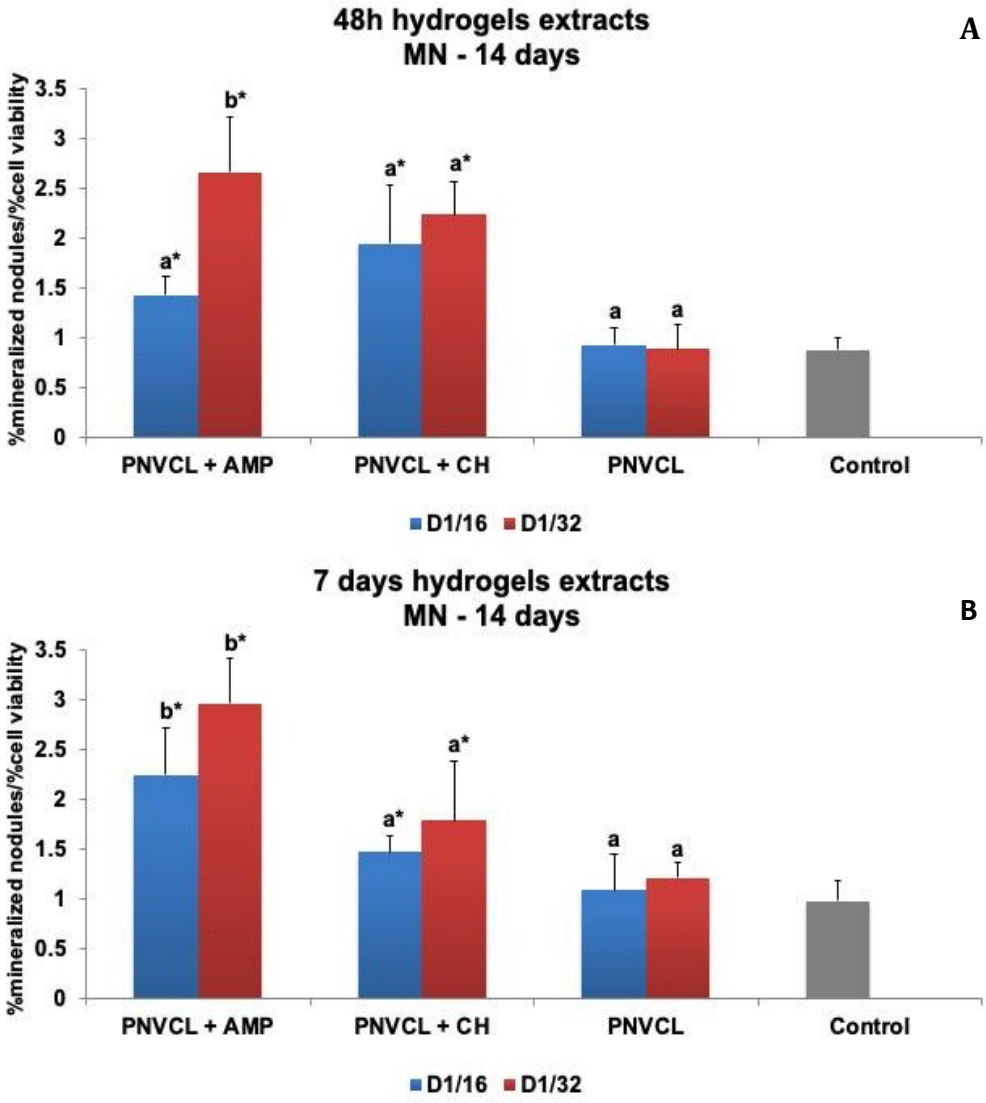

Figure 9. Mineralized nodules deposition by MDPC-23 cells at 14 days of evaluation after 48h treatments with 48h (A) and 7 days (B) hydrogels extracts containing or not ampelopsin (AMP) and calcium hydroxide (CH). The values are expressed in means/standard deviations.

[a]Different letters show statistical difference among the concentrations, considering each PNVCL hydrogel separately, according to t Student test ($p<0.05$).* Statistical difference from each experimental group and the control group, according to Dunnett test ($p<0.05$)

Discussion

Considering the wide range of therapeutic properties of the flavonoids and the search for new biomolecules for regenerative endodontic applications, this study evaluated the cytotoxicity and effect of three flavonols derivatives: ampelopsin (AMP) or dihydromyricetin and the glycosylated flavonols – isoquercitrin (ISO) and rutin (RUT) on mineralization marker of odontoblast-like cells. All flavonols were cytocompatible when applied on MDPC-23 at the concentrations tested (between 100 and 25 µM), maintaining cell viability above 70%, independent on the timepoint evaluated. AMP has demonstrated cytoprotective effects on porcine intestinal columnar epithelial cell IPEC-J2 at 20 and 40 µM, when these cells were exposed to the mycotoxin deoxynivalenol (Long et al., 2021) and increased HaCaT cells viability and suppressed UVA-induced production of inflammatory cytokines (He et al., 2016). ISO and RUT also acted as a neuroprotective agents on pheochromocytoma PC12 cells exposed to 6-hydroxydopamine at concentrations between 10 and 100 µM (Magalingam et al., 2014, 2016). Rutin has also been demonstrated as a cytoprotective flavonoid capable of attenuating the inflammatory response on macrophage cells (Huang et al. 2020), keratocytes and fibroblasts (Gęgotek et al., 2019).

Alkaline phosphatase (ALP) and mineralized nodules deposition are considered useful markers of biomineralization in osteoblast and odontoblast cells (Goldberg et al., 2011). Alkaline phosphatase (ALP) activity is the initial phase of the dentin matrix biomineralization and the formation of mineralization nodules is the final product of cell differentiation (Hoemann et al., 2009). In this present study, all flavonoids induced ALP activity and mineralized nodules, at specific concentrations. Among the flavonoids tested, AMP showed the greater effect on mineralization markers, differing from the control at their highest concentrations. AMP also exhibited no cytotoxic effect on human bone narrow mesenchymal stem cells (BMSCs) from 0.1 to 50 µM and increased ALP activity, osteoblast-specific gene expression and mineral deposition revealing enhancing osteogenic differentiation (Zhang et al., 2016). In another study, RUT induced cell proliferation from 0.1 to 100 µM concentrations and protected periodontal ligament stem cells (PDLSCs) from the damages induced by LPS. However, different from the present study, RUT at 10 µM had the greater effects on ALP activity and stimulated osteogenic differentiation of PDLSCs (Zhao et al., 2020). ISO, in this study, significantly increased ALP activity and NM deposition at 50 and 25 µM in MDPC-23

cells, different from this study, ISO demonstrated osteogenic differentiation and mineral deposition at the concentrations between 0.1 to 10 μM by MC3T3-E1 and BMSCs stem cells (Li et al., 2018, 2019).

Considering the results observed by AMP, it was chosen to be incorporated in PNVCL hydrogel (PNVCL+AMP) and evaluated for cytotoxicity and effects of hydrogels extracts on mineralization markers in comparison to PNVCL containing calcium hydroxide (PNVCL + CH). The PNVCL hydrogel is a thermosensitive polymer capable of acting as drug delivery system (Sponchioni et al., 2019). It has been considered biocompatible when exposed to different types of cells, such as human mesenchymal stem cells (MSCs), encapsulated bovine chondrocytes (Sala et al., 2017), Caco-2 and pulmonary Calu-3 cell lines (Vihola et al., 2005). In this study, 48 h and 7 days PNVCL hydrogels extracts containing or not AMP or CH were applied for 48 h and evaluated in different time points – 48 h, 8 and 14 days. At low concentrations (from dilution 1/8 to 1/32), PNVCL+AMP and PNVCL+CH extracts were cytocompatible and induced mineralization markers (at dilutions 1/16 and 1/32).

Similar to our results, other studies have shown cytocompatibility of PNVCL hydrogels (Sala et al., 2017; Parameswarn-Thankan et al., 2018; Fallon et al., 2019). Injectable thermo responsive hydroxypropyl guar-graft-poly(N-vinylcaprolactam) or HPG-g-PNVCL was studied for sustainable release of ciprofloxacin and revealed biocompatibility for being used as scaffold for osteogenic cellgrowth (Parameswarn-Thankan et al., 2018). Chondrocytes and mesenchymal stem cells were encapsulated in PNVCL hydrogels and observed cell viability higher than 80 %. In this same study, three-dimensional constructs of chondrocytes and PNVCL hydrogels demonstrated time-dependent increase in glycosaminoglycans and collagens distribution in areas of implants in vivo (Sala et al., 2017). PNVCL and itaconic acid at 02 to 1 mg/mL were synthetized and presented dual pH and temperature sensitive properties and cytocompatibility on immortalized hepatic cell line Hep G2 cells (Fallon et al., 2019). This is the first study evaluating ampelopsin loaded PNVCL hydrogel. However, therapeutic effects of AMP have enhanced when it was incorporated in different hydrogels (Dalcin et al., 2021; Lyu et al., 2022). AMP (or dihydromyricetin) was incorporated in cationic nanocapsules for safe topical use in photoprotection against UV-induced DNA damage. This system showed 99.9% protections against DNA lesion induction and cytocompatibility when appliedin 3T3 mouse fibroblasts, human fibroblasts and HaCaT human epithelial cells (at 10 μg/mL) (Dalcinet al.,

2021). Recently, AMP was added to a self-microemulsion delivery system d-α-tocopheryl polyethylene glycol 1000 succinate - quillaja saponin) and this system enhanced the absorptivity andability of AMP to prevent hyperlipidemia in mice model (Lyu et al., 2022). In view of the results presented, the flavonoid AMP demonstrated potential for clinical use in endodontic procedures, overcoming deficiencies in the materials already used. However, in vivo studies are necessary to confirm its safety and efficacy for clinical application.

In conclusion, AMP was cytocompatible and induced the highest levels of mineralized markers in MDCP-23. Low concentrations of AMP-loaded PNVCL extracts were biocompatible and able to induce ALP activity and mineralized nodules deposition.

Acknowledgements

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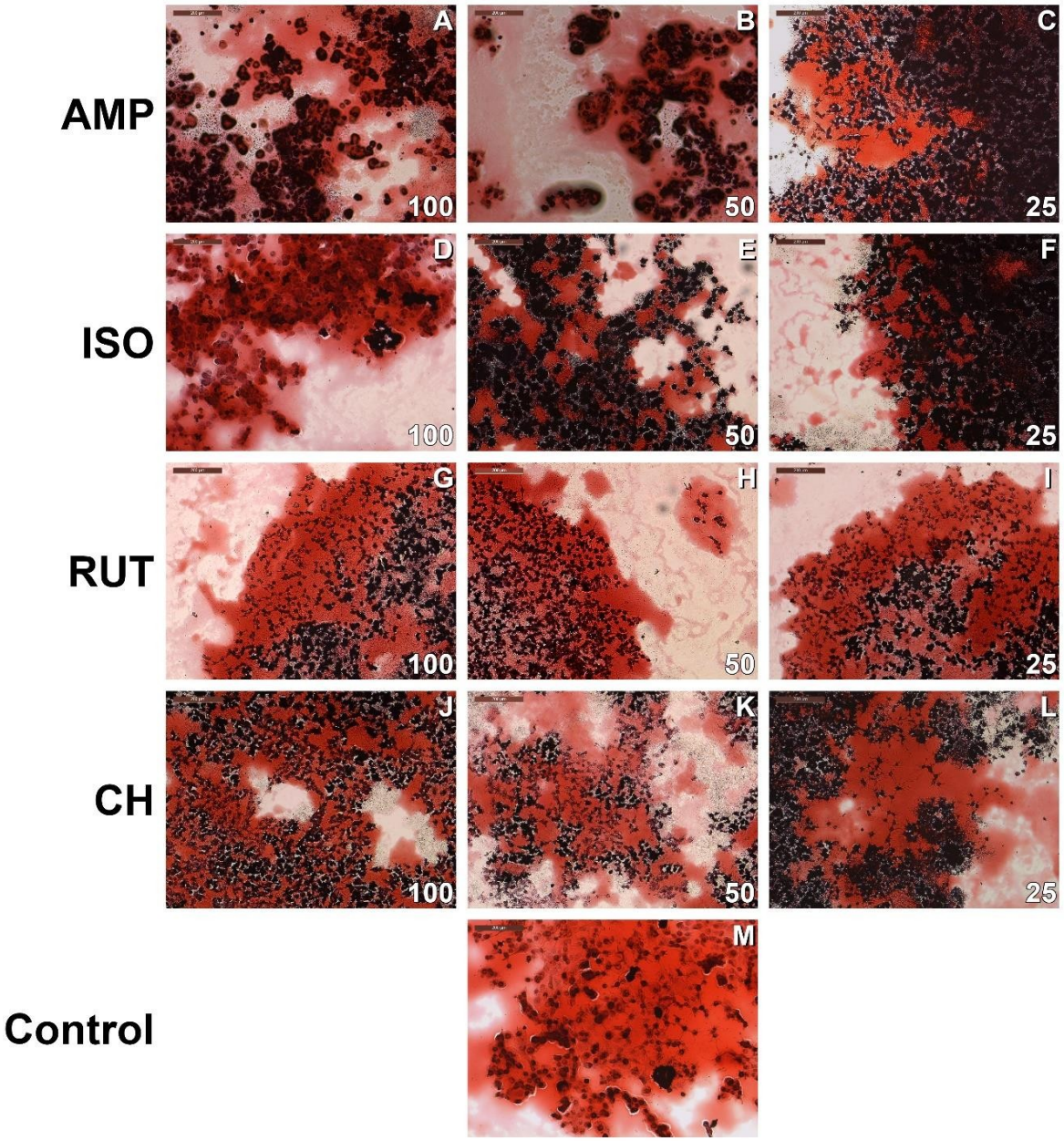
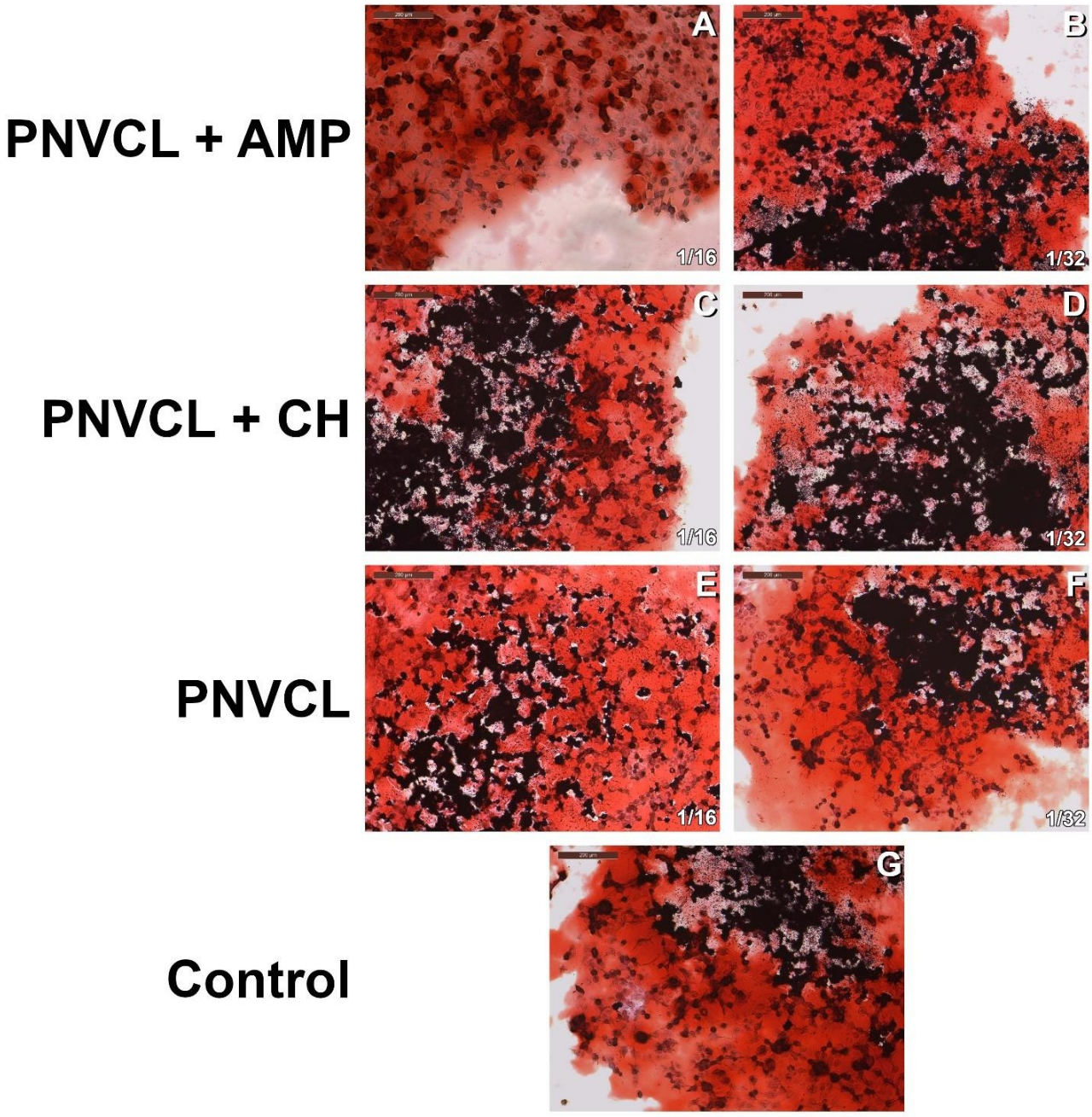
Supplementary material



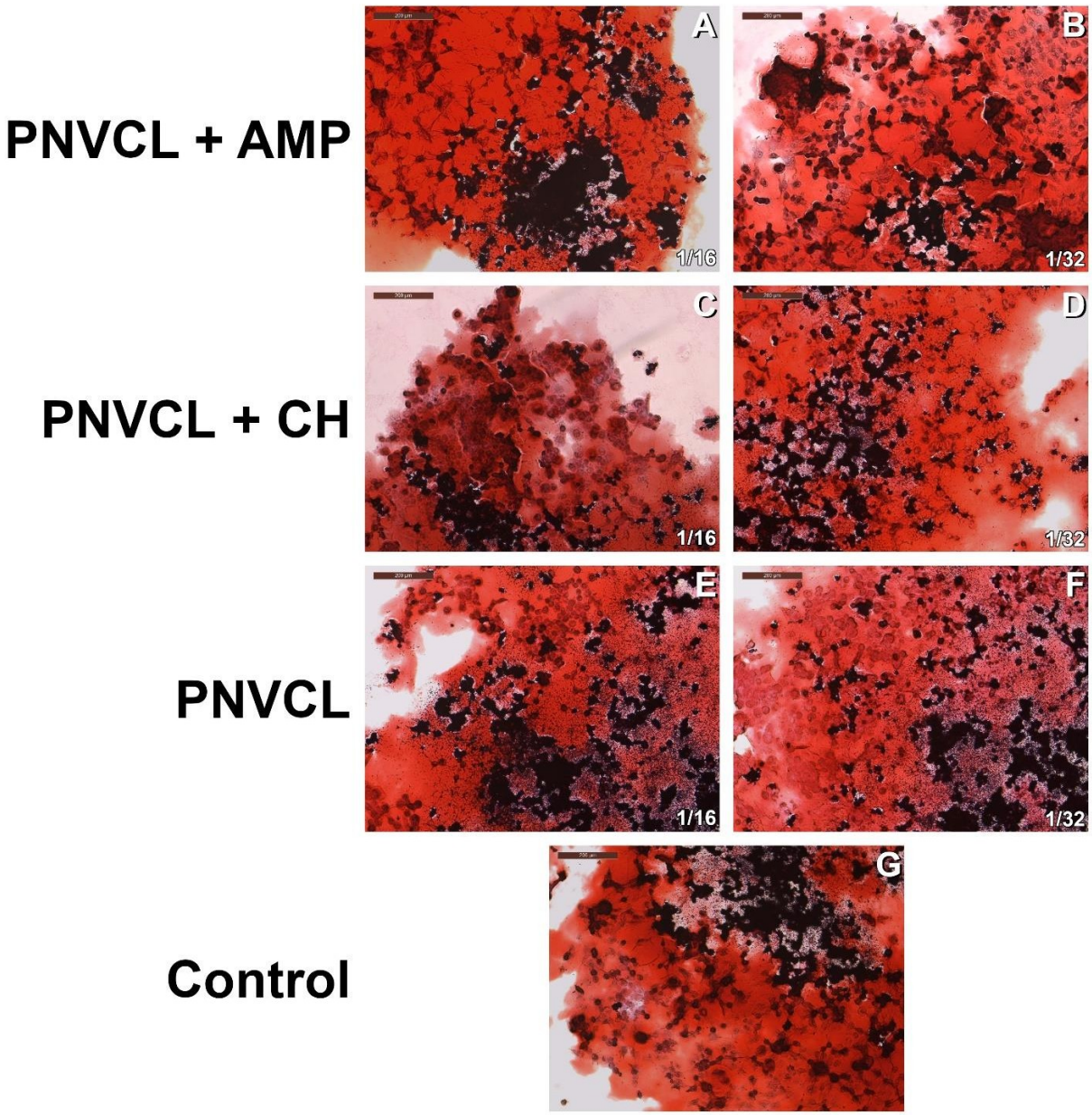
Suppl.1. Images of alizarin red staining obtained by inverted light microscope, indicating the bioactive effect of flavonoids on the MDPC-23 cells relative to mineralized nodules formations. All groups induced high nodules deposition dependent on the concentration.

AMP at 10 µM (A), AMP at 50 µM (B), AMP at 25 µM (C), ISO at 100 µM (D), ISO at 50 µM (E), ISO at25 µM (F), RUT at 100 µM (G), RUT at 50 µM (H), RUT at 25 µM (I) and CH at 100 µM (J), CH at 50 µM (K), CH at 25 µM (L) Control DMEM (M). 4x magnification.



Suppl.2. Images of alizarin red staining obtained by inverted light microscope, indicating the bioactive effect of 48 h hydrogels extracts on the MDPC-23 cells relative to mineralized nodules formations. The greatest nodules deposition can be seen in the groups PNVCL+AMP 1/32 and bothdilutions of PNVCL+CH (1/16 and 1/32).

PNVCL+AMP 1/16 (A), PNVCL+AMP 1/32 (B), PNVCL+CH 1/16 (C), PNVCL+CH 1/32 (D), PNCVL 1/16 (E), PNVCL 1/32 (F) and control DMEM (G). 4x magnification.



Suppl.3. Images of alizarin red staining obtained by inverted light microscope, indicating the bioactive effect of 7 days hydrogels extracts on the MDPC-23 cells relative to mineralized nodules formations. The greatest nodules deposition can be seen in the groups PNVCL + AMP and PNVCL + CH , both at 1/16 and 1/32 dilutions.

PNVCL+AMP 1/16 (A), PNVCL+AMP 1/32 (B), PNVCL+CH 1/16 (C), PNVCL+CH 1/32 (D), PNCVL 1/16 (E), PNVCL 1/32 (F) and control DMEM (G). 4x magnification.

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ANEXOS

REFERÊNCIAS – INTRODUÇÃO GERAL

ANEXO A

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ANEXO B

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- Open Access
- Peer Review and Ethics
- Preparing Your Paper
  - Structure
  - Word Limits
  - Style Guidelines
  - Formatting and Templates
  - References
  - Taylor & Francis Editing Services
  - Checklist: What to Include
- Using Third-Party Material
- Submitting Your Paper
- Data Sharing Policy
- Publication Charges
- Copyright Options
- Complying with Funding Agencies
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ANEXO D

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