Karina Sampaio Caiaffa

EFEITO ISOLADO OU COMBINADO DE FLAVONOIDES E PEPTÍDEOS CATIÔNICOS SOBRE BIOFILME ENDODÔNTICO E SUA INFLUÊNCIA NA VIABILIDADE CELULAR, CAPACIDADE DE MIGRAÇÃO E INIBIÇÃO DE CITOCINAS EM FIBROBLASTOS

Araçatuba – SP
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Orientadora: Profª Drª Cristiane Duque

Coorientador: Prof. Dr. Luciano Tavares Angelo Cintra

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Karina Sampaio Caiaffa

Nascimento	02.06.1989 – Petrópolis - RJ
Filiação	Gerson da Silva Caiaffa Irani Sampaio Caiaffa
2008/2013	Curso de Graduação em Odontologia pela Faculdade de Odontologia da Universidade Federal Fluminense – Polo Universitário de Nova Friburgo, FOUFF/NF.
2010/2012	Desenvolvimento de Projeto de Iniciação Científica, com auxílio do Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq.
2013/2014	Desenvolvimento de Projeto de Mestrado com auxílio do Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq.
2014/2015	Desenvolvimento de Projeto de Mestrado com auxílio da Fundação de Amparo à Pesquisa do Estado de São Paulo.
2015/2017	Desenvolvimento de Projeto de Doutorado com auxílio CAPES.
2018/2019	E Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq.
2017/2017	Desenvolvimento de Projeto de Doutorado Sanduíche com auxílio CAPES/ Programa de Doutorado Sanduíche no Exterior - PDSE.
Associações	CROSP - Conselho Regional de Odontologia de São Paulo. SBPqO - Sociedade Brasileira de Pesquisa Odontológica.

COMISSÃO EXAMINADORA

TESE PARA OBTENÇÃO DO GRAU DE DOUTOR

Profª. Drª. Cristiane Duque - Orientadora. Professora Associada do Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba, São Paulo.

Prof. Dr. João Eduardo Gomes Filho - Professor Titular do Departamento de Odontologia Restauradora, Disciplina de Endodontia da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba, São Paulo.

Profª. Drª. Thais Marchini de Oliveira Valarelli - Professora Doutora do Departamento de Odontopediatria, Ortodontia e Saúde Coletiva, Disciplina de Odontopediatria da Faculdade de Odontologia de Bauru, USP – Universidade de São Paulo, Bauru, São Paulo.

Profª. Drª. Mariana Emi Nagata - Professora Colaboradora do Departamento de Odontologia, Disciplina de Clínica Integrada Infantil I e II da UENP - Universidade Estadual do Norte do Paraná, Jacarezinho, Paraná.

Profª. Drª. Christine Men Martins - Professora Titular Universitária do Departamento de Odontologia, Disciplina de Endodontia e Clínica Odontológica da Faculdade de Odontologia de Presidente Prudente, NOESTE - Universidade do Oeste Paulista, Presidente Prudente, São Paulo.

"Feliz aquele que transfere o que sabe e aprende o que ensina." (Cora Coralina)

Dedicatória

Dedico este trabalho,

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Meus amores, vocês são tudo para mim!

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"O amor não vê com os olhos, vê com a mente; por isso é alado, é cego e tão potente."

Willian Shakespeare

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"Aprenda a viver dentro das suas possibilidades. Buscar uma vida de aparências, fora da sua realidade, só o levará para o abismo sem volta. Construa a sua vida aos poucos, lutando a cada dia e extraindo da vida o que ela tem de melhor: a Simplicidade." Chico Xavier

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"Fundamental é mesmo o amor, é impossível ser feliz sozinho." Tom Jobim

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"Ninguém cruza nosso caminho por acaso
e nós não entramos na vida de ninguém sem nenhuma razão."
"Embora ninguém possa voltar atrás e fazer um novo começo,
qualquer um pode começar agora e fazer um novo fim." Chico Xavier

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"Depois de um tempo você aprende que verdadeiras amizades continuam a crescer mesmo a longas distâncias".

Willian Shakespeare

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Karina Sampaio Caiaffa

A arte de ser feliz

Houve um tempo em que minha janela se abria
sobre uma cidade que parecia ser feita de giz.
Perto da janela havia um pequeno jardim quase seco.
Era uma época de estiagem, de terra esfarelada,
e o jardim parecia morto.
Mas todas as manhãs vinha um pobre com um balde,
e, em silêncio, ia atirando com a mão umas gotas de água sobre as plantas.
Não era uma rega: era uma espécie de aspersão ritual, para que o jardim não morresse.
E eu olhava para as plantas, para o homem, para as gotas de água que caíam de seus dedos
magros e meu coração ficava completamente feliz.
Às vezes abro a janela e encontro o jasmineiro em flor.
Outras vezes encontro nuvens espessas.
Avisto crianças que vão para a escola.
Pardais que pulam pelo muro.
Gatos que abrem e fecham os olhos, sonhando com pardais.
Borboletas brancas, duas a duas, como refletidas no espelho do ar.
Marimbondos que sempre me parecem personagens de Lope de Vega.
Ás vezes, um galo canta.
Às vezes, um avião passa.
Tudo está certo, no seu lugar, cumprindo o seu destino.
E eu me sinto completamente feliz.
Mas, quando falo dessas pequenas felicidades certas,
que estão diante de cada janela, uns dizem que essas coisas não existem,
outros que só existem diante das minhas janelas, e outros,
finalmente, que é preciso aprender a olhar, para poder vê-las assim.
Cecília Meireles

Resumo Geral

Caiaffa KS. Efeito isolado ou combinado de flavonoides e peptídeos catiônicos sobre biofilme endodôntico e sua influência na viabilidade celular, capacidade de migração e inibição de citocinas em fibroblastos. 115 f. [Tese] Doutorado em Ciência Odontológica, área Endodontia, Araçatuba: Universidade Estadual Paulista, 2019.

Resumo Geral

Este trabalho foi dividido em dois capítulos que objetivou avaliar: 1) o efeito isolado ou combinado do flavonoide epigallocatechin-3-gallate (EGCG) em associação com o peptídeo LL-37 e seu análogo KR-12-a5 sobre a viabilidade celular de fibroblastos e sobre cultura planctônica, biofilme simples, dual-espécies e túbulos dentináios e 2) as interações sinérgicas do EGCG e proantocianidina do oxicoco (A-type cranberry proanthocyanidins, AC-PAC), quando usado em combinação com LL-37 ou KR-12-a5 sobre a viabilidade celular, a capacidade de migração e inibição das citocinas em cultura de fibroblastos (HGF-1), quando estimuladas ou não pelo lipopolissacarídeo de *A. actinomycetencomitans* (LPS). No capítulo 1, a concentração inibitória mínima (MIC), a concentração bactericida mínima (MBC) e concentração inibitória fracionária (FIC) de EGCG, LL-37 e KR-12-a5 foram determinadas a partir de valores decrescentes dos compostos por meio dos métodos de microdiluição e checkerboard contra *Streptococcus mutans*, *Enterococcus faecalis*, *Actinomyces israelii* e *Fusobacterium nucleatum* após 24 horas de tratamento. Fibroblastos da linhagem L-929 foram expostos a combinações de EGCG com peptídeos em diferentes concentrações e o metabolismo celular avaliado por ensaios de MTT. Os compostos com melhor efeito antimicrobiano e citotóxico foram avaliados por 24-36h, isoladamente ou em combinação, em biofilmes individuais ou biofilmes de dual-espécies com *E. faecalis* formados em placas de poliestireno por 48h por meio de contagem bacteriana. Os biofilmes de *E. faecalis* também foram cultivados em túbulos dentinários por 2 semanas, tratados com EGCG, KR-12-a5 e EGCG + KR-12-a5 e a porcentagem de células mortas foi determinada pela análise de imagens usando Microscopia Confocal. No capítulo 2, a linhagem celular de fibroblastos gengivais humanos primários HGF-1 foi pré-tratada durante 2 h com EGCG ou AC-PAC a 25 e 12,5 µg / mL, LL-37 ou KR-12-a5 a 0,06 e 0,03 µM ou com uma combinação de EGCG + AC-PAC; AC-PAC + KR-12-a5; AC-PAC + LL-37; EGCG + KR-12-a5 ou EGCG + LL-37, nas

mesmas concentrações. As culturas celulares foram então estimuladas com 50 μg/mL de LPS por 24-48h. A viabilidade celular e migração foram analisadas usando ensaios colorimétricos e fluorescentes, respectivamente. A quantificação de citocinas foi determinada por ensaios multiplex ELISA. Os resultados mostraram que em condições planctônicas, EGCG + KR-12-a5 apresentaram efeito sinérgico ou aditivo contra todas as bactérias testadas, com FIC menor que os valores de MIC obtidos pelos compostos isolados. As combinações de EGCG e peptídeos testados não foram tóxicas para os fibroblastos, uma vez que o crescimento celular foi superior a 70%. Em condições de biofilme simples, EGCG + KR-12-a5 eliminou *S. mutans* e *A. israelii* e reduziu *E. faecalis* e *F. nucleatum*. Para biofilmes de duas espécies, quando *E. faecalis* foi combinado com *S. mutans*, EGCG + KR-12-a5 teve efeito sinérgico eliminando *S. mutans* e reduzindo estatisticamente as contagens de *E. faecalis*. Em biofilmes associando *E. faecalis* e *A. israelii* ou *F. nucleatum,* EGCG + KR-12-a5 eliminaram *E. faecalis* e promoveram redução de *A. israelii* e *F. nucleatum*, embora não tenha sido observada diferença estatística entre os compostos. EGCG + KR-12-a5 reduziu mais de 80% dos biofilmes de *E. faecalis* nos túbulos dentinários. Dentre os grupos experimentais estudados, o EGCG, principalmente a 25 e 12,5 μg/mL estimulou o crescimento de fibroblastos, protegendo-os dos efeitos do LPS. Efeito sinérgico entre EGCG + AC-PAC, EGCG + LL-37 e EGCG + KR-12-a5 no metabolismo celular também foi observado na presença de LPS. Combinações do EGCG com AC-PAC ou KR-12-a5 e AC-PAC com LL-37 foram capazes de aumentar estatisticamente a migração celular. EGCG, AC-PAC, LL-37 e KR-12-a5 promoveram a redução de citocinas individualmente ou em combinação (EGCG + AC-PAC e EGCG + KR-12-a5) mais especificamente para IL-6, IL-8, GM- CSF e TNF-α. Conclui-se que a associação de EGCG e KR-12-a5 é citocompatível e promove um efeito sinérgico contra bactérias associadas a infecções endodônticas, sob condições planctônicas e de biofilme. O EGCG, isoladamente ou associado ao AC-PAC e ao KR-12-a5, aumenta a viabilidade e migração celular, bem como a inibição de citocinas por fibroblastos estimulados por LPS. A associação de EGCG com KR-12-a5 poderia ser uma opção de princípio ativo em medicações para fins endodônticos.

Palavras-chave: Peptídeos Catiônicos Antimicrobianos, Flavonoides, Biofilmes, Citocinas.

General Abstract

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General Abstract

This study was divided in two chapters that aimed to evaluate: 1) the effect of flavonoid epigallocatechin-3-gallate (EGCG), cationic peptide LL-37 peptide and its analogue KR-12-a5, alone or in combination, on fibroblast cell viability and on bacteria in planktonic and single/dual-species biofilms/dentin tubules; 2) the synergistic interactions of EGCG and cranberry proanthocyanidins (A-type cranberry proanthocyanidins, AC-PAC), when used in combination with LL-37 or KR-12-a5 on cell viability, the ability to induce cell migration and inhibit cytokines in culture of fibroblasts (HGF-1) when stimulated or not by the lipopolysaccharide of *A. actinomycetencomitans* (LPS). For the chapter 1, Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and fractional inhibitory concentration (FIC) of EGCG, LL-37 and KR-12-a5 were determined from decreasing values of the compounds by *Streptococcus mutans, Enterococcus faecalis, Actinomyces israelii* and *Fusobacterium nucleatum* against microdilution and checkerboard after 24 hours of treatment. L-929 fibroblasts were exposed to combinations of EGCG with peptides at different concentrations and cell metabolism assessed by MTT assays. The compounds if the best antimicrobial and cytotoxic effect were also evaluated for 24-36h, alone or in combination, in 48h single- or dual-species biofilms with *E. faecalis* formed on polystyrene plates by bacterial counting. *E. faecalis* biofilms were also cultured in dentin tubules for 2 weeks and treated with EGCG, KR-12-a5 and EGCG + KR-12-a5 to determine the percentage of dead cells by analysis of images using Confocal Microscopy. For the chaper 2, primary human gingival fibroblast HGF-1 cell line was pretreated for 2 h with either EGCG or AC-PAC at 25 and 12.5 μg/mL, LL-37 or KR-12-a5 at 0.03 and 0.06 μM or with a combination of EGCG + AC-PAC; AC-PAC + KR-12-a5; AC-PAC + LL-37; EGCG + KR-12-a5 or EGCG + LL-37, at the same concentrations. Cell cultures were then stimulated with 50 μg/mL LPS for 24-48h. Cell viability and migration were analyzed using colorimetric and

fluorescent assays, respectively. Quantification of cytokines was determined by multiplex ELISA assays. The results show that in planktonic conditions, EGCG + KR-12-a5 showed a synergistic or additive effect against all the bacteria tested, with FIC lower than the MIC values obtained by the compounds alone. Combinations of EGCG and peptides tested were not toxic to fibroblasts, since cell growth was higher than 70%. Under single biofilm conditions, EGCG + KR-12-a5 eliminated *S. mutans* and *A. israelii* and reduced *E. faecalis* and *F. nucleatum*. For dual- species biofilms, when *E. faecalis* was combined with *S. mutans*, EGCG + KR-12-a5 had a synergistic effect by eliminating *S. mutans* and statistically reducing *E. faecalis* counts. In biofilms associated with *E. faecalis* and *A. israelii* or *F. nucleatum*, EGCG + KR-12-a5 eliminated *E. faecalis* and promoted reduction of *A. israelii* and *F. nucleatum*, although no statistical difference was observed between the compounds. EGCG + KR-12-a5 reduced more than 80% of the *E. faecalis* biofilms in the dentin tubules. Among the experimental groups studied, EGCG, mainly at 25 and 12.5 μg/mL stimulated the growth of fibroblasts, protecting them from the effects of LPS. Synergistic effect between EGCG + AC-PAC, EGCG + LL-37 and EGCG + KR-12-a5 on cell metabolism was also observed in the presence of LPS. Combinations of EGCG with AC-PAC or KR-12-a5 and AC-PAC with LL-37 were able to increase statistically cell migration. EGCG, AC-PAC, LL-37 and KR-12-α5 promoted cytokine reduction individually or in combination (EGCG + AC-PAC and EGCG + KR-12-a5) more specifically for IL-6, IL-8, GM-CSF and TNF-α. The association of EGCG and KR-12-a5 was cytocompatible and promoted a synergistic effect against bacteria associated with endodontic infections under planktonic and biofilm conditions. EGCG, alone or in combination with AC-PAC and KR-12-a5, increases cell viability and migration, as well as inhibition of cytokines by LPS-stimulated fibroblasts. The association of EGCG with KR-12-a5 could be an option as active principle for medications to be used for endodontic purposes.

Key words: Antimicrobial Cationic Peptides, Flavonoids, Biofilms, Cytokines.

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Introdução Geral

Introdução Geral

O tratamento de dentes permanentes jovens que sofreram danos irreversíveis devido à infecção ou trauma local antes do fechamento fisiológico normal do ápice radicular representa um verdadeiro desafio clínico. Após a erupção completa do dente na cavidade bucal ainda são necessários 3 a 4 anos para que ocorra o desenvolvimento completo dos canais e o fechamento dos ápices radiculares (Bhasker, 1991). Durante este período, se ocorrer algum tipo de trauma ou infecção, esses processos são interrompidos e o fechamento natural do ápice não acontece. As paredes dentinárias finas divergentes ou paralelas do dente imaturo dificultam a desinfecção e a execução dos procedimentos endodônticos convencionais, comprometendo o resultado a longo prazo (Rafter, 2005; Wang *et al*., 2010; Iglesias-Linares *et al*., 2013). Além disso, devido à complexa malha de canais secundários e acessórios e a persistência de algumas espécies microbianas, o preparo químico-mecânico não possibilita a total desinfecção do sistema de canais radiculares, levando à necessidade do uso de medicação intracanal (Byström e Sundqvist, 1981; Chávez De Paz *et al*., 2003).

Bactérias Gram-negativas, membros comuns das infecções primárias, são geralmente eliminadas após procedimento de tratamento químico-mecânico. Exceções podem incluir alguns bacilos anaeróbios como *Fusobacterium nucleatum*, *Prevotella* species e *Campylobacter rectus*, que estão entre as espécies encontradas em amostras após instrumentação/medicação. Entretanto, a maioria dos estudos tem revelado que as bactérias Gram-positivas são as mais frequentemente presentes nestes casos, incluindo estreptococos (*S. mitis, S. gordonii, S. anginosus, S. sanguinis, S. oralis*), *P. micra*, *Actinomyces* species (*A. israelii* e *A. odontolyticus*), *Propionibacterium* species (*P. acnes* e *P. propionicum*), *P. aeruginosa*, *P. alactolyticus*, lactobacilos, *Enterococcus faecalis*, etc (Siqueira e Rôças, 2009).

Mesmo eliminando as bactérias, diversas toxinas são liberadas no canal radicular durante a infecção e quando em contato com as células do hospedeiro levam a resposta inflamatória. Entre esses fatores de virulência, está o lipopolissacarídeo (LPS). Embora o mecanismo exato de como o LPS atua não esteja completamente elucidado, ele é reconhecido e age ativando fagócitos mononucleares (monócitos e macrófagos), que consequentemente aumentam sua atividade fagocítica e a secreção de citocinas pró-inflamatórias, tais como, fator de necrose tumoral (TNF-α), interleucinas (IL-1β, IL-6,

entre outras), além das prostaglandinas (PGE2). Sabe-se que a resposta inflamatória é uma aliada ao processo de reparo, entretanto, a secreção descontrolada dessas citocinas induz a expressão de RANKL (ligante do receptor de NF-κβ), um crítico fator de diferenciação dos osteoblastos em osteoclastos, podendo levar à destruição tecidual (Raetz e Whitfield, 2002; Walsh *et al*., 2006). Assim, uma importante característica para uma medicação intracanal é a neutralização dessas toxinas bacterianas, pois somente o preparo químico-mecânico (com hipoclorito de sódio, por exemplo) não tem se mostrado efetivo em eliminar LPS (De Oliveira *et al*., 2007).

Idealmente, a medicação intracanal deve promover a eliminação e impedir a proliferação de microrganismos remanescentes, atuar como barreira físico-química contra a infecção ou a reinfecção por microrganismos, reduzir a inflamação perirradicular, neutralizar produtos tóxicos, controlar a reabsorção dentinária inflamatória externa, além de estimular a reparação por tecido mineralizado (Siqueira e Lopes, 1999). Em casos onde o comprometimento pulpar evolui para necrose pulpar o tratamento mais indicado é realizado através da técnica de apicificação, que se baseia na indução do fechamento do forame apical, por meio da deposição de uma barreira de tecido duro, na região apical ou ainda pela indução do desenvolvimento apical, o que se encontra diretamente relacionado à manutenção da bainha epitelial de Hertwig (American Association of Endodontics 2004; Rafter 2005; Soares *et al*., 2008).

Os materiais sintéticos mais indicados para o tratamento de dentes permanentes com comprometimento pulpar/periapical são o hidróxido de cálcio (HC) e agregado de trióxido mineral (MTA). Diversos trabalhos têm mostrado que estes materiais apresentam biocompatibilidade e atividade antimicrobiana, além de estimular a apicificação em dentes permanentes jovens (Sheehy e Roberts, 1997; Shabahang e Torabinejad, 2000; Rafter, 2005, Parirokh e Torabinejad, 2010, da Silva *et al*., 2010). Porém, na técnica de apicificação com o hidróxido de cálcio (HC) ocorre um processo chamado de selamento biológico originando a formação de um tecido duro, semelhante ao cemento. Entretanto, a capacidade indutora de mineralização do HC é dependente da manutenção de sua alcalinidade durante todo o período de tratamento, onde são necessária trocas periódicas deste material para que ocorra constante renovação de suas propriedades, principalmente de sua propriedade alcalina, o tratamento pode varia de 8 – 12 meses (Soares *et al*., 2008). O fato da necessidade de múltiplas sessões pode

aumentar o risco de contaminação, além da formação de paredes dentinárias delgadas e raiz encurtadas, tornando o dente suscetível à fratura (Hargreaves *et al*., 2013).

Outra técnica utilizada para alcançar a apicificação é através de materiais à base de agregado de trióxido mineral (MTA) sendo empregada por meio da colocação de uma barreira apical artificial (plug de MTA) que permite a obturação do canal radicular na mesma sessão (American Association of Endodontists, 2004). A indicação do MTA tem sido preoconizada principalmente em casos onde há a impossibilidade de acompanhamento a longo prazo, e seu efeito tem mostrado induzir a formação da barreira apical, semelhante ao HC (Shabahang *et al.,* 1999). O emprego do MTA também tem sido associado a um menor número de fraturas (Jeeruphan *et al*., 2012), devido à redução no número de sessões durante o tratamento (Damle *et al*., 2012; Marí-Beffa *et al*., 2017).

A técnica de apicificação, seja com HC ou plug de MTA não pode ser considerada completamente biológica, pois não permite a regeneração pulpar e o desenvolvimento radicular completo, tanto em largura quanto em comprimento (Jeeruphan *et al*., 2012). Independente do material, a apicificação leva ao encurtamento da raiz e à manutenção das paredes dentinárias finas, o que aumenta o risco de fratura radicular no dente permanente jovem (Andreasen *et al*., 2002; Doyon *et al*., 2005). Além disso, o contato desses materiais sintéticos com o tecido periapical vital residual pode causar impacto negativo sobre a viabilidade das células da bainha epitelial de Hertwig, o que indubitavelmente inibirá sua capacidade de estimular as células indiferenciadas da papila apical em se diferenciar em odontoblastos (Banchs e Trope, 2004). Embora esses tratamentos frequentemente conduzam à resolução dos sinais e sintomas das doenças pulpares (Sheehy e Roberts, 1997; Bose *et al*., 2009; Parirokh e Torabinejad, 2010), promovem pouco benefício para a continuidade do desenvolvimento radicular, manutenção da nocicepção pulpar normal e defesa imune esperada para um dente permanente jovem (Ruparel *et al*., 2012; Althumairy *et al.,* 2014).

Atualmente estão surgindo novas propostas para tratamento de dentes imaturos como alternativas aos tratamentos convencionais, visando a resolução da periodontite apical e mantendo as funções fisiológicas pulpares normais. Uma pasta tripla de antibióticos (metronidazol, ciprofloxacina, minociclina) vem sendo utilizada em diversos estudos clínicos (Banchs e Trope, 2004; Thibodeaud *et al*., 2007; Jung *et al*., 2008; Ding *et al*., 2009; Taneja *et al*., 2010) alcançando bons resultados clínicos, com a eliminação

das bactérias e promoção do reparo dos tecidos periapicais. O primeiro estudo com esta pasta triantibiótica foi realizado por Hoshino *et al*. (1996) que verificaram que nenhuma dessas drogas isoladamente foi eficaz em eliminar bactérias de dentina infectada, polpas infectadas e lesões periapicais, entretanto, em combinação foram capazes de esterilizar todas as amostras. Metronidazol é um composto nitroimidazol que exibe ampla atividade antiprotozoária, antibacteriana contra bacilos Gram-negativos anaeróbios, bacilos Gram-positivos esporulados e cocos anaeróbios. Ciprofloxina é uma fluoroquinolona que apresenta ação bactericida, principalmente contra Gram-negativas. Minociclina é um derivado semi-sintético da tetraciclina com amplo espectro de ação, similar ao metronidazol (Vijayaraghavan *et al*., 2012). Uma crítica sobre o uso da pasta de antibióticos refere-se à sua resistência bacteriana. Em amostras de infecções endodônticas crônicas ou agudas, genes de resistência antibiótica foram detectados antes e após tratamento. Os genes mais prevalentes foram blaTEM (24%) (beta-lactâmicos) e ermC (24%) (eritromicina) nos abscessos e tetM (42%) e tetW (29%) (tetraciclinas) em casos assintomáticos. Outra crítica em relação ao uso da pasta tripla antibiótica é em relação ao uso da minociclina, que causa manchamento do dente, devido à sua reação com o cálcio via quelação formando um complexo insolúvel (Tanase *et al*., 1998) e ainda foi observada uma significativa redução na viabilidade de fibroblastos do ligamento periodontal após sua utilização (Yadlapati *et al*., 2014).

Produtos naturais derivados de plantas medicinais são fontes abundantes de compostos biologicamente ativos que poderiam ser base para o desenvolvimento de novos medicamentos. Em Odontologia, produtos derivados de plantas estão sendo incorporados em dentifrícios, enxaguatórios e materiais dentários, devido às suas propriedades anti-inflamatórias, antibacterianas e antifúngicas, além de não promoverem resistência antibiótica e serem biocompatíveis em concentrações terapêuticas, criando novas perspectivas biológicas integrando o tratamento odontológico (Hotwani *et al*., 2014). Estudos têm demonstrado a eficácia antimicrobiana e anti-inflamatória de extratos de plantas isolados e/ou combinados com outras substâncias naturais ou sintéticas contra patógenos da cárie dentária e periodontais (Chandra *et al*., 2015; Panche *et al.,* 2016). No caso de tratamento de dentes permanentes jovens, fitoterápicos poderiam auxiliar no controle da infecção microbiana e inflamação, estimulando a regeneração tecidual e a completa formação radicular.

Devido à natureza polimicrobiana e a consequente liberação de componentes inflamatórios nas infecções endodônticas, a combinação de drogas poderia tornar possível alcançar melhores resultados de tratamento com doses mais baixas de agentes terapêuticos (Bedran *et al*., 2014 e 2015). Fitoquímicos são moléculas promissoras que estão sendo estudadas na prevenção e tratamento de doenças bucais, pois atuam tanto na eliminação dos patógenos quanto no controle da resposta inflamatória do organismo (Palaska *et al*., 2013). Flavonoides são compostos fenólicos encontrados em células vegetais, detectados nas frutas, sementes, nozes, caules e flores, bem como nos derivados das plantas, como chás, vinho, própolis e mel e representam um constituinte comum da dieta humana (Cushnie e Lamb, 2005). Os flavonoides apresentam estrutura química comum, sendo constituídos por dois anéis aromáticos (A e B) unidos por três carbonos que formam um anel heterocíclico, denominado de anel C. De acordo com as variações no anel C, os flavonoides são divididos em seis classes principais: flavonóis, flavonas, flavanonas, flavanóis (ou catequinas), isoflavonoides e antocianidinas. Substituições dos anéis A e B originam diferentes compostos dentro de cada classe de flavonoides (Cushnie e Lamb, 2005). Diversas são as propriedades clínicas dos flavonoides já relatadas na literatura, entre elas, atividade antioxidante, inibição enzimática, atividade anti-inflamatória, atividade vascular, estrogênica, anti-tumor e atividade antimicrobiana. Assim, não é surpresa que o interesse pela pesquisa com essas substâncias tem crescido na área biomédica (Harborne e Willians, 2000).

O chá verde é composto por 24 a 40% de flavonoides, incluindo flavanóis, flavonols, flavanonas, e ácidos fenólicos (Nkhili *et al*., 2009). O galato de epigalocatequina (epigallocatechin-3-gallate, EGCG) é uma das mais abundantes e importantes catequinas, componente bioativo do chá verde (Harborne e Williams, 2000). O EGCG é produzido a partir da folha da planta *Camellia sinensis* e tem sido consumido por humanos há muitos anos (Saito *et al*., 2009). Pesquisas mostram os variados efeitos benéficos do EGCG incluindo ação anti-inflamatória, anti-tumoral, diminuição da absorção de gordura, anti-envelhecimento, prevenção e proteção de doenças cardiovasculares, efeitos imunoreguladores e neuroprotetores, além da inibição de infecção por vírus e bactérias (Chacko *et al*., 2010; Steinmann *et al*., 2013; Legeay *et al*., 2015), bem como efeito anticariogênico, auxílio no tratamento da halitose, prevenção e regressão de tumores bucais (Nawrotzki *et al*., 2012; Morin *et al*., 2015). Apresentando ainda atividade antimicrobiana contra *Enterococcus faecalis*, tanto em células

planctônicas como em biofilme (Lee e Tan, 2015). EGCG também pode inibir a adesão de *Streptococcus mutans* de maneira dose-dependente e suprimir a expressão de genes relacionados com a formação de biofilme (Xu *et al.*, 2012). Além de inibir o crescimento de agentes patogênicos bucais, o EGCG reduz a secreção de citocinas pró-inflamatórias por fibroblastos gengivais, células epiteliais, e por células endoteliais (Wheeler *et al.*, 2004; Lee *et al.*, 2009; Hosokawa *et al.*, 2010a e 2010b; Asahi *et al.*, 2014). A presença de EGCG tem reduzido significativamente, de modo dependente da concentração, a expressão de IL-6 e IL-8 em células de polpa dentária expostas ao LPS (Nakanishi *et al.*, 2010). EGCG demonstrou capacidade para inibir a produção citocinas e quimiocinas em fibroblastos, impedindo a fosforilação da MAPK / ERK e vias JNK, componentes chave na transdução de sinais celulares (Hosokawa *et al.*, 2009, 2010a e 2010b).

O *American Cranberry* (*Vaccinium macrocarpon*), também conhecido como oxicoco, é um fruto/planta que apresenta metabolismo secundários bioativos com implicação importante à saúde humana. O Cranberry é um flavonoide polifenólico conhecido por possuir atividade farmacológica e largo potencial terapêutico composto por tipos flavonóis, antocianidinas e de proantocianidinas, bem como ácidos fenólicos (Wang *et al.*, 2017). As Protocianidinas de *Cranberry* (PAC) são oligômeros de Flavan-3-ols que apresentam, além da reconhecida atividade antioxidante, propriedades imunoestimulantes, anti-carcinogênicas, antialérgicas, anti-inflamatórias, antimicrobianas e efeitos cardioprotetores (Fine, 2000). Estudos têm relatado a ação de de proantocianidinas de *cranberry* na inibição da secreção de citocinas inflamatórias pelas células imunes e das mucosas (Bodet *et al.*, 2006; La *et al.*, 2010).

A Protocianidina de Cranberry do tipo A (PAC) apresenta efeito inibitório sobre a adesão e proliferação de bactérias formadoras de biofilme dental (Koo et al., 2010; Feng *et al.*, 2013). Essa atividade biológica foi atribuída à atividade de PAC sobre fatores de virulência específicos de *Streptococcus mutans*, como a inibição da atividade de glicosiltransferases (Gtf) (Gregoire *et al.*, 2007; Yoo, Murata e Duarte, 2011). Outros estudos apontam também benefícios do *Cranberry* na prevenção e tratamento da periodontite, uma doença inflamatória de origem bacteriana que afeta os tecidos de suporte dentário, exercendo potente ação inibitória na formação de biofilme por *Porphyromonas gingivalis* e impedindo significativamente a ligação de *P. gingivalis a* superfícies revestidas com colágeno tipo I, fibrinogênio ou soro humano. Dentre os mecanismos de ação dos PAC de *cranberry* estão a inibição de enzimas proteolíticas

bacterianas, da resposta inflamatória do hospedeiro e da diferenciação e atividade de osteoclastos (Feghali *et al.,* 2011).

Outros agentes biológicos que também apresentam efeito terapêutico são os peptídeos catiônicos antimicrobianos (PCAM) quem vêm sendo estudados em diversos campos da Medicina e, atualmente, na Odontologia (Hölzl et al., 2008; Gorr e Abdolhosseini, 2011; Caiaffa *et al.,* 2017; Sierra *et al.,* 2017). Dentre os PCAMs, o peptídeo catiônico humano (hCAP-18) é a única catelicidina identificada em seres humanos isolada primeiramente em grânulos de neutrófilos. hCAP-18 é produzida também por células epiteliais do pulmão, intestino, cavidade bucal e trato urogenital, sendo encontrada no plasma seminal e plasma sanguíneo. Após a secreção, ocorre a quebra de hCAP-18 pela ação de proteases em pequenos fragmentos de peptídeos RK-31 e KS-30 e em um peptídeo ativo de cadeia longa LL-37, todos com ação antimicrobiana. Esse último fragmento do peptídeo hCAP-18, o LL-37, é um modulador multifuncional da imunidade inata, envolvendo a função antibacteriana, estímulo de angiogênese, cicatrização cutânea e quimiotaxia de células inflamatórias e do sistema imune. LL-37 atenua a produção de citocinas induzidas por LPS e a expressão de quimiocinas por fibroblastos (Jönsson e Nilsson, 2012). A ação antimicrobiana de LL-37 está relacionada à formação de poros na membrana das bactérias e a lise celular, entretanto em altas concentrações (>13µM) pode se tóxica para as células eucarióticas (Johansson *et al*., 1998; Zhang *et al*., 2008; Mccormick e Weinberg, 2010). LL-37 tem mostrado excelente ação antimicrobiana contra patógenos orais, incluindo bactérias Gram-positivas e Gram-negativas facultativas ou estritas, como *A. actinomycetemcomitans, P. gingivalis, P. intermedia, F. nucleatum, S. mutans, S. sobrinus, S. salivarius, S. sanguis, S. mitis e L. casei* (Ouhara *et al*., 2005) e fungos, como *C. albicans* (Harder *et al*., 2001; Wong *et al*., 2011). Além disso, esses PCAM tem mostrado ação antimicrobiana e atividade neutralizante de LPS de *A. actinomycetemcomitans, P. gingivalis, P. intermedia e F. nucleatum* para LL-37 (Suphasiriroj *et al*., 2013).

Devido à dificuldade e alto custo da sua síntese, diversos estudos têm proposto reduções e/ou modificações na estrutura dos PCAMs. KR-12 é o menor fragmento com similar atividade antimicrobiana do seu original LL-37 (Wang, 2008). Análogos de KR-12 foram propostos por Jacob *et al*., (2013) e apresentaram potente ação antimicrobiana contra cepas de *S. aureus* meticilina-resistentes e mantiveram a ação neutralizante contra LPS, sem causar toxicidade às células eucarióticas. Destes, o fragmento KR-12-a5

apresentou a maior atividade antiendotóxica, bastante similar à do próprio LL-37. Caiaffa *et al*., (2017) mostraram reduzido efeito citotóxico do fragmento KR-12-a5 sobre a viabilidade de fibroblastos da linhagem L-929 e reduzidos valores de MIC/MBC, mostrando sua excelente ação antimicrobiana, além de efeito sobre biofilme de *S. mutans* e *E. faecalis*, importantes microrganismos associados às infecções endodônticas.

Estudos têm apontado que a associação entre os flavonoides ou peptídeos antimicrobianos e flavonoides melhoram seus efeitos antimicrobianos e anti-inflamatórios (Harasstani *et al*., 2010; Feldman e Grenier, 2012; Bedran *et al*., 2015). A ação sinérgica entre proantocianidinas e licocalcona A, ambos os flavonoides de *cranberry* e alcaçuz, respectivamente, levou a uma redução do crescimento de biofilme de *P. gingivalis*, bem como a redução da secreção de mediadores pró-inflamatórios em macrófagos induzidos por LPS (Feldman e Granier, 2012). O único estudo encontrado que avaliou sinergismo entre flavonoides e peptídeos antimicrobianos foi conduzido por Bedran *et al*., (2015) mostrando que a combinação de LL-37 e EGCG ou proantocianidinas reduziu a secreção de várias citocinas induzida por LPS em modelo de co-cultura de células da mucosa bucal.

Baseado na filosofia de se utilizar materiais biocompatíveis com propriedades antimicrobianas, anti-inflamatórias e ainda que possam estimular a completa formação apical, novos protocolos de tratamento estão surgindo para dentes permanentes jovens (Iglesias-Linares *et al*., 2013; Moreno-Hidalgo *et al*., 2013). Assim, o presente trabalho pretende avaliar: 1) o efeito isolado ou combinado do flavonoide galato de epigalocatequina (epigallocatechin-3-gallate, EGCG) em associação aos peptídeos LL-37 ou KR-12-a5 sobre a viabilidade de fibroblastos da linhagem L-929, sobre cultura planctônica e biofilme simples e misto de bactérias de interesse endodôntico e 2) investigar o efeito sinérgico do galato de epigalocatequina (epigallocatechin-3-gallate, EGCG) e proantocianidinas do oxicoco (A-type cranberry proanthocyanidins, AC-PAC) em combinação com LL-37 ou KR-12-a5 sobre a viabilidade celular, a capacidade de migração e inibição das citocinas em cultura de fibroblastos da linhagem HGF-1, quando estimuladas ou não com LPS.

Hipóteses Nulas

Hipótese Nula 1: EGCG, isolado ou combinado com os peptídeos LL-37 e ao KR-12-a5 não apresentarão efeito inibitório sobre o crescimento planctônico e sobre biofilme de microrganismos de interesse endodôntico, apresentando efeito tóxico sobre fibroblastos da linhagem L-929;

Hipótese Nula 2: EGCG, AC-PAC, LL-37 e KR-12-a5 individualmente ou em combinação, na presença ou ausência de LPS, não influenciarão a viabilidade, a migração celular e a secreção de citocinas inflamatórias em cultura de fibroblastos gengivais humanos primários da linhagem HGF-1.

*Referências da Introdução Geral em Anexo A

Capítulo 1

Cytocompatibility and synergy of EGCG and cationic peptides against bacteria related to endodontic infections, in planktonic and biofilm conditions

Karina Sampaio Caiaffa[1,2], Vanessa Rodrigues dos Santos[2], Gabriel Flores Abuna[3], Norival Alves Santos-Filho[4], Eduardo Maffud Cilli[4], Vivien Thiemy Sakai[5], Luciano Tavares Angelo Cintra[1], Cristiane Duque[2]*

[1]Department of Endodontics, São Paulo State University (UNESP), Araçatuba Dental School, Araçatuba, São Paulo, Brazil.
[2]Department of Pediatric Dentistry and Public Health, São Paulo State University (UNESP), Araçatuba Dental School, Araçatuba, São Paulo, Brazil.
[3]Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas (UNICAMP), Piracicaba, São Paulo, Brazil.
[4]Department of Biochemistry and Chemical Technology, Institute of Chemistry (UNESP), University of São Paulo State, Araraquara, São Paulo, Brazil.
[5]Department of Clinics and Surgery, School of Dentistry, Federal University of Alfenas (UNIFAL-MG), Alfenas, Minas Gerais, Brazil.

***Corresponding author:**
Department of Pediatric Dentistry and Public Health, São Paulo State University (UNESP), Araçatuba Dental School.
Address: R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil.
Tel: (+55) 1836363315
E-mail: cristianeduque@yahoo.com.br, cristiane.duque@unesp.br

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Abstract

Introduction: The objectives of the study were to investigate the cytocompatibility and synergistic interactions of the flavonoid epigallocatechin-3-gallate (EGCG) with the peptide LL-37 and its analogue KR-12-a5, on bacteria related to endodontic infections, under planktonic and biofilm conditions.

Material and Methods: Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and Fractional inhibitory concentration (FIC) of EGCG, LL-37, and KR-12-a5 were determined against *Streptococcus mutans, Enterococcus faecalis, Actinomyces israelii, and Fusobacterium nucleatum* by microdilution and checkerboard methods, after 24h of treatment. L-929 fibroblasts were exposed to combinations of EGCG with peptides in different concentrations and cell metabolism evaluated by MTT assays. The effect of compounds for 24-36h, alone or in combination, was also evaluated on 48 h single biofilm or dual-species biofilms with *E. faecalis* formed in polystyrene plates by means of bacterial counting. *E. faecalis* biofilms were also grown in dentinal tubules for 2 weeks, treated with EGCG + KR-12-a5 and percentage of dead cells was determined by analysis of images using Confocal Microscopy.

Results: In planktonic conditions, EGCG + KR-12-a5 showed synergistic or addictive effects against all bacteria tested, with FIC lower than MIC values obtained from compounds alone. EGCG and peptide combinations tested were not toxic to fibroblasts since cell growth was higher than 70%. In single-biofilm conditions, EGCG + KR-12-a5 eliminated *S. mutans* and *A. israelii* and reduced *E. faecalis* and *F. nucleatum.* For dual-species biofilms, when *E. faecalis* was combined with *S. mutans*, EGCG + KR-12-a5 had a synergistic effect, eliminating *S. mutans* and statistically reducing *E. faecalis* counts. In biofilms associating *E. faecalis* and *A. israelii* or *F. nucleatum*, EGCG + KR-12-a5 eliminated *E. faecalis* and promoted a reduction in *A. israelii* and *F. nucleatum*, although no statistical difference was observed among the compounds. EGCG + KR-12-a5 reduced more than 80% of *E. faecalis* biofilms in dentin tubules.

Conclusion: The association of EGCG and KR-12-a5 was cytocompatible and promoted a synergistic effect against bacteria associated with endodontic infections, under planktonic and biofilm conditions, suggesting that these compounds in combination could be used as antimicrobial medication for endodontic purposes.

Keywords: EGCG; Cationic Antimicrobial Peptides; Biofilms; Cell Culture; Endodontics.

Introduction

New biological proposals are now emerging for endodontic treatment of young permanent teeth as alternatives to conventional treatments. Bioactive and multifunctional compounds with biocompatibility have been studied aiming at the resolution of endodontic infections, preserving and stimulating the continuity of root formation. Studies have demonstrated the antimicrobial and anti-inflammatory efficacy of plant extracts individually and/or in combination with other natural or synthetic substances against dental caries and periodontal pathogens (Chandra *et al*., 2015). Due to the polymicrobial nature and consequent release of inflammatory components in endodontic infections, the combination of drugs could make it possible to achieve better treatment results with lower doses of therapeutic agents (Bedran *et al*., 2015). Phytochemicals are promising molecules that have been studied in the prevention and treatment of oral diseases, since they act both in the elimination of pathogens and in the control of the inflammatory response of the organism (Palaska *et al*., 2013).

Epigallocatechin gallate (epigallocatechin-3-gallate, EGCG) is one of the most abundant and important catechins, a bioactive component of green tea (Harborne e Williams, 2000). Green tea is composed of 24 to 40% flavonoids, including flavanols, flavonols, flavanones, and phenolic acids (Nkhili *et al*., 2009). EGCG is produced from the leaf of the *Camellia sinensis* plant of green tea and has been consumed by humans for many years (Saito *et al*., 2009). Several investigations have pointed out the variety of beneficial effects of EGCG including anti-inflammatory and anti-tumoral action, prevention and protection from cardiovascular diseases, and immunoregulatory and neuroprotective effects, as well as inhibition of virus and bacterial infection (Chacko *et al*., 2010; Legeay *et al*., 2015; Song *et al*., 2017). EGCG has demonstrated antimicrobial activity against *Enterococcus faecalis*, both in planktonic cells and biofilm (Lee e Tan, 2015) and inhibited the adhesion of *Streptococcus mutans* in a dose-dependent manner by means of suppression of genes related to biofilm formation (Xu *et al*., 2012).

Antimicrobial cationic peptides (PCAM) have been studied with respect to the elimination of microorganisms in several fields of Medicine and, currently, in Dentistry (Hözl *et al*., 2008; Gorr and Abdolhosseini, 2011). Among the PCAMs, LL-37, a fragment originated from the proteolytic cleavage of the C-terminal human cationic peptide (hCAP-18), is the only cathelicidin identified in humans in neutrophil granules (Dürr,

Sudheendra and Ramamoorthy, 2006). The antimicrobial action of LL-37 is related to the formation of pores in the bacterial membrane and cell lysis, however, in high concentrations (> 13μM) it can be toxic to eukaryotic cells (Zhang *et al*., 2008; Mccormick and Weinberg, 2010). LL-37 has shown excellent antimicrobial action against oral pathogens, including facultative or strict Gram-positive and Gram-negative bacteria, such as *A. actinomycetemcomitans, P. gingivalis, P. intermedia, F. nucleatum, S. mutans, S. sobrinus, S. salivarius, S. sanguis, S. mitis,* and *L. casei* (Ouhara *et al*., 2005, Caiaffa *et al.*, 2017) as well as fungi, such *C. albicans* (Harder *et al*., 2001; Wong *et al*., 2011). In addition, LL-37 has also shown LPS neutralizing activity for *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia,* and *F. nucleatum* (Suphasiriroj *et al*., 2013).

Due to the difficulty and high cost of their synthesis, several studies have proposed reductions and / or modifications in the structure of the PCAMs. KR-12 is the smallest fragment with the same antimicrobial activity as its original LL-37 (Wang, 2008). KR-12 analogues have been proposed and presented improved antimicrobial and neutralizing action against LPS without causing toxicity to eukaryotic cells (Jacob *et al*., 2013). Of these, the KR-12-a5 fragment showed the highest anti-endotoxic activity, similar to LL-37 (Jacob *et al*., 2013). Caiaffa *et al*. (2017) demonstrated low cytotoxic effects of KR-12-a5 on fibroblast viability and potent antimicrobial and anti-biofilm action on *S. mutans* and *E. faecalis*, important microorganisms isolated from endodontic infections. Considering the therapeutic potential of both flavonoids and antimicrobial cationic peptides, some studies have explored the synergism between them. Their combination has shown increased antimicrobial and anti-inflammatory effects in low concentrations (Harasstani *et al*., 2010; Feldman and Grenier, 2012; Bedran *et al*., 2015; Guo *et al*., 2016), however no study was found evaluated the effect of these combinations on endodontics microorganisms. The objectives of the study were to investigate the compatibility on fibroblast cells and synergistic interactions of the flavonoid EGCG with the peptide LL-37 and its analogue KR-12-a5, on bacteria related to endodontic infections, under planktonic and biofilm conditions. The null hypothesis is that EGCG, individually or combined with the peptides, will not have cytocompatibility, antimicrobial activity, or an effect on biofilms of oral pathogens.

Material and Methods

Compounds and peptides synthesis (Anexo B)

EGCG (#E4143 - HPLC ≥ 95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in distilled water at a concentration of 4 mg/mL. The peptides used in this study were cathelicidin LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRI-TES) (Ji *et al.,* 2007) and KR-12-a5 (LL-37 analogue - KRIVKLILKWLR-NH2) (Jacob *et al.,* 2013). All peptides were synthesized manually at the Institute of Chemistry - UNESP, (Araraquara, SP, Brazil) following the methodology described by Caiaffa *et al.* (2017) using Fmoc (9-fluorenylmethyloxycarbonyl) protocols on Rink-amide resin. Molecular masses of peptides were estimated by mass spectrometry, using positive ion-mode electrospray ionization (ESI) apparatus (Bruker, Germany) and were in agreement with corresponding calculated values. Purification of synthesized peptides was performed in a semi-preparative HPLC Beckman System Gold on a reverse phase C18 column (2.1 × 25 cm, Phenomenex, Torrance, CA, USA). Final purity levels of peptides were determined at least 95% on a Shimadzu chromatograph equipped with an analytical C18 reverse phase column (0.46 × 25 cm, Kromasil, Bohus, Sweden).

Peptides were weighed with an analytical scale (OHAUS Corporation, Parsippany, NJ, USA) at a concentration of 4 mg/mL and dissolved in sterile deionized water. For all experiments, Chlorhexidine digluconate (CHX) (Manipullis Pharmacy, Araçatuba, SP, Brazil) was used as positive control and dissolved in sterile deionized water at 20 mg/mL. All solutions were sterilized on syringe filters using 0.2 µm Millipore membranes (Kasvi, Curitiba, PR, Brazil). Subsequent experiments were performed in triplicate, in three independent assays.

Antimicrobial activity

Microbial strains and growth conditions

The following standard strains were used for microbiological assays: *Enterococcus faecalis* (ATCC 51299), *Streptococcus mutans* (UA 159), *Actinomyces israelii* (ATCC 12102), and *Fusobacterium nucleatum* (NCTC 11326). All strains were kindly provided by the Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, São Paulo, Brazil). Microbial suspensions were prepared from culture previously grown in Mitis Salivarius Agar (Difco Laboratories, Kansas City, MO, USA) with 0.2U/mL bacitracin (Sigma-

Aldrich) for *Streptococcus mutans*, 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 Blood BHI Agar (Difco Laboratories) containing 5 mg/mL hemin, 10mg/mL menadione, 0.5% yeast extract powder (YE, Difco Laboratories), and 5% defibrinated sheep blood at 37° C in an anaerobic system (AnaeroGen, Oxoid, Hampshire, UK).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Anexo C)

MICs and MBCs were determined by the microdilution method using 96- well microplates, based on the criteria by the Clinical and Laboratory Standard Institute M7-A9 (CLSI, 2012) for bacteria and according to Mor *et al*. (1994) with modifications. After reactivation of *S. mutans, A. israelii,* and *E. faecalis* in solid medium, 5-10 representative colonies were cultured in BHI broth at 37°C for 24 h in 5% CO_2. *F. nucleatum* was grown in BHI broth containing 5 mg/mL hemin, 10mg/mL menadione, and yeast extract in an anaerobic chamber for 48 h. Microbial cultures were grown until reaching the standard optical density - OD (0.5) 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 1-5x10^6 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, in order to obtain concentrations ranging from 0.00024mg/mL to 2mg/mL. The microbial suspensions were inoculated in each well containing the previously diluted flavonoids and peptides. 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 4 h to determine cell viability. After incubation, the last blue well (MIC) 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 anaerobic bacteria for 48h 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 90% of the tested microbial strains. All experiments were performed in triplicate.

Fractional inhibitory concentration (FIC) (Anexo D)

In order to analyze the synergistic effect of EGCG with LL-37 or KR-12-a5, the microdilution method was conducted on a checkerboard, according to Tong *et al*. (2011) and Kim *et al*. (2017). Briefly, the same concentrations of EGCG (from 0.00781mg/mL to 1mg/mL), 4X concentrated from the original MIC values obtained by each bacterium, were pipetted into all lines (x-axis) of the 96-well microplates. In the columns (y-axis), peptide LL-37 or KR-12- a5, concentrated 4X from the original MIC values were added at concentrations from 0.00195mg/mL to 1mg/mL. Thereafter, bacterial cultures were added at the final concentration of 1-5x10^6 CFU/mL. The plates were subsequently inoculated at 37 ◦C for 24 h. The samples were plated onto MH agar plates to obtain the rates of cell survival. The combination values were derived from the highest dilution of the antimicrobial combination that did not show bacterial growth. The FIC index (FICI) was calculated using the following formula: FICI = (MIC of antimicrobial A in combination/MIC of A alone) + (MIC of antimicrobial B in combination/MIC of B alone). Synergy was interpreted for FICI ≤ 0.5, addictive for 0.5 < FICI < 1, indifferent for 1 < FICI < 4, and antagonism for FICI > 4.

Cell Viability assays

Fibroblast cells and growth conditions (Anexo E)

Fibroblast cells from L-929 lines 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 37ºC (Isotemp Fisher Scientific, Pittsburgh, PA, USA). Cell cultures were sub-cultured every 2 days until reaching the formation of a monolayer with an adequate number of cells for subsequent assays.

Analysis of cell metabolism

Fibroblast cells were submitted to trypsin treatment (0.25% Trypsin-EDTA-1X, Gibco, Life Technologies Inc.) for 5 min at 37ºC. Proteases were then inactivated by adding DMEM, and the cells harvested by centrifugation (3000 x *g* for 5 min), suspended in fresh medium, seeded in 96-well microplates (100μL/well, 1x10^4 cells/well) and incubated for 24h at 37°C in a 5% CO_2 atmosphere to allow cell adhesion before stimulation. After that, cells were stimulated with EGCG combinations with LL-37 or KR-12-a5 and the control CHX at concentrations from 0.00375 to 0.5 mg/mL for 24 h. The colorimetric methyl tetrazolium (MTT) assay was used to evaluate cell metabolism by succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial cell respiration.

After antimicrobial agent stimulation, the culture medium was aspirated and 90 μL of fresh DMEM and 10 μL of MTT solution (5 mg/mL in phosphate-buffered saline) (Sigma-Aldrich) were added to each well and incubated at 37ºC for 4 h. Thereafter, the culture medium with MTT solution was aspirated, and the formazan crystals, resulting from the cleavage of the MTT salt ring by the SDH enzyme, were solubilized with 200 μL/well acidified isopropanol solution (0.04 N HCl) for 30 minutes in agitation. A spectrophotometer plate reader (Eon Microplate Spectrophotometer) was used to assess cell viability proportional to the absorbance value determined by spectrophotometry at a 570 nm wavelength. The means were calculated for the groups and transformed into percentages of cell viability in relation to the negative control (DMEM), which was defined as having 100% cell metabolism (Soares *et al.,* 2014).

Anti-biofilm activity

Single-biofilm assays in polystyrene microplates (Anexo F)

EGCG, LL-37, and KR-12-a5 were selected for biofilm assays based on the results observed in the previous assays: MTT and FIC. The assays were conducted according to Massunari *et al*. (2017) and with single-biofilms of *Enterococcus faecalis (ATCC 51299), Streptococcus mutans* (UA 159), *Actinomyces israelii* (ATCC 12102), and *Fusobacterium nucleatum* (NCTC 11326). Bacterial cultures were individually centrifuged at 5000 x *g* at 4°C for 5 minutes and the supernatant discarded. The pellet was washed twice with sterile saline solution (0.9% NaCl), resuspended in broth BHI and pre-adjusted to an OD

0.5. In sterile U-shaped bottom polystyrene 96-well microplates, a pretreatment with 200µL/well of artificial saliva (composition: 800mL of deionized water, 1.6g of yeast extract, 4g of peptone, 0.28g of NaCl, 1.6g of glucose or 3.2g of sucrose, 0.16g of CaCl2, 0.16g of KCl and 0.8g of mucin) was applied for 4 hours at 37°C in a 5% CO_2 atmosphere (coating phase). After the incubation period, the saliva was removed and 200µl of each bacterial culture were inserted in each well at a dilution of 1000X (approximately 1-5x10^6CFU/mL). BHI broth supplemented with 0.5% sucrose (for *S. mutans*) or 1% glucose (the other bacterial strains). Plates were incubated at 37°C in an atmosphere of 5% CO_2 for 48 h, except for *F. nucleatum,* which was incubated for 72 hours in an anaerobic system (AnaeroGen, Oxoid, Hampshire, UK). After these periods, the culture medium was removed and the wells were washed with sterile saline (0.9% NaCl) for subsequent addition of 150µl in each well of the compounds at predefined concentrations determined from the FIC results: EGCG at 0.6 mg/mL, KR-12-a5 at 0.3 mg/mL, and a combination of both at the same concentrations. Chlorhexidine digluconate (0.05 and 0.5mg/mL) was considered as a positive control and biofilm in culture medium without antimicrobial agents as a negative control. The microplates were incubated for 24 hours under the same conditions as previously described. Then the treatments were removed and 150µl of 0.9% saline solution were added to each well for further dilution, plated in BHIA, and incubated for 48 hours. After this period, the colony forming units/mL - CFU/mL - were determined.

Dual-biofilm assays in polystyrene microplates (Anexo G)

EGCG, KR-12-a5, and EGCG + KR-12-a5 at the same concentrations used in the previous assays were evaluated on dual-species biofilm associating *E. faecalis* with *S. mutans* or *A. israelii* or *F. nucleatum* as proposed by Gao *et al*. (2016). Briefly, bacterial cultures at a dilution of 100X from OD 0.3 (1-5x10^3 CFU/mL) was mixed in equal proportions in BHI broth supplemented with 1% glucose. After 48h biofilm formation, the treatments were performed as described for single-biofilm assays and plates incubated for 36h. Aliquots from wells were plated on BHI agar for counting of total bacteria and in parallel on BHI plates containing 0.1 mg/mL (*E. faecalis* + *S. mutans*) or 0.5 mg/mL (*E. faecalis* + *A. israelii*) of cefuroxime (Sigma-Aldrich) for counting of *E. faecalis*. All plates were incubated for 24 hours for further CFU/mL counts.

E. faecalis biofilms assays on dentin tubules and analysis by Confocal Microscopy (Anexo H)

This study was approved by the Animal Committee of Araçatuba Dental School, UNESP, Brazil (FOA: 01194-2017 – Anexo J) 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 1mm below the cement-enamel junction. The roots were fixed to acrylic plates, then with two diamond discs of 0.6 mm (Isomet 5000, BuehlerLtd, LakeBluff, IL) at 1000 rpm under water irrigation using a precision saw (IsoMet 1000, Buehler, Lake Bluff, IL, USA), a 4mm 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 half-cylindrical halves with a 0.6mm 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 20s.

Dentin infection

E. faecalis cultures were centrifuged and the counting adjusted to 1-5X10^6 CFU/mL. In the microtubes with the dentin specimens, 500 µl 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 EGCG 0.6 mg/mL, KR-12-a5 0.3

mg/mL, EGCG 0.6 + KR-12-a5 0.3 mg/mL and CHX at 0.05 and 0.5 mg/mL as positive controls and sterile water as negative controls. Each sample was immersed in 350μl 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).

Analysis by Confocal Microscopy

This analysis was performed at the Piracicaba School of Dentistry – UNICAMP. 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 and 490/635 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 of 1024 by 1024 pixels. Ten-micrometer-deep scans were obtained with the CLSM from two randomly selected places. In order to analyze the Live/Dead cells 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.

Statistical analysis

In virtue of amplitude of CFU/mL counts, 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, considering each concentration separately. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analysis.

Results

Antimicrobial activity

Table 1 shows the antimicrobial effect of EGCG, KR-12-a5, and LL-37, alone or in combination, against oral bacteria. Among the experimental groups, KR-12-a5 demonstrated the highest bactericidal activity on all bacteria tested, with MIC/MBC ranging from 0.003 to 0.06 mg/mL. For LL-37, MIC/MBC ranged from 0.007 to 2 mg/mL and *E. faecalis* was the most resistant bacteria. EGCG had a bactericidal effect against all bacteria, with lowest MIC/MBC values for *A. israelii* and *F. nucleatum.* When associated with LL-37 or KR-12-a5, EGCG presented a synergic effect against *S. mutans* and *A. israelii*. However, EGCG associated with KR-12-a5 presented FIC values much lower than MIC values for compounds alone (EGCG or KR-12-a5). EGCG+KR-12-a5 also presented an addictive effect against *E. faecalis* and *F. nucleatum.* CHX, as a positive control, had the lowest MIC/MBC values for all bacteria.

Cell Viability

The cytotoxicity effect of EGCG and KR-12-a5 associations in comparison with EGCG and LL-37 combinations was evaluated on fibroblastic cell culture, as shown in **Figure 1.** Cell viability remained above 70% in all EGCG and peptide combinations, showing cytocompatibility. The highest concentrations of EGCG and KR-12-a5 in combination (EG 0.5 + KR 0.25; EG 0.25 + KR 0.125; EG 0.125 + KR 0.062) stimulated cell metabolism around 150%. CHX was the most cytotoxic compound tested in this study. Its cytotoxic effect was reduced only in concentrations below 0.007 mg/mL. The presence of 5% DMSO in the culture media did not affect cell growth.

Anti-biofilm activity on polystyrene plates

The concentration of EG+KR for the biofilm assays was 10x FIC value obtained against *E. faecalis* (EGCG 0.6 mg/mL and KR-12-a5 0.3 mg/mL), considering posterior assays with dual-species biofilms containing *E. faecalis*. The effect of EGCG and KR-12-a5, alone or in combination was observed on 48h single biofilms of *S. mutans, E. faecalis, A. israelii,* and *F. nucleatum* (**Figure 2**). *S. mutans* biofilms were completely eliminated by EGCG or KR-12-a5 or their combination. EGCG associated with KR-12-a5 significantly reduced *E. faecalis* biofilm (5.16 – 5.50 log CFU/mL), similar to CHX 0.05 mg/mL (5.64

log CFU/mL), similar to KR-12-a5 (4.39 log CFU/mL). However, KR-12-a5 associated or not with EGCG eliminated *A. israelii* biofilms, compared to EGCG and CHX, which reduced them around 2 log CFU/mL. For *F. nucleatum*, only the EGCG and KR-12-a5 combination demonstrated an antibiofilm effect and both CHX concentrations eliminated the biofilm.

The effect of EGCG, KR-12-a5, and their combination on dual-species biofilm can be observed in **Figure 3.** When *E. faecalis* was mixed with *S. mutans*, there was a significant reduction in *E. faecalis* counts. EGCG combined with KR-12-a5 reduced 3.92 log CFU/mL *E. faecalis* compared to 2.7 for EGCG and 2.9 log CFU/mL for KR-12-a5. *S. mutans* was eliminated for all groups. For *E. faecalis* and *A. israelii* dual-species biofilms, there was a significant reduction in *A. israelii* and elimination of *E. faecalis*, independent of the group. When combined with *F. nucleatum, E. faecalis* was completely eliminated by all groups. *F. nucleatum* counts were reduced in 2 log CFU/mL by EGCG and between 4-5 log CFU/mL for KR-12-a5, EGCG + KR-12-a5, and CHX 0.05 mg/mL.

Effect on biofilms in dentin tubules and CLSM analysis

The effect of EGCG, KR-12-a5 and their combination was also observed on *E. faecalis* biofilm formed in dentinal tubules, as show on **Figure 4.** There was a significant reduction on *E. faecalis* biofilm for all groups, with 37.92%, 31.09%, 88.03% of dead cells on dentinal tubules for EGCG, KR-12-a5 and EGCG+KR-12-a5, respectively in comparison with 49.72% and 89.41% for CHX 0.05 and 0.5 mg/mL. The effect of combination against *E. faecalis* biofilms was statistically higher than compounds alone and similar to CHX 0.5 mg/mL. **Figure 5** shows representative images of these groups, demonstrating superior bactericidal effect of EGCG and KR-12-a5 combination.

Discussion

The present study showed the antimicrobial effect of EGCG, LL-37, and KR-12-a5 against all bacteria tested, rejecting the null hypothesis of the study. The antimicrobial effect of EGCG has been previously tested against *E. faecalis and S. mutans,* with MIC and MBC values between 5-31.25 μg/mL and 20-62.5 μg/mL, respectively (Xu *et al.*, 2012; Lee and Tan, 2015). KR-12-a5 demonstrated an inhibitory effect on *S. mutans, A. israelii, E. faecalis, P. gingivalis, and F. nucleatum* with MIC and MBC values between 3.91 – 62.5 μg/mL (Caiaffa *et al.*, 2017). LL-37 was also tested against the same bacterial species

and demonstrated an antimicrobial effect, however, in higher concentration than its analogue KR-12-a5 with MIC/MBC between 7.81 – 2000 µg/mL (Caiaffa *et al*., 2017). The same was observed in the present study with the following sequence of antimicrobial effect: KR-12-a5> EGCG> LL-37.

In order to increase the antimicrobial effect of compounds, this study proposed the combination of EGCG with cationic peptides. Synergism between EGCG + KR-12-a5 and EGCG + LL-37 was observed for *S. mutans* and *A. israelii* (FICI = 0.5). An addictive effect was observed on *E. faecalis* for both combinations and on *F. nucleatum* only for EGCG + KR-12-a5 (FICI > 0.5 < 4). This is the first study to combine EGCG with KR-12-a5. No study was found evaluating the combination of EGCG and peptides in planktonic conditions. Previous studies have demonstrated the synergistic and addictive effect of EGCG or cationic peptides only with antibiotics. The combination of EGCG at 12.5 µg/mL with penicillin showed potent synergy against in vitro penicillinase-producing *S. aureus.* KR-12-a5 and its analogs were combined with three conventional antibiotics: chloramphenicol, ciprofloxacin, and oxacillin and demonstrated an effect against multidrug-resistant *Pseudomonas aeruginosa*, with FICI values from 0.3 to 0.5 (Kim *et al*., 2017). In the same study, LL-37 did not show synergy with any antibiotic tested, but an additive effect with chloramphenicol and ciprofloxacin, with FICI values of 0.7 and 1, respectively (Kim *et al*., 2017).

Besides the antimicrobial effect, a primordial property of a material indicated for endodontic treatment, especially for young permanent teeth, is the cytocompatibility. The wide root canal and open apex promotes more contact of materials with periapical cells, including fibroblasts. In this study, all EGCG and peptide combinations tested were not toxic to fibroblasts considering that cell growth was higher than 70%. EGCG (1 and 5 µg/m) and LL-37 (0.1 and 0.2 µM) individually and in combination have previously been tested on co-culture of fibroblasts and epithelial cells and cell viability was above 90% when compared with untreated control cells (Bedran et al., 2014). Higher concentrations of EGCG and peptides (EG 0.5 + KR 0.25; EG 0.25 + KR 0.125; EG 0.125 + KR 0.062) were considered for the present study, aiming at evaluating cell viability at concentrations that will be chosen for biofilm assays. These concentrations were not toxic to cells, stimulating cell growth around 150%.

This is the first study evaluating EGCG on mixed biofilms. Based on the antimicrobial and cytocompatibility results, EGCG (at 0.6 mg/mL) and KR-12-a5 (at 0.3 mg/mL) individually and in combination were tested on single and dual-species biofilm assays. These concentrations represent 10x the highest FIC obtained by compounds considering posterior assays with dual-species biofilms containing *E. faecalis*. EGCG + KR-12-a5 completely eliminated *S. mutans* and *A. israelii* biofilms and significantly reduced *E. faecalis* and *F. nucleatum* biofilms. In dual-species biofilms with *E. faecalis*, EGCG + KR-12-a5 also eliminated *S. mutans* and reduced *A. israelii* and *F. nucleatum.* Previous studies have evaluated the effect of KR-12 or its analogues on *S. mutans* biofilms (da Silva *et al*., 2017) and multidrug-resistant *P. aeruginosa* strains - MDRPA (Kim *et al*., 2017). [W7] KR12-KAEK, an analogue of KR-12, significantly reduced 50–100% of *S. mutans* biofilm biomass (da Silva *et al.*, 2017). KR-12-a5 and its analogs showed 4- to 8-fold increased antibiofilm activity against MDRPA compared to LL-37 (Kim *et al*., 2017). When combined with EGCG, LL-37 effectively enhanced the bactericidal activity of EGCG against *S. mutans* biofilm formation and preformed biofilms as determined by quantitative crystal violet staining and electron microscopy (Guo *et al*., 2016).

Confocal microscopy has been used as a tool for analyzing the antimicrobial effectiveness of materials on dentin tubules (Ma *et al*., 2014; Caiaffa *et al.,* 2017). In the present study, EGCG + KR-12-a5 reduced around 90% of *E. faecalis* counts in dentin tubules, more than CHX (0.05 and 0.5 mg/mL). A reduction of 70% of *E. faecalis* biofilms in dentin tubules was previously observed by KR-12-a5 at 0.5 mg/mL, similar to CHX at the same concentration. The ability of *E. faecalis* to produce biofilms is primordial for the resistance of this species inside dentin tubules (Caiaffa *et al*., 2017). A non-piliated mutant of *E. faecalis* was unable to produce biofilm, demonstrating the importance of pili for biofilm formation in enterococci (Budzik and Schneewind, 2006). Other virulence factors are related to the ability of *E. faecalis* to survive a range of stresses and hostile environments, including variations in temperatures (5-65° C) and pH (4.5-10.0) (van den Berghe *et al*., 2006). Temperature resistance is associated with membrane composition, rich in lipid and fatty acids. The survival of *E. faecalis* in an extreme pH has been associated with relative impermeability to acid and alkali and the presence of membrane-bound H^{+} -ATPase activity (Nakajo *et al.,* 2005). When *E. faecalis* was

associated with other oral species in multi-species biofilm, such as *F. nucleatum*, a significant reduction in the number of *E. faecalis* cells present was observed, which is possibly related to reduction in biological space (nutrient source, space for adherence) available for *E. faecalis* to be present in the same number (Yap *et al*., 2014).

The antimicrobial action of cationic peptides, such as LL-37, can occur by membrane permeabilization in a nonpore carpet-like mechanism of action. The detection of transmembrane pores seems also to be induced by LL-37 (Lee *et al*., 2011). Electrostatic and hydrophobic interactions between peptides and bacteria can facilitate the penetration of peptides into cell membranes (Nguyen *et al*., 2011). The increase in the ratio between hydrophobicity and the charge of peptides improves antimicrobial effects, as observed for KR-12-a5 and its analogues (Jacob *et al.,* 2013; Caiaffa *et al.,* 2017), which caused depolarization of the cytoplasmic membrane faster than that seen with LL-37 (Kim *et al.,* 2017). EGCG presents a different mechanism of action dependent on the bacteria. EGCG has been demonstrated to induce damage to the cell wall and interference with its biosynthesis through direct binding with peptidoglycan in Gram-positive bacteria and in Gram-negative bacteria can go through the outer membrane via porin pores (Yoda *et al*., 2004; Cui *et al*., 2012). In the present study, the synergism between EGCG and KR-12-a5 could be explained by the combination of different mechanisms of action, promoting selective bacterial damage even in low concentrations. Our study confirms that combination therapy is preferred over monotherapy in treating biofilm-related diseases, such as endodontic infections.

Conclusion

The association of EGCG and KR-12-a5 was cytocompatible and promoted a synergistic effect against bacteria associated with endodontic infections, under planktonic and biofilm conditions, suggesting that these compounds in combination could be used as antimicrobial medication for endodontic purposes.

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Results

Table 1. MIC, MBC and FIC values (mg/mL) obtained for EGCG, KR-12-a5 and LL-37 against oral bacteria.

Compounds (mg/mL)	*E. faecalis*	*S. mutans*	*A. israelii*	*F. nucleatum*
EGCG MIC (MBC)	0.25 (1)	0.25 (0.5)	0.03 (0.06)	0.03 (0.03)
KR12-a5 MIC (MBC)	0.06 (0.06)	0.003 (0.007)	0.003 (0.003)	0.007 (0.007)
LL-37 MIC (MBC)	2 (2)	0.25 (1)	0.007 (0.007)	0.25 (0.25)
EGCG + KR-12-a5 FIC	0.06 EGCG 0.03 KR-12-a5 FICI = 0.7	0.06 EGCG 0.001 KR-12-a5 FICI = 0.5	0.0002 EGCG 0.002 KR-12-a5 FICI = 0.5	0.03 EGCG 0.002 KR-12-a5 FICI = 1
EGCG + LL-37 FIC	0.06 EGCG 1 LL-37 FICI = 0.7	0.06 EGCG 0.06 LL-37 FICI = 0.5	0.007 EGCG 0.002 LL-37 FICI = 0.5	FICI > 1
CHX MIC (MBC)	0.007 (0.015)	0.00024 0.007	0.0009 (0.0009)	0.001 (0.001)

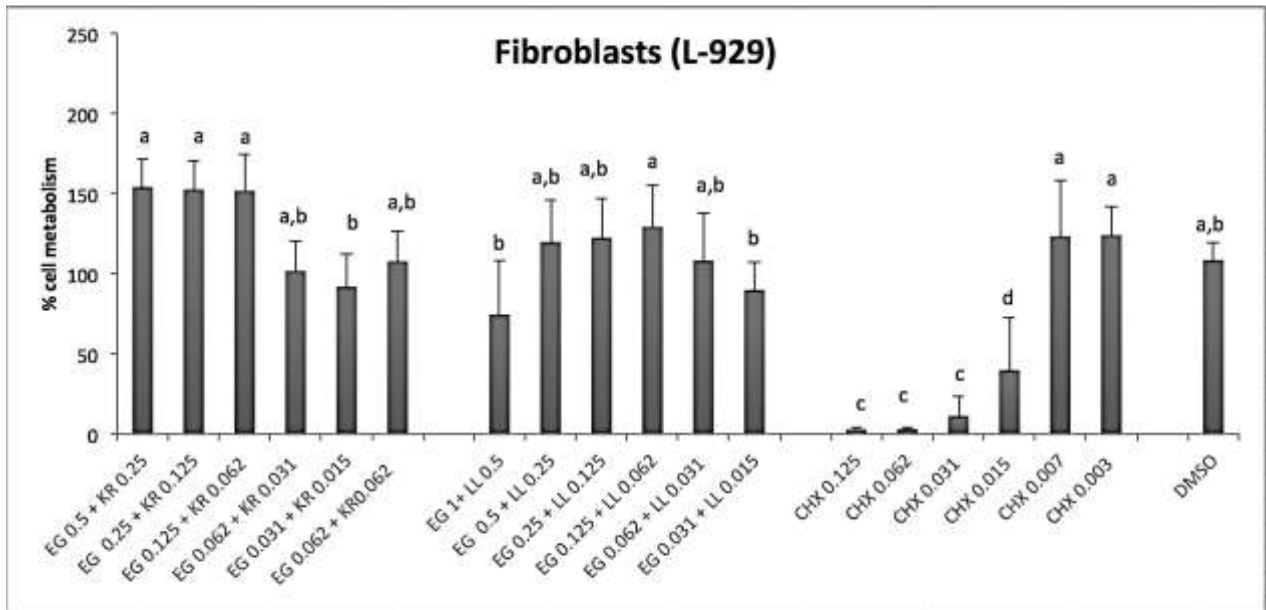

Figure 1. Effect of EGCG combinations with the peptides KR-12-a5 and LL-37 on the viability of fibroblasts (L-929). The results are expressed in means/SDs. Means were calculated for the groups and transformed into percentage of cell viability in relation to negative control (untreated cells), which was defined as having 100% cell metabolism. DMSO: culture media with 5% DMSO.

[a] Different lower letters show statistical differences among all groups, considering each concentration separately, according to ANOVA and Tukey tests ($p<0.05$).

*EG = EGCG; KR = KR-12-a5; LL = LL-37 and CHX = Chlorhexidine digluconate. The concentrations of compounds are expressed in mg/mL.

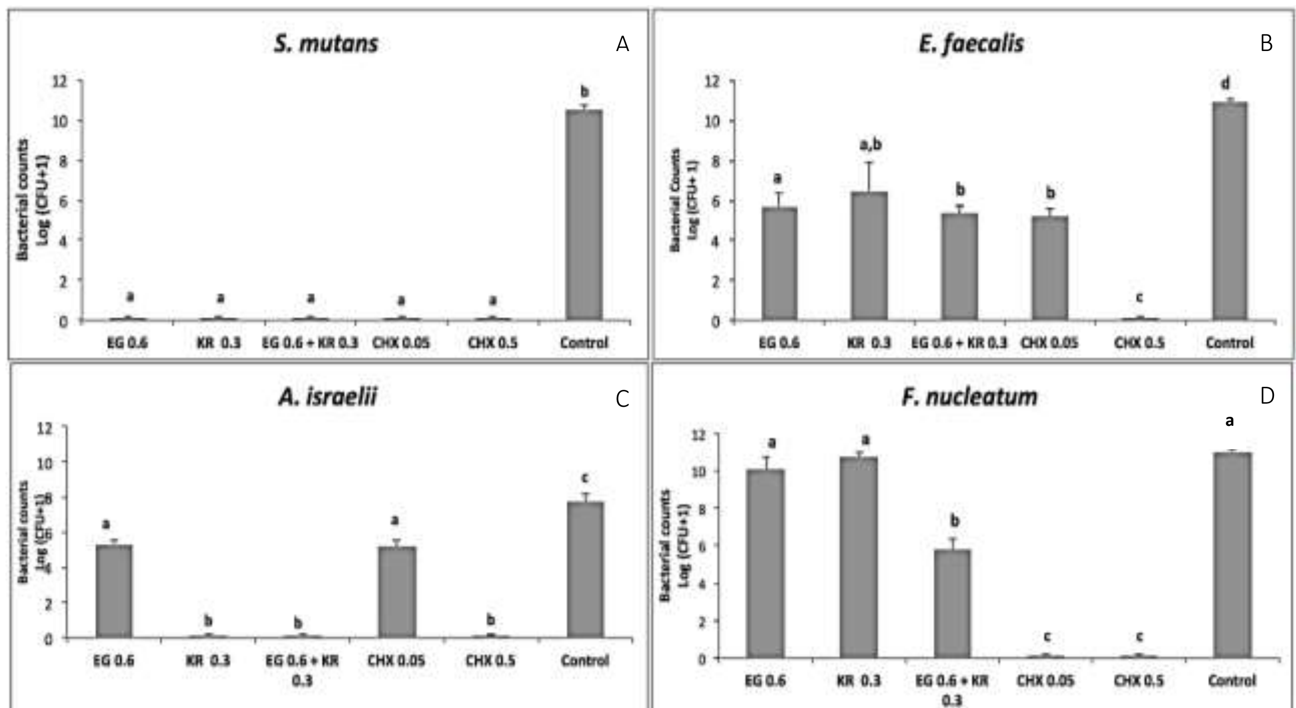

Figure 2. Effect of EGCG and KR-12-a5 treatments, alone or in combination, on 48 h-single species biofilm of *S. mutans* (A), *E. faecalis* (B), *A. israelii* (C) and *F. nucleatum* (D) for 24 h. Results are expressed in means/SDs of bacterial counts (transformed in Log (CFU+1)). Control: untreated cells.

[a] Different lower letters show statistical differences among all groups, considering each concentration separately, according to ANOVA and Tukey tests ($p<0.05$).

*EG = EGCG; KR = KR-12-a5 and CHX = Chlorhexidine digluconate. The concentrations of compounds are expressed in mg/mL.

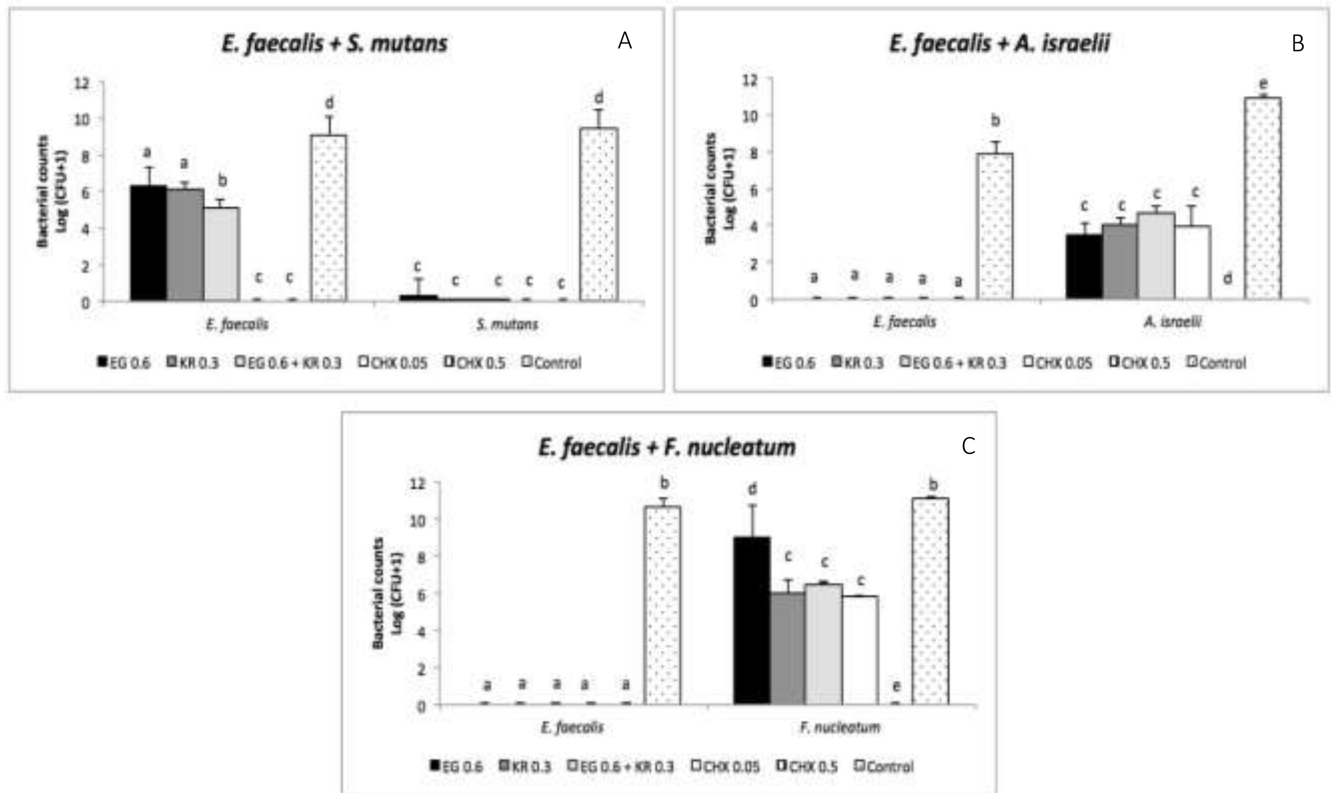

Figure 3. Effect of EGCG and KR-12-a5 treatments, alone or in combination, on 48 h dual-species biofilm of *E. faecalis* associated with *S. mutans (A), A. israelii* (B) and *F. nucleatum* (C) for 36 h. Results are expressed in means/SDs of bacterial counts (transformed in Log (CFU+1)). Control: untreated cells.

[a] Different lower letters show statistical differences among all groups, considering each concentration separately, according to ANOVA and Tukey tests ($p<0.05$).

*EG = EGCG; KR = KR-12-a5 and CHX = Chlorhexidine digluconate. The concentrations of compounds are expressed in mg/mL.

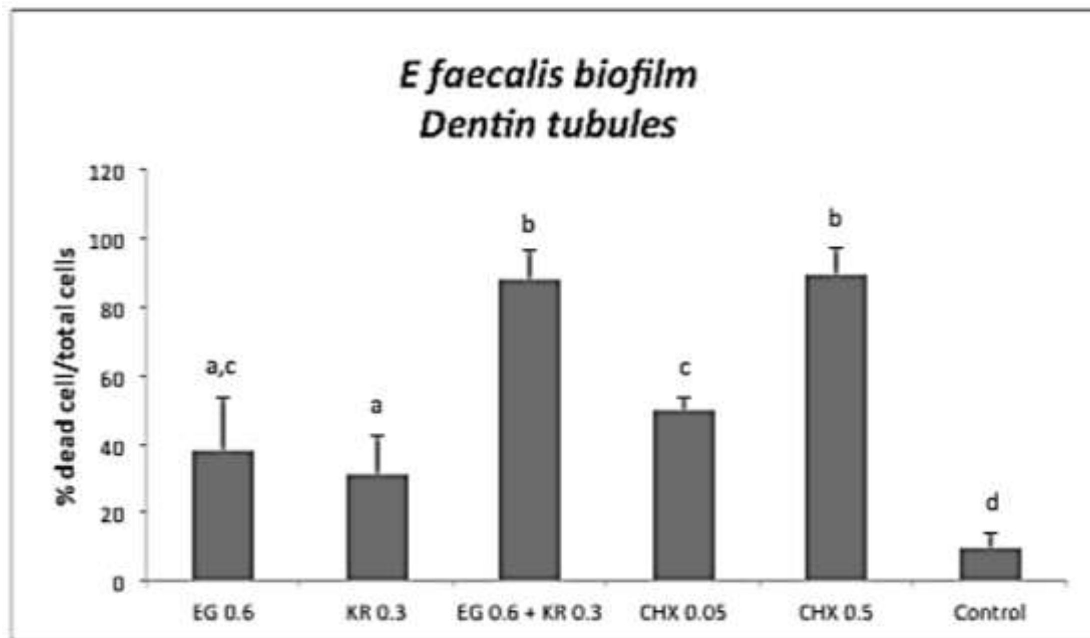

Figure 4. Proportion of dead cells obtained after CLSM analysis of *E. faecalis* biofilms on dentin blocks after exposure to EG, KR, EG+KR and CHX. The results are expressed as means/SDs. Control: untreated cells.

[a] Different lower letters show statistical differences among all groups, considering each concentration separately, according to ANOVA and Tukey tests ($p<0.05$).

*EG = EGCG; KR = KR-12-a5 and CHX = Chlorhexidine digluconate. The concentrations of compounds are expressed in mg/mL.

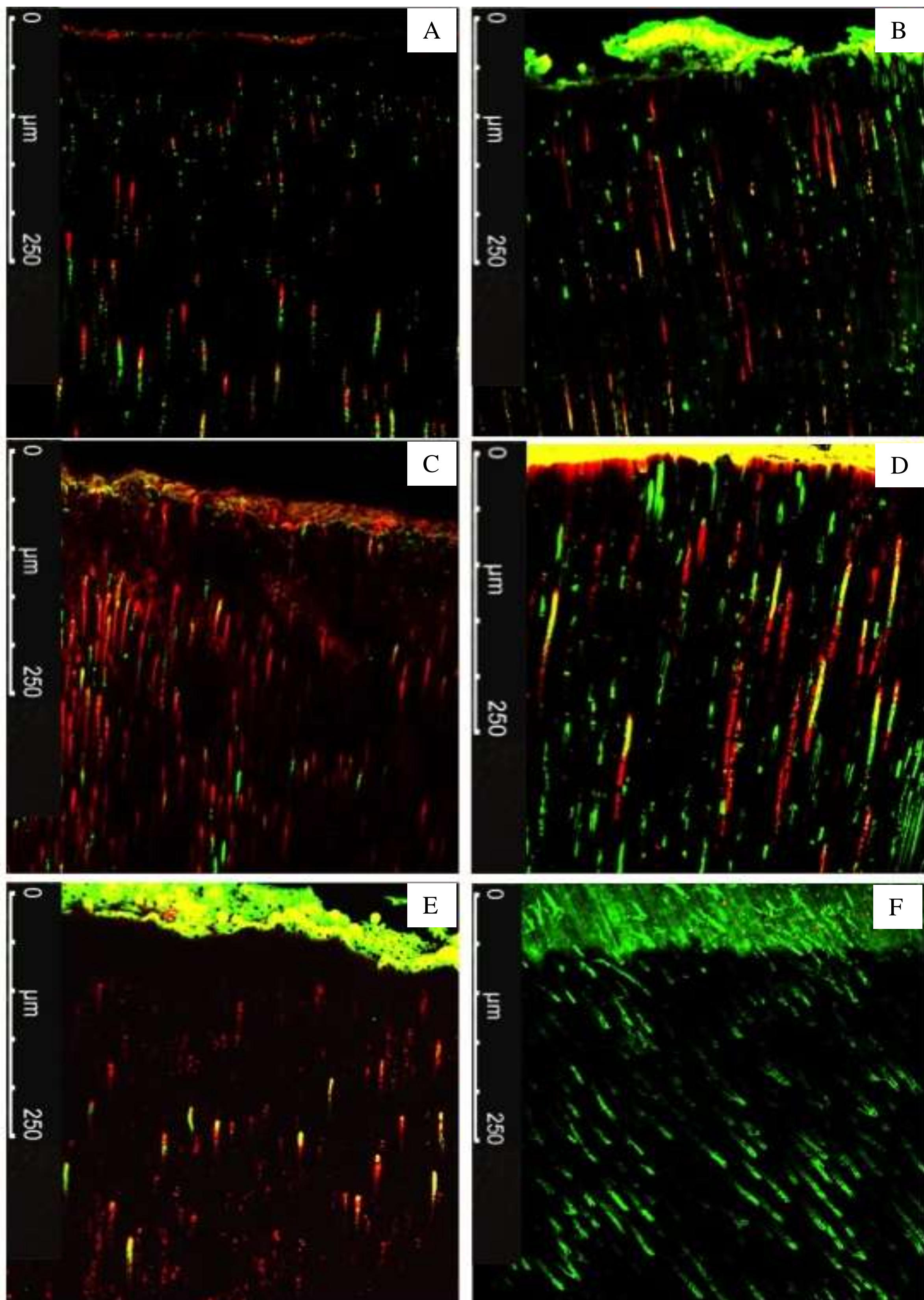

Figure 5. Representative 2-D CLSM images of *E. faecalis* biofilms on dentin blocks exposed to (A) EGCG 0.6 mg/mL; (B) KR-12-a5 0.3 mg/mL; (C) EGCG 0.6 mg/mL + KR-12-a5 0.3 mg/mL; (D) CHX 0.05 mg/mL; (E) CHX 0.5 mg/mL and (F) Control *E. faecalis*.

Capítulo 2

Synergistic effect between flavonoids and cationic peptides on cell viability, migration capacity and cytokine inhibition in LPS-stimulated fibroblasts

Karina Sampaio Caiaffa[1], Amel Bem Lagh[2], Daniel Granier[2], Juliana Nascimento Santos[2], Cristiane Duque[3]*

[1]Department of Endodontics, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba, São Paulo, Brazil.
[2]Oral Ecology Research Group, Faculty of Dentistry, Universite´ Laval, Quebec City, QC, Canada.
[3]Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba, São Paulo, Brazil.

***Corresponding author:**
Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP)
Address: R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil
Tel: (+55) 1836363315
E-mail: cristianeduque@yahoo.com.br, cristiane.duque@unesp.br

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Abstract

Introduction: Studies have pointed out that the synergism between flavonoids or flavonoids and antimicrobial peptides improves their therapeutic effects. This study aimed to investigate the synergistic interactions of EGCG and A-type cranberry proanthocyanidins (AC-PAC) when used in combination with LL-37 or KR-12-a5 on cell viability, migration capacity, and inhibition of cytokines in culture of fibroblasts stimulated with LPS.

Material and Methods: Primary human gingival fibroblast cell line HGF-1 was pre-treated for 2 h with EGCG or AC-PAC at 25 and 12.5 μg/mL, LL-37 or KR-12-a5 at 0.06 and 0.03 μM, or with a combination of EGCG + AC-PAC; AC-PAC + KR-12-a5; AC-PAC + LL-37; EGCG + KR-12-a5 or EGCG + LL- 37, at the same concentrations. The cell cultures were then stimulated with 50 μg/mL of *A. actinomycetemcomitans* LPS for 24-48h. Cell viability and migration were analyzed using colorimetric and fluorescent assays, respectively. Cytokine quantification was determined by multiplex ELISA assays.

Results: Among the experimental groups studied, EGCG, mainly at 25 and 12.5 μg/mL stimulated fibroblast growth, protecting them from LPS effects. Synergistic effects between EGCG + AC-PAC, EGCG + LL-37, and EGCG + KR-12-a5 on cell metabolism were also observed in the presence of LPS. Combinations of EGCG with AC-PAC or KR-12-a5 and AC-PAC with LL-37 were able to statistically increase cell migration. EGCG, AC-PAC, LL-37, and KR-12-a5 promoted cytokine reduction individually or in combination (EGCG + AC-PAC and EGCG + KR-12-a5), more specifically for IL-6, IL-8, GM-CSF, and TNF-α.

Conclusion: EGCG, alone or associated with AC-PAC and KR-12-a5 increases cell viability and migration, as well as cytokine inhibition by LPS-stimulated fibroblasts.

Keywords: Cationic Antimicrobial Peptides, Flavonoids, Cell Viability, Migration, Cytokines

Introduction

Dental caries and trauma can trigger cellular and molecular responses in pulp tissue, leading to inflammatory and/or regenerative events (Cooper *et al*., 2014). In response to microbial invasion, pulp cells (odontoblasts, fibroblasts, stem cells, and tissue-resident immune cells) release host-signaling factors such as chemokines and cytokines which lead to the recruitment of inflammatory and immune cells to the site of infection or injury (Horst *et al.,* 2011). These generate a complex signaling network and binding to their receptors, present on several cell types, can result in amplification of the inflammatory response within the tissue. Key and well-characterized cytokines and chemokines include interleukin (IL)-1α, IL-1β, tumor necrosis factor alpha (TNF-α), IL-4, IL-6, IL-8, and IL-10 (McLachlan *et al*., 2005). Clearly, the roles of these cytokines at any given point within the disease and repair process may be context and concentration dependent. Evidence supports that repair events occur only when the infection and inflammation are under control (Cooper *et al*., 2014).

Antimicrobial peptides are also recruited to the site of infection, targeted at killing the invading microbes. Cathelicidins are one of the most common and important cationic antimicrobial peptides (CAMP) found in the oral cavity. The peptide fragment LL-37 is provided by the action of proteases on human cationic peptide-18 and also produced by epithelial cells. LL-37 has broad antimicrobial activity (Vandamme *et al.,* 2012) and exerts immunomodulatory effects, including the chemotactic effect of neutrophils and eosinophils (Tjabringa *et al.,* 2006), prostaglandin E2 production in gingival fibroblasts (Chotjumlong *et al.,* 2013), attenuation of cytokine production induced by lipopolysaccharides (LPS), and the expression of chemokines in fibroblasts (Jönsson and Nilsson, 2012). Short peptides or analogues of peptides based in original CAMP have attracted attention because of their potentially low cost of production and optimization of antimicrobial and immunological properties (Taylor *et al.,* 2008; Wang, 2008; Jacob *et al.,* 2013). The analogue of LL-37, KR-12-a5, exhibited potent antimicrobial activity against important Gram-positive and Gram-negative bacteria and high LPS-binding activity and an inhibitory effect on LPS-stimulated TNF-α production, more than the original LL-37 and their tested analogues (Jacob *et al.,* 2013).

Flavonoids are heterocyclic organic compounds found in several plants, vegetables, and herbal medicines, which have biological properties such as

antimicrobial, antioxidant, anti-inflammatory, anticancer, and other activities (Pieta, 2000, Havsteen, 2002, Tripoli *et al.,* 2007, Chushnie and Lamb, 2011). There are 14 classes of flavonoids differentiated on the basis of the chemical nature and position of aromatic rings. Among them, six classes have attracted attention by their antibacterial properties related to skeleton structure or isoflavonoid counterparts; flavones, chalcones, flavonols, catechins, flavanones, and anthocyanidines (Chushnie and Lamb, 2011).

Epigallocatechin-3-gallate (EGCG) is one of the most abundant catechins and a major bioactive component of green tea (Harborne and Williams, 2000). EGCG inhibits the growth of oral pathogens and reduces the secretion of pro-inflammatory cytokines by gingival fibroblasts, epithelial cells, and endothelial cells (Wheeler *et al.,* 2004, Lee *et al.,* 2009; Hosokawa *et al.,* 2010; Asahi *et al.,* 2014). The presence of EGCG significantly reduced, in a concentration-dependent manner, the expression of IL-6 and IL-8 in dental pulp cells exposed to LPS (Nakanishi *et al.,* 2010). EGCG inhibited cytokine/chemokine production in fibroblasts by preventing phosphorylation of MAPK/ERK and JNK pathways, key components in the transduction of cell signals (Hosokawa *et al.,* 2009, 2010).

Proanthocyanidins (PAC) are a group of polyphenolic bioflavonoids that are known to possess broad pharmacological activity and therapeutic potential. Besides antioxidant activity, proanthocyanidins exhibit immunostimulating, anticarcinogenic, anti-allergic, anti-inflammatory, antimicrobial, and cardio protective effects (Fine, 2000). Cranberry proanthocyanidins present a unique oligomeric structure with A-linkage that differentiates them from B-type proanthocyanidins found in other berry fruits. Cranberry A-type proanthocyanidins (AC-PAC) were able to inhibit matrix metalloproteinase (MMP) production by human macrophages stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharides (LPS), as well as reducing MMP-1 and -9 catalytic activities (Grenier *et al.,* 2011). Studies have demonstrated the ability of cranberry proanthocyanidins on inhibition of inflammatory cytokine secretion by immune and mucosal cells (Bodet *et al.,* 2006; La *et al*., 2010).

Studies have pointed out that the synergism between flavonoids or flavonoid and antimicrobial peptides improves their antimicrobial and anti-inflammatory effects (Harasstani *et al.,* 2010; Feldman and Grenier, 2012; Bedran *et al.,* 2015). The synergistic

action between proanthocyanidins and licochalcone A, both flavonoids from cranberry (cranberry) and licorice respectively, led to a reduction in the growth and biofilm of *P. gingivalis*, as well as reducing the secretion of pro-inflammatory mediators in an LPS-induced macrophage model (Feldman and Grenier, 2012). The only study evaluating synergism between flavonoids and antimicrobial peptides was conducted by Bedran *et al.* (2015) showing that the combination of LL-37 and EGCG (epigallocatechin 3-gallate) or proanthocyanidins reduced the secretion of various cytokines induced by an LPS co-culture model of oral mucosal cells.

This study aimed to investigate the synergistic interactions of EGCG and A-type cranberry proanthocyanidins (AC-PAC) when used in combination with LL-37 or KR-12-a5 on cell viability, migration capacity, and inhibition of cytokines in culture of fibroblasts stimulated with LPS. The null hypothesis is that EGCG, AC-PAC, LL-37, and KR-12-a5, individually or in combination, will not influence cell viability and migration, or cytokine inhibition by LPS-stimulated fibroblasts.

Material and Methods

Flavonoids, peptides and LPS

EGCG (#E3893) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A fraction of cranberry proanthocyanidins (AC-PAC) was isolated from cranberry fruit (*Vaccinium macrocarpon*) by means of solid-phase chromatography (La *et al.,* 2010) kindly provided by A. Howell (Rutgers, The State University of New Jersey, USA) (Bedran *et al.,* 2015). The EGCG was dissolved in distilled water at a concentration of 1 mg/mL and sterilized by filtration through a 0.2-mm pore size membrane. The AC-PAC was dissolved in 70% ethanol at a final concentration of 3 mg/mL and stored at 4° C, protected from light, for up to one month. The peptides used in this study and their amino acid sequence are: cathelicidin LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) (Ji *et al.,* 2007) and KR-12-a5 (LL-37 analogue - KRIVKLILKWLR) (Jacob *et al.,* 2013). All peptides were purchased from Biomatik (Cambridge, ON, Canada). The peptides were dissolved in sterile UltraPure DNase/RNase-free distilled water (Life Technologies Inc., Canada) at a concentration of 1 mM and stored at -20° C until use. *A. actinomycetemcomitans* (ATCC 29522) LPS isolated using the protocol described by Darveau and Hancock (1983) was dissolved at a

concentration of 1 mg/mL in sterile distilled water and stored at -20° C.

Culture of fibroblasts and stimulation with LPS

The primary human gingival fibroblast cell line HGF-1 (CRL-2014; American Type Culture Collection [ATCC]; Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine (HyClone Laboratories, Logan, UT, USA), 10% heat-inactivated fetal bovine serum (FBS), and 100 mg/mL of penicillin G–streptomycin. Cells were incubated at 37° C in a 5% CO_2 atmosphere until they reached confluence. The culture was pre-treated for 2 h with EGCG or AC-PAC at 25 and 12.5 μg/mL, LL-37 or KR-12-a5 at 0.06 and 0.03 μM, or with a combination of EGCG + AC-PAC; AC-PAC + KR-12-a5; AC-PAC + LL-37; EGCG + KR-12-a5, or EGCG + LL- 37, at the same concentrations. The concentrations were chosen based on the study of Bedran *et al.* (2015). The culture was then stimulated with 50μg/mL of *A. actinomycetemcomitans* LPS for 24h at 37° C in a 5% CO_2atmosphere. Cultures with LPS (without treatments) were used as controls. All experiments were performed in duplicate assays from two independent experiments.

Determination of cell viability (Anexo I)

The effect of AC-PAC, EGCG, KR-12-a5, LL-37, and LPS, individually and in combination, on the viability of HGF-1 cells was evaluated. The cells ($3x10^4$ cells/mL) were seeded in the wells of 96-well microplates and incubated for 48 h at 37° C in a 5% CO_2 atmosphere to allow cell adhesion. The culture medium was removed and the cells were pre-treated for 2h with compounds individually or in combination. *A. actinomycetemcomitans* LPS (50 μg/mL) was then added to the wells, and the cells were incubated for an additional 24h at 37°C in a 5% CO_2 atmosphere. A colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany) using 3-[4,5-diethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide as substrate was performed according to the manufacturer's protocol. The means were calculated for the groups and transformed into percentage of cell viability in relation to the negative control (DMEM), which was defined as having 100% cell viability.

Migration Assay

Cell migration was assessed using Oris™ Cell Migration Assay – Collagen I Coated, Product n°: CMACC1.101 & CMACC5. 101, (Platypus Technologies, Madison, WI, USA), according to the manufacturer's instructions (https://www.platypustech.com/wp-content/uploads/Oris-COL-CMA protocol_SP0061.07.pdf). The cells ($3x10^4$ cells/mL) were seeded on 96-well microplates with the stoppers for 48h at 37°C in a 5% CO_2atmosphere until reaching 80% cell confluence. Then the stoppers were removed and cells were pre-treated with AC-PAC, EGCG, KR-12-a5, and LL-37 in combination for 2h. Subsequently, they were stimulated with 50 µg/mL of *A. actinomycetemcomitans* LPS for 24h at 37° C. The wells were fluorescently stained with Calcein AM using a microplate reader with mask for quantification at 485 and 528nm.

Determination of cytokine secretion by multiplex ELISA assays

Briefly, the cells ($3x10^5$ cells/mL) were seeded in the wells of 96-well microplates and incubated for 48 h at 37° C in a 5% CO_2 atmosphere to allow cell adhesion. The culture was pre-treated with compounds, individually or in combination for 2 h and then LPS 50 µg/mL was added to the wells for 48 h. The supernatants were collected, centrifuged (1000 x *g* for 5 min at 4° C), and stored at -80° C. These samples were sent to Eve Technologies (Calgary, AB, Canada; http://www.evetechnologies.com) for multiplex ELISA assays. The Bio-Plex Suspension Array System was used to quantify the following cytokines: granulocyte-macrophage colony-stimulating factor [GM-CSF], interferon gamma [IFNγ], interleukin-1 beta [IL-1β], interleukin-2 [IL-2], interleukin-4 [IL-4], interleukin-5 [IL-5], interleukin-6 [IL-6], interleukin-8 [IL-8], interleukin-10 [IL-10], interleukin-12 [IL-12 p70], interleukin-13 [IL-13], monocyte chemoattractant protein-1 [MCP-1] and tumor necrosis factor-alpha [TNF-α].

Statistical analysis

Data obtained from MTT assays are expressed in means (standard deviations) of percentage of cell viability in relation to control (DMEM - 100% of cell growth) and submitted to ANOVA and Tukey tests ($p<0.05$). SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to run the statistical analyses.

Results

Analysis of cell viability (MTT assays)

Figures 1A and **1B** show the effect of treatment with EGCG, AC-PAC, KR-12-a5, and LL-37 individually on fibroblast viability (HGF-1), after exposure or not to *A. actinomycetemcomitans* LPS at 50 μg/ml. In the absence of LPS, all experimental groups, independent of the concentration, presented cell viability above 70%. For EGCG, KR-12-a5, and LL-37 groups, under this condition, the percentage of HGF-1 metabolism was over 90%. Among the experimental groups studied, EGCG (25 and 12.5 μg/mL) in the presence of LPS was able to stimulate cell metabolism above 100%. Cell metabolism of groups treated with peptides was reduced in the presence of LPS. Under the effect of LPS, the metabolism of cells treated with AC-PAC reduced from 48.45 to 61.26%, although no statistical difference was observed for the concentrations 25 and 6.25 mg/mL when compared to the same groups without LPS.

Figure 2 shows the combined effect of the compounds on the percentage of cellular metabolism of HGF-1. Cell viability remained around 80% even in the presence of LPS for all the experimental groups. The combinations that stimulated the metabolism even in the presence of LPS, maintaining above 90% viable cells were the combinations: EGCG and AC-PAC (EG 25 + PAC 25; EG 12.5 + PAC 25); AC-PAC and KR-12-a-5 (PAC 25 + KR 0.06); EGCG and KR-12-a-5 (EG 12.5 + KR 0.03); and the concentrations of EGCG and LL-37 (EG 12.5 + LL 0.03).

Migration assay

In the migration assay shown in **Figure 3**, combinations of EGCG and AC-PAC (EG 25 + PAC 12.5) and AC-PAC and LL-37 (PAC 25 + LL 0.06, PAC 25 + LL 0.03) compounds were able to statistically increase proliferation in the presence of LPS. EG 12.5 + KR 0.03 promoted cell migration similar to control without LPS, and superior to control with LPS. For the other combinations, there was no statistical difference between the groups exposed or not to LPS.

Determination of cytokine secretion by multiplex ELISA assays

Table 1 shows the effect of compounds alone or in combination on cytokines after exposure to LPS. EGCG, AC-PAC, KR-12-a5, and LL-37, when used individually,

reduced the secretion of cytokine produced by the LPS-stimulated fibroblasts, more specifically IL-6, IL-8, GM-CSF, and TNF-α. The combinations with the highest synergistic effect observed were: EGCG 12.5 and KR-12-a5 0.03, which reduced the secretion of cytokines as follows IL-8 > IL-6 > GM-GSF > TNF-α > INFγ > IL-1β, followed by combinations of EGCG 25 and AC-PAC 25 which reduced the secretion of cytokines as follows IL-8 > IL-6 > GM-GSF > TNF-α > MCP-1 > IL-1β. The cytokines IL-5, IL-10, IL-12, and IL-13 were not detected by Multiplex, considering the standard curve used by the manufacturer.

Discussion

In the present study, synergism was observed between flavonoids and peptides, improving cell viability and migration and decreasing cytokine secretion by LPS-stimulated fibroblasts, rejecting the null hypothesis. Independent of the concentration, all groups tested individually maintained cell viability above 70%, in the absence of LPS, showing no toxicity in the concentrations tested. EGCG had the best effect on fibroblast viability, even in the presence of LPS. Both peptides and AC-PAC had no protective effect on cell viability when exposed to LPS, without statistical difference from control with LPS. Similar to our study, Liu *et al*. (2013) evaluated the impact of EGCG as pre-treatment for 2h on LPS-stimulated hepatocytes L02 for 48h and observed no cytotoxicity until 50 µM (22.9 µg/mL).

Compounds that can prevent LPS effects have potential therapeutic application in the reduction of inflammation. Bacterial lipopolysaccharides (LPS) are the major structural component of the outer wall of all Gram-negative bacteria and a potent activator of the immune system and inflammation. The architecture of LPS consists of three separate “building blocks” as follows: lipid A, an inner core region, and the *O*-specific side chain. These separate building blocks have widely different compositions and structures that are reflected in their biological activities (Henderson *et al*., 1996). LPS quickly stimulate the production of reactive oxygen species (ROS) possibly by induction of NADPH oxidases present in mitochondria. The most important ROS are free radicals such as superoxide anion ($O2^{-}$) and hydroxyl radical (-OH) or non-radicals such as hydrogen peroxide (H_2O_2). These produce adverse modifications to cell components, such as lipids, proteins, and DNA. A variety of antioxidants is produced by the organism

to control the levels of ROS, such as enzymes superoxide dismutase, catalase, or non-enzymatic agents such as vitamins C and E (Birben *et al*., 2012). Most of the protective effects of EGCG are related to its intrinsic antioxidant properties, including the free radical scavenging activity, as well as its ability to modulate many cellular enzyme functions (Cia *et al*., 2014). In the current study, EGCG, mainly at 25 and 12.5 µg/mL stimulated fibroblast growth, protecting them from LPS effects. Corroborating with our results, a protective effect of EGCG on culture of rat retinal pigment epithelial cells (REP) was observed at concentrations of 10-50 µM (4.5 – 22.9 µg/mL), a range of concentrations reported in the literature to have an antioxidant effect *in vitro* (Cia *et al*., 2014).

Besides the individual protective effect of EGCG, synergistic action between EGCG and the other compounds (AC-PAC, LL-37 and KR-12) on cell metabolism was also observed in the presence of LPS. Few studies were found evaluating the synergism between flavonoids or between flavonoids with peptides for anti-inflammatory purposes. Shao *et al*. (2004) combined the Chinese herb medicine *Scutellaria baicalensis* (SbE) and grape seed proanthocyanidins and observed that this combination potentially enhances their antioxidant efficacy, acting synergistically to scavenge ROS at lower dosages of each drug. One investigation demonstrated that PAC inhibits the binding of LPS to the surface of human embryonic kidney (HEK) cells expressing LPS receptors (TLR4/MD2 and CD14) and consequently the endocytosis of LPS (Delehanty *et al*., 2007). Similar to our results, Bedran *et al*. (2015) combined AC-PAC (25 and 50µg/mL), EGCG (1 and 5 µg/mL), and LL-37 (0.1 and 0.2 µM) in the presence of 1µg/mL of LPS and none of the combinations had cytotoxic effects on co-culture of fibroblast and epithelial cells, and viability remained above 90% in all groups compared to the untreated control cells. The mechanism of peptides for LPS neutralization is not completely elucidated, however, some studies have shown that peptides bind to the LPS and change the structure from cubic lamellar to multilamellar form, thereby preventing the binding of LPS to host proteins involved in inflammation pathways (Rosenfeld *et al*., 2006; Bhattacharjya, 2010). Among the KR-12 analogues evaluated by Jacob *et al*. (2013), KR-12-a5 displayed the highest LPS-binding activity.

Migration assays are used to evaluate the ability of materials to induce cell proliferation and healing. In the current study, some combinations of the EGCG + AC-

PAC, AC-PAC + LL-37, and EG 12.5 + KR 0.03 increased cell migration in comparison with control (LPS only). Extracts from wild blueberry pomace, rich in anthocyanins, increased cell migration after 22 hours of treatment in fibroblasts (Hoskin *et al*., 2019). Anthocyanins potentiate cell migration in fibroblasts and keratinocytes by several mechanisms, including the increase in angiogenesis by stimulation of vascular endothelial growth factor (VEGF) and suppression of ROS production (Nizamutdinova *et al*., 2009).

The effect of EGCG, AC-PAC, LL-37, and KR-12-a5 on cytokine reduction was observed individually or in combination (EG 25 + PAC 25 and EG 12.5 + KR 0.03) more specifically for IL-6, IL-8, GM-CSF, and TNF-α. An investigation reported that EGCG significantly reduced, in a concentration-dependent manner, the expression of IL-6 and IL-8 in dental pulp cells exposed to LPS (Nakanishi *et al.,* 2010). The combination of proanthocyanidins and licochalcone A led to a reduction in the secretion of pro-inflammatory mediators in an LPS-induced macrophage model (Feldman and Grenier, 2012). The only study evaluating synergism between flavonoids and antimicrobial peptides was conducted by Bedran *et al.* (2015) showing that the combination of LL-37 and EGCG (epigallocatechin 3-gallate) or proanthocyanidins reduced the secretion of various cytokines induced by an LPS co-culture model of oral mucosal cells. Polyphenols such as AC-PAC and EGCG inhibit the secretion of pro-inflammatory cytokines through the regulation of the NF-kB, phosphorylation of MAPK/ERK and JNK pathways, key components in the transduction of cell signals (Bremmer *et al*., 2002; Hosokawa et al*.,* 2009, 2010), while LL-37 binds to TLR1/2 and TLR4 cell surface receptors, preventing LPS binding and cell activation (Into *et al*., 2010)

Conclusion

EGCG, alone or associated with AC-PAC and KR-12-a5 increases cell viability and migration, as well as cytokine inhibition by LPS-stimulated fibroblasts.

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Results

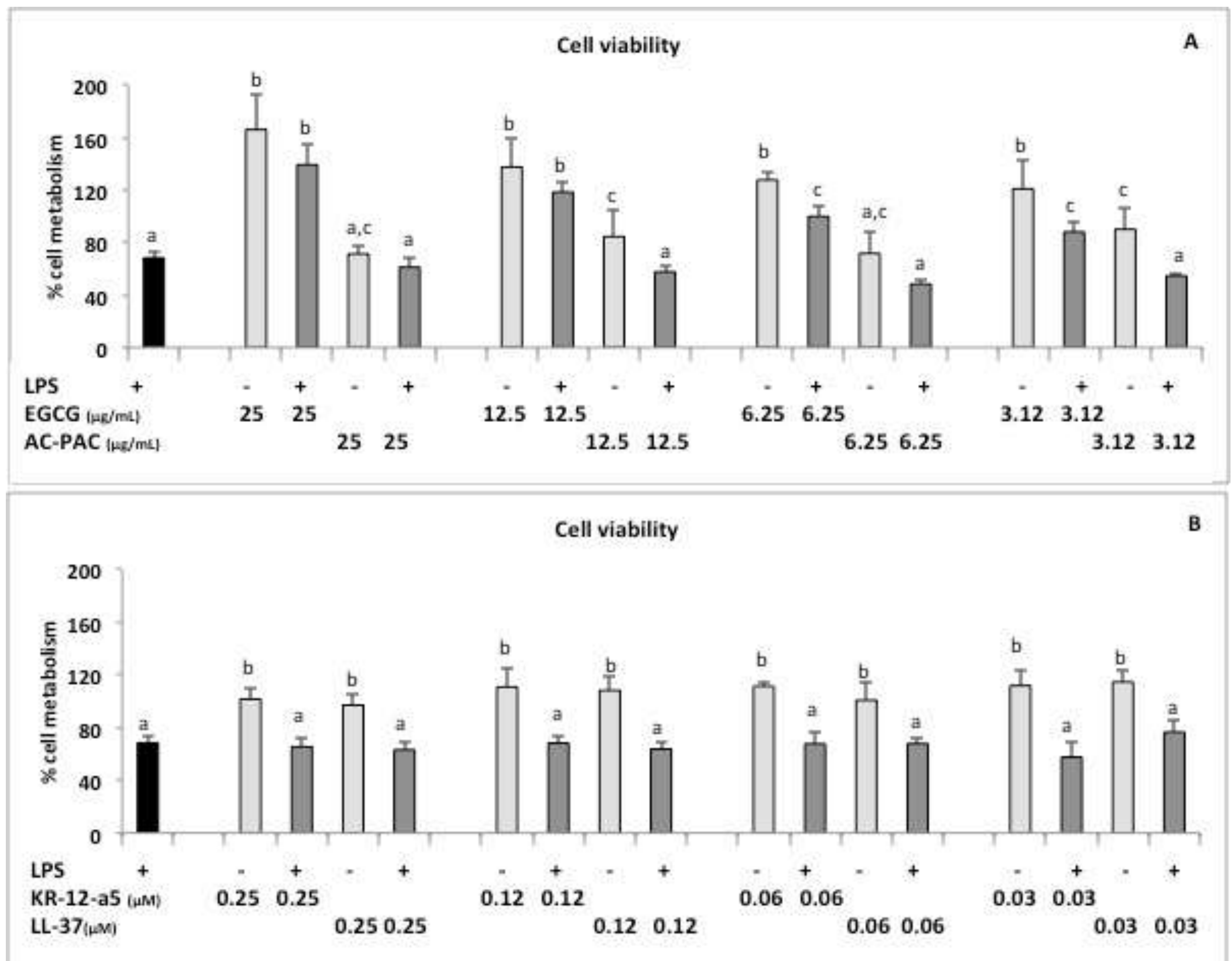

Figure 1. Effect of EGCG and AC-PAC (A), KR-12-a5 and LL-37 (B) on fibroblasts viability (HGF-1), after exposure or not of LPS. The results are expressed in means/SDs. Values were transformed into percentage of cell viability in relation to negative control (untreated cells), which was defined as having 100% cell metabolism. LPS: lipopolysaccharide.

[a] Different lower letters show statistical differences among all groups, considering each concentration separately, according to ANOVA and Tukey test ($p<0.05$).

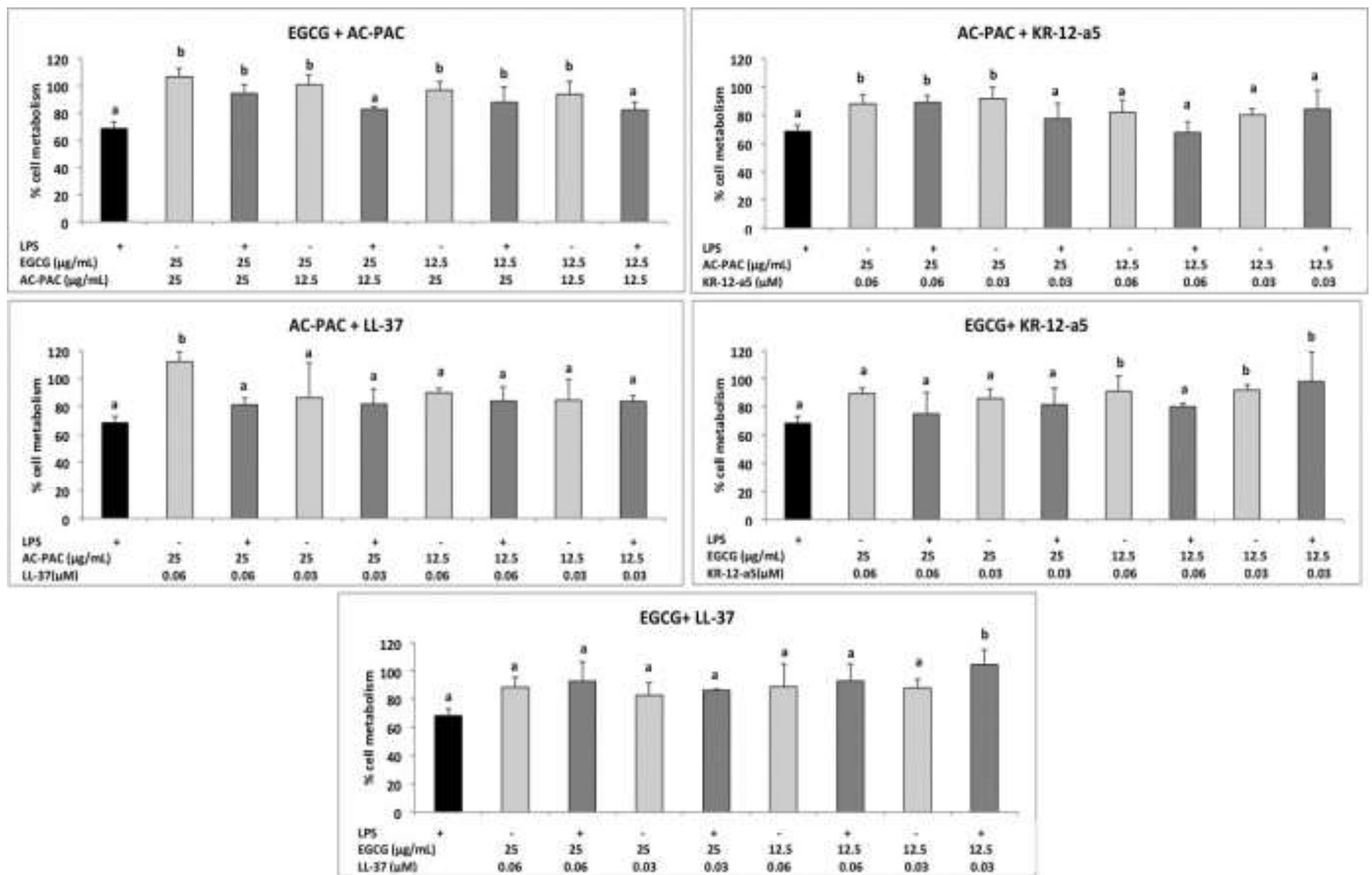

Figure 2. Effect of EGCG, AC-PAC, KR-12-a5 and LL-37 combinations on fibroblasts viability (HGF-1), after exposure or not of LPS. The results are expressed in means/SDs. Values were transformed into percentage of cell viability in relation to negative control (untreated cells), which was defined as having 100% cell metabolism. LPS: lipopolysaccharide.

[a] Different lower letters show statistical differences among all groups, considering each concentration separately, according to ANOVA and Tukey tests ($p<0.05$).

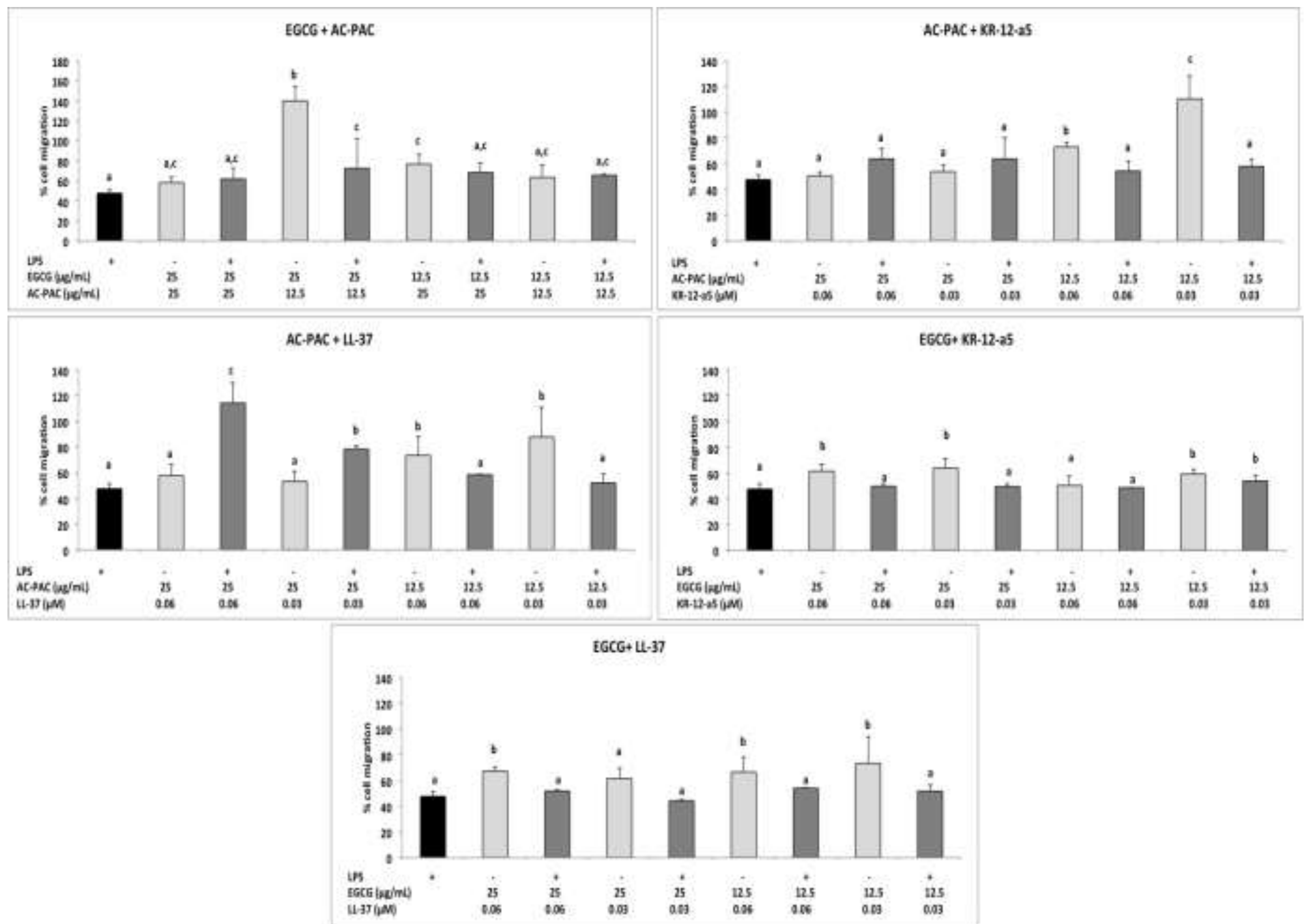

Figure 3. Effect of EGCG, AC-PAC, KR-12-a5 and LL-37 combinations on fibroblasts migration (HGF-1), after exposure or not of LPS. The results are expressed in means/SDs. Values were transformed into percentage of cell migration in relation to negative control (untreated cells), which was defined as having 100% cell migration. LPS: lipopolysaccharide.

[a] Different lower letters show statistical differences among all groups, considering each concentration separately, according to ANOVA and Tukey tests ($p<0.05$).

Table 1. Effect of EGCG, AC-PAC, KR-12-a5 and LL-37, alone or in combination, on cytokines levels (% reduction), after exposure of LPS. The results are expressed in means of percentage of reduction in relation to LPS group.

	GM-CSF	INFγ	IL-1β	IL-4	IL-6	IL-8	MCP-1	TNF-α
EG 25	91.3	Nd	8.9	< 0	96.1	98.6	< 0	84.2
EG 12.5	78.4	49.5	< 0	< 0	87.4	94.8	< 0	74.4
PAC 25	89.6	79.5	25.7	65	94.1	98.8	64.3	90
PAC 12.5	62.2	72.1	22.7	< 0	76	87.5	< 0	63.2
KR 0.03	50.2	41.7	8.9	9.1	63.7	45.1	< 0	63.4
LL 0.03	49.6	< 0	< 0	< 0	61	65.3	< 0	34.4
EG 25 + PAC 25	95.3	Nd	8.9	< 0	98	99.7	81.8	85
EG 12.5 + PAC 12.5	94.4	Nd	< 0	< 0	96.6	99.1	< 0	86
EG 12.5 + KR 0.03	85.2	49.5	31.6	< 0	95	96.8	< 0	79.2
EG 12.5 + LL 0.03	85.2	60.8	< 0	< 0	90	95.5	7.1	79
PAC 12.5 + KR 0.03	75.4	53.4	29.7	< 0	86.7	89	19.5	62
PAC 12.5 + LL 0.03	79.8	79.5	< 0	< 0	87	92.6	1.8	60.2

* The values for IL-5, IL10, IL12, IL-13 were lower than those detectable by standard curve.

*Nd = No detected

* < 0 are values of stimulation above of LPS control.

*EG = EGCG; KR = KR-12-a5; LL = LL-37; PAC = AC-PAC; CHX = Chlorhexidine digluconate; LPS = lipopolysaccharide.

Anexos

ANEXO A

REFERÊNCIAS INTRODUÇÃO GERAL

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ANEXO B

Análise da Síntese de LL-37

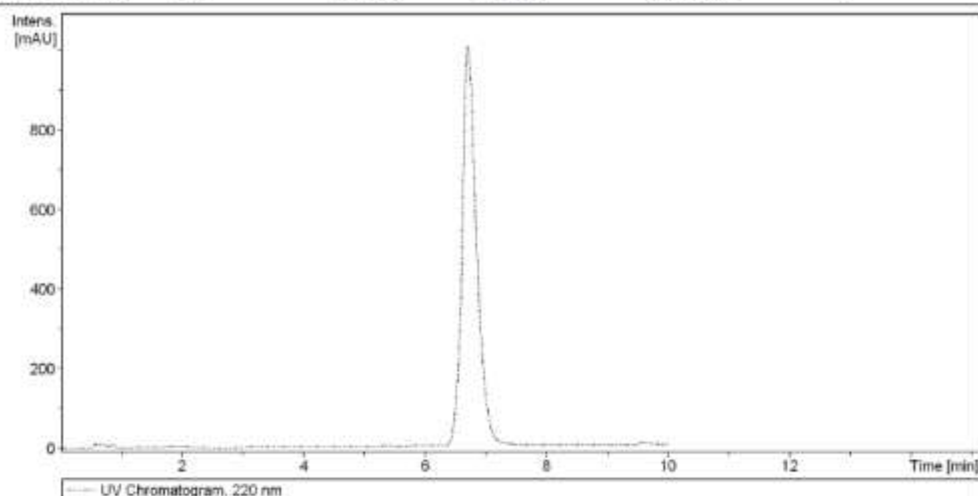
Display Report

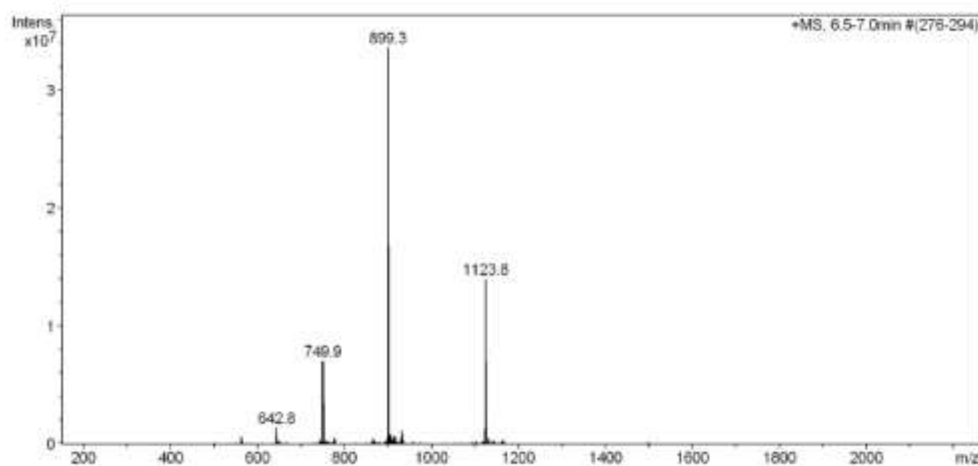
Analysis Info

Analysis Name	D:\Data\Norival\2014\NA1350_1-2_01_1811.d	Acquisition Date	9/26/2014 12:10:40 PM
Method	1811.m	Operator	emcilli
Sample Name	NA1350	Instrument	amaZon SL
Comment			

Acquisition Parameter

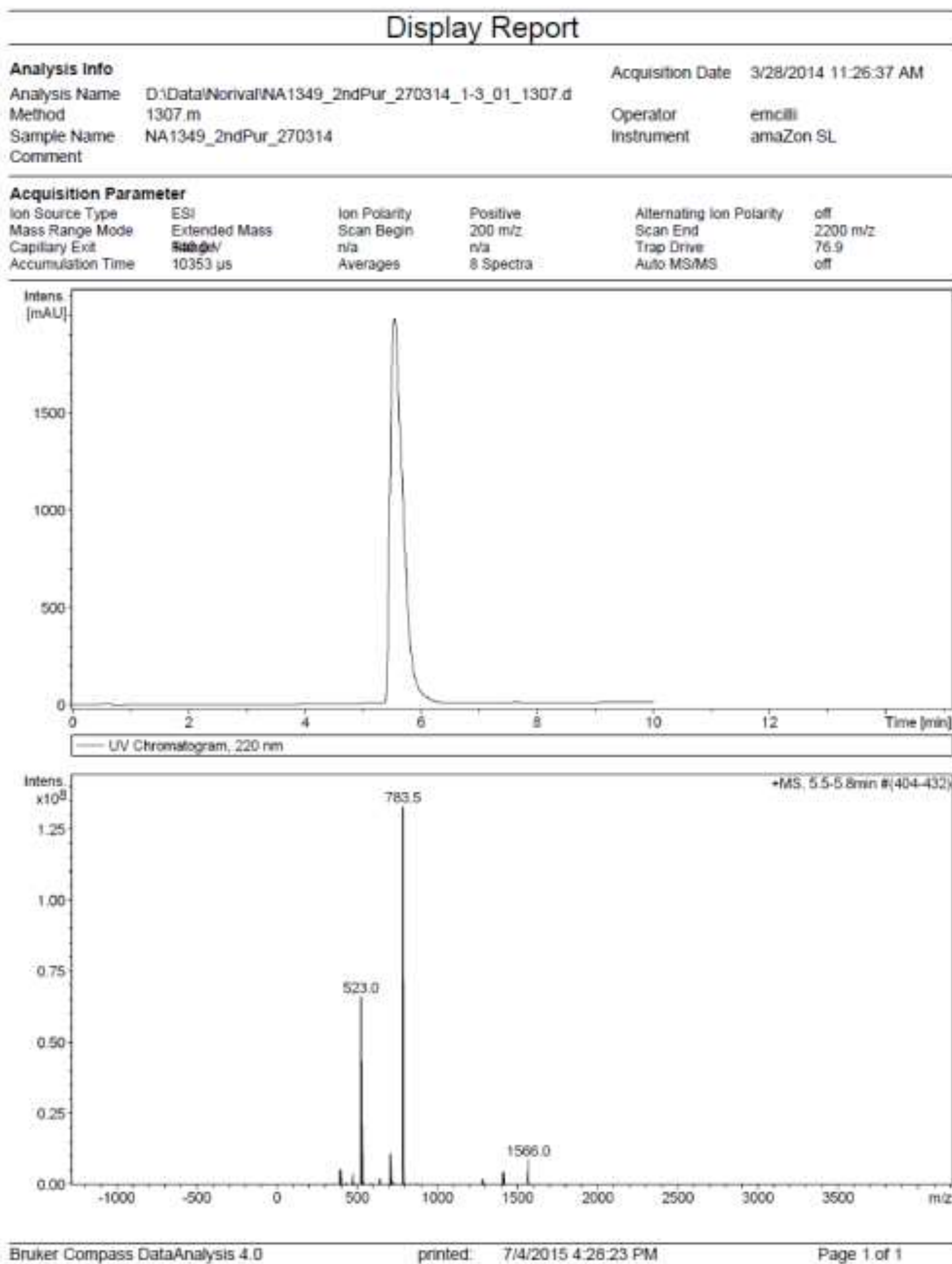
Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	off
Mass Range Mode	Enhanced	Scan Begin	70 m/z	Scan End	2200 m/z
Capillary Exit	[illegible]	n/a	n/a	Trap Drive	76.9
Accumulation Time	100 µs	Averages	5 Spectra	Auto MS/MS	off





Bruker Compass DataAnalysis 4.0 printed: 7/4/2015 4:53:20 PM Page 1 of 1

Análise da Síntese de KR-12-a5



ANEXO C

Protocolo de Determinação da MIC e MBC

1º dia experimental: Proceder com a reativação do microrganismo (MO) em Mitis Agar Salivarius Agar (Difco, Kansas City, MO, USA) com 0,2U/mL de bacitracina para *S. mutans*, Brain Heart Infusion Agar – BHIA (Difco) para *A. israelii e E. faecalis, BHIA sangue* para *F. nucleatum* (estriar 15 µL da cultura estoque em Técnica de esgotamento) e incubar de acordo com as exigências de ambiente requeridas para cada microrganismo e experimento.

2º dia experimental: Repicar o microrganismo da placa para o respectivo meio de cultura em caldo (5 a 10 UFC em 5mL) e incubar.

3º dia experimental: Diluição dos antimicrobianos teste:

→ Pipetar 50 µL do peptídeo e controle de Clorexidina, ressuspendidos na concentração inicial que se deseja (mg/mL) no primeiro e segundo poço de uma placa de 96 poços.

→ Pipetar 50 µL de água deionizada estéril em cada poço, exceto no primeiro.

→ Ressuspender o segundo poço e transferir 50 µL para o terceiro poço, e assim sucessivamente (Técnica de diluição seriada).

* Preparar o inóculo de acordo com a curva de crescimento: D.O. 0,5 para as bactérias facultativas *e* para *F. nucleatum*

* Mueller-Hinton caldo para as bactérias anaeróbias facultativas e BHI caldo suplementado com Hemina e Menadiona para as bactérias anaeróbias estritas.

→ Diluir 1000X no meio de cultura 2X concentrado (500 µL do inoculo em 4,5ml de meio).

→ Ressuspender 50 µL do inoculo em cada poço; tampar a placa e incubar por 24h em condições específicas para cada microrganismo.

4º dia experimental: Pipetar 15 µL de solução de Resazurina 0,01% em cada poço e incubar por 4h.

→ Plaquear o último poço com coloração azulada (MIC) e, no mínimo, 2 poços anteriores para se determinar a MBC (Diluição de 10 para 90 µL/ 0-7 quadrantes).

5º dia experimental: Leitura das placas em absorbância de 550 a 600 nm (dependo do MO analizado) e plaqueamento / contagem das UFC/mL.

ANEXO D

Protocolo de Determinação da FIC

1º e 2º dia experimental: Proceder conforme o protocolo de MIC e MBC.

3º dia experimental: Diluição dos compostos antimicrobianos teste:

DEVE-SE MULTIPLICAR POR 4, O VALOR DO MIC DE CADA DROGA (ESTA SERÁ SUA PRIMEIRA SOLUÇÃO, DEPOIS É SÓ IR DILUINDO)		
Ex. OS VALORES DE MIC PARA E. FAECALIS FORAM: COMPOSTO 1 (0,25mg/mL) E COMPOSTO 2 (0,03125MG/ML)		
Solução Composto 1 (LETRAS)		Em cada poço
A	1mg/ml	25µl COMPOSTO 1
B	0,5mg/ml	25µl COMPOSTO 2
C	0,25mg/ml	50µl BAC
D	0,125mg/ml	
E	0,0625mg/ml	
F	0,03125mg/ml	
G	0,015625mg/ml	
H	0,007813mg/ml	
SOLUÇÃO Composto 2 (NÚMEROS)		
1	0,125mg/ml	
2	0,0625mg/ml	
3	0,03125mg/ml	
4	0,015625mg/ml	
5	0,007813mg/ml	
6	0,000977mg/ml	
7	0,000488mg/ml	
8	0,000244mg/ml	

Ordem de Pipetagem:

E.faecalis	1	2	3	4	5	6	7	8	9	10	11	12
A	25ul SOL A+ 25ul SOL1	25ul SOL A+ 25ul SOL 2	25ul SOL A+ 25ul SOL 3	25ul SOL A+ 25ul SOL 4	25ul SOL A+ 25ul SOL 5	25ul SOL A+ 25ul SOL 6	25ul SOL A+ 25ul SOL 7	25ul SOL A+ 25ul SOL 8				
B	25ul SOL B+ 25ul SOL 1	25ul SOL B+ 25ul SOL 2	25ul SOL B+ 25ul SOL 3	25ul SOL B+ 25ul SOL 4	25ul SOL B+ 25ul SOL 5	25ul SOL B+ 25ul Quer 6	25ul SOL B+ 25ul SOL 7	25ul SOL B+ 25ul SOL 8				
C	25ul SOL C+ 25ul SOL 1	25ul SOL C+ 25ul SOL 2	25ul SOL C+ 25ul SOL 3	25ul SOL C+ 25ul SOL 4	25ul SOL C+ 25ul SOL 5	25ul SOL C+ 25ul Quer 6	25ul SOL C+ 25ul SOL 7	25ul SOL C+ 25ul SOL 8				
D	25ul SOL D+ 25ul SOL 1	25ul SOL D+ 25ul SOL 2	25ul SOL D+ 25ul SOL 3	25ul SOL D+ 25ul SOL 4	25ul SOL D+ 25ul SOL 5	25ul SOL D+ 25ul Quer 6	25ul SOL D+ 25ul SOL 7	25ul SOL D+ 25ul SOL 8				
E	25ul SOL E+ 25ul SOL 1	25ul SOL E+ 25ul SOL 2	25ul SOL E+ 25ul SOL 3	25ul SOL E+ 25ul SOL 4	25ul SOL E+ 25ul SOL 5	25ul SOL E+ 25ul Quer 6	25ul SOL E+ 25ul SOL 7	25ul SOL E+ 25ul SOL 8				
F	25ul SOL F+ 25ul SOL 1	25ul SOL F+ 25ul SOL 2	25ul SOL F+ 25ul SOL 3	25ul SOL F+ 25ul SOL 4	25ul SOL F+ 25ul Quer 5	25ul SOL F+ 25ul SOL 6	25ul SOL F+ 25ul SOL 7	25ul SOL F+ 25ul SOL 8				
G	25ul SOL G+ 25ul SOL 1	25ul SOL G+ 25ul SOL 2	25ul SOL G+ 25ul SOL 3	25ul SOL G+ 25ul SOL 4	25ul SOL G+ 25ul Quer 5	25ul SOL G+ 25ul SOL 6	25ul SOL G+ 25ul SOL 7	25ul SOL G+ 25ul SOL 8				
H	25ul SOL H+ 25ul SOL 1	25ul SOL H+ 25ul SOL 2	25ul SOL H+ 25ul SOL 3	25ul SOL H+ 25ul SOL 4	25ul SOL H+ 25ul Quer 5	25ul SOL H+ 25ul SOL 6	25ul SOL H+ 25ul SOL 7	25ul SOL H+ 25ul SOL 8		50ul bac + 50ul água	50ul bac + 50ul 2XMIC COMPOSTO 1	50ul bac + 50ul 2XMIC COMPOSTO 2

Concentrações finais dos compostos em cada poço:

E. faecalis

Composto 1	1	2	3	4	5	6	7	8	9	10	11	12
A	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25				
B	0,125	0,125	0,125	0,125	0,125	0,125	0,125	0,125				
C	0,0625	0,0625	0,0625	0,0625	0,0625	0,0625	0,0625	0,0625				
D	0,0313	0,0313	0,0313	0,0313	0,0313	0,0313	0,0313	0,0313				
E	0,0156	0,0156	0,0156	0,0156	0,0156	0,0156	0,0156	0,0156				
F	0,0078	0,0078	0,0078	0,0078	0,0078	0,0078	0,0078	0,0078				
G	0,0039	0,0039	0,0039	0,0039	0,0039	0,0039	0,0039	0,0039				
H	0,002	0,002	0,002	0,002	0,002	0,002	0,002	0,002				

Composto 2	1	2	3	4	5	6	7	8	9	10	11	12
A	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				
B	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				
C	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				
D	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				
E	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				
F	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				
G	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				
H	0,0313	0,0156	0,0078	0,0039	0,002	0,001	0,0005	0,0002				

→Aplicar 50 µL da cultura microbiana em cada poço e incubar por 24 horas

4º dia experimental: Pipetar 15 µL de solução de Resazurina 0,01% em cada poço e incubar por 4h.

→ Plaquear os últimos poços com coloração azulada (no sentido diagonal da placa) - Diluição de 10 para 90 µL/ 0-7 quadrantes.

5º dia experimental: Realizar a contagem das UFC/mL.

ANEXO E

ENSAIO DE MTT (L-929) – Análise de Viabilidade celular (24 HORAS)

1. Realizar plantio das células (Fibroblasto L-929) semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços (100µL → 1 X 10^4 por poço);
2. Incubar por 24 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar a mistura de DMEM com as concentrações dos antimicrobianos teste (em combinação) e controle de CHX sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro);
5. Incubar por 24 horas para análise da viabilidade celular.
6. Após incubação, aspirar o meio e acrescentar a 100µL solução de MTT (10 µL da solução de MTT em uma concentração de 5mg/mL em PBS 1X e 90 µL de DMEM)
7. Incubar por 4 horas;
8. Aspirar a solução de MTT;
9. Acrescentar 100µL em cada poço de solução de Isopropanol acidulada;
10. Agitar por 30 minutos;
11. Homogeneizar e transferir 100 µL para uma nova placa de 96 poços;
12. Leitura da absorbância em 570 nm em espectrofotômetro.

Anexo F

Protocolo de Ensaio de Biofilme

Protocolo de Biofilme Simples formado em fundo de placa de poliestireno

(Concentrações isololadas, combinadas e controle de clorexidina são correspondentes à 10X à Concentração Inibitória Fracionada - FIC)

→ Coletar uma alçada de microrganismos (5 – 10 UFC) para um tubo contendo 5 mL de BHI caldo (quando para MO anaeróbios suplementar com Hemina e Menadiona e Extrato de levedura) e incubar por 24 horas em condições favoráveis de crescimento para cada MO testado.

→ Para crescimento da D.O 0.5, após 24 horas de crescimento, coletar uma alíquota de 500 µL da cultura de *E. faecalis*, *F. nucleatum* e *A. israelii* e acrescentar em tubos contendo 5 mL de BHI caldo com glicose 1% e de *S. mutans* em BHI caldo com scarose a 0,5 %, incubar por 4 horas e 30 min. aproximadamente em CO_2 a 37º C e 24 horas em sistema de anaerobiose a 37º C (*F. nucleatum*).

→ Realizar uma diluição de 1000x em 4,5 mL de BHI caldo (supl.) com glicose ou sacarose em concentração normal (1x concentrado).

Montagem da placa:

→ Pré-tratar uma placa de 96 poços com saliva artificial (Composição: 800 mL de água deionizada, 1,6 g de extrato de levedura, 4 g de peptona, 0,28 g de NaCl, 1,6 g de glicose ou 3,2 g de sacarose, 0,16 g de $CaCl_2$, 0,16 g de KCl e 0,8 g de mucina) por 4 horas;

→ Remover a saliva e acrescentar 200 µL da cultura diluída em cada poço;

→ Incubar por 48 horas;

→ Retirar o meio de cultura dos poços e lavar uma vez com 100 µL de salina 0,9%.

→ Acrescentar aos poços a concentração exata dos antimicrobianos (isoladas e em combinação) e controles (controle positivo: clorexidina, controle negativo: BHI caldo);

→ Incubar por 24 horas;

Para a contagem das UFC:

→ Remover os tratamentos e lavar com 100 µL de solução salina 0,9%;

→ Adicionar 150 µL de solução salina 0,9% e ressuspender o biofilme;

→ Realizar uma diluição seriada de 10 µL para 90 µL/ 0-7 quadrantes;

→ Fazer plaqueamento em BHI Agar.

→ Incubar em condições favoráveis para cada MO estudado e realizar a contagem das UFC/mL.

Anexo G

Protocolo de Ensaio de Biofilme

Protocolo de Biofilme Dual-espécies formado em fundo de placa

(Concentrações isololadas, combinadas e controle de clorexidina são correspondentes à 10X à Concentração Inibitória Fracionada - FIC)

→ Coletar uma alçada de microrganismos (5 – 10 UFC) para um tubo contendo 5 ml de BHI caldo (quando para MO anaeróbios suplementados com Hemina e Menadiona e Extrato de levedura) e incubar por 24 horas em condições favoráveis de crescimento para cada MO testado.
→ Para crescimento da D.O 0.3, após 24 horas de crescimento, coletar uma alíquota de 500 µL da cultura de *E. faecalis*, *F. nucleatum* e *A. israelii* e acrescentar em tubos contendo 5 mL de BHI caldo com glicose 1% e de *S. mutans* em BHI caldo com scarose a 0,5 %, incubar por 4 horas e 30 min. aproximadamente em CO_2 a 37º C e 24 horas em sistema de anaerobiose a 37º C (*F. nucleatum*).
→ Realizar uma diluição de 100x em 4,5 mL de BHI caldo (supl.) com glicose ou sacarose em concentração normal (1x concentrado). Obs. As associações das culturas de microrganismo são realizadas em proporções de iguais volumes.

Montagem da placa:
→ Pré-tratar uma placa de 96 poços com saliva artificial (Composição: 800 mL de água deionizada, 1,6 g de extrato de levedura, 4 g de peptona, 0,28 g de NaCl, 1,6 g de glicose ou 3,2 g de sacarose, 0,16 g de $CaCl_2$, 0,16 g de KCl e 0,8 g de mucina) por 4 horas;
→ Remover a saliva, acrescentar 100 µL de cada cultura diluída (volume final de 200 µL) em cada poço nas seguintes associações: *E. faecalis* + *S. mutans, E. faecalis* + *A. israelii* e *E. faecalis* + *F. nucleatum*;
→ Incubar por 48 horas;
→ Retirar o meio de cultura dos poços e lavar uma vez com 100 µL de salina 0,9%.
→ Acrescentar aos poços a concentração exata dos antimicrobianos (isoladas e em combinação) e controles (controle positivo: clorexidina, controle negativo: BHI caldo);

→ Incubar por 36 horas;

→ Após incubar, retirar a solução dos poços e lavar com 100 µL de salina 0,9%.

Para a contagem das UFC:

→ Remover os tratamentos e lavar com 100 µL de salina 0,9%;

→ Adicionar 150 µL de solução salina 0,9% e ressuspender o biofilme;

→ Realizar uma diluição seriada de 20 µL para 180 µL/ 0-7 quadrantes;

→ Fazer plaqueamento em BHI Agar e BHI Agar + Cefuroxima para isolar a espécie *E. faecalis.* Em cada quadrante pipetar 2 gotas de 25 µL.

→ Incubar em condições favoráveis para cada MO estudado e realizar a contagem das UFC/mL.

ANEXO H

BIOFILME FORMADO EM TÚBULOS DENTINÁRIOS

OBTENÇÃO E PREPARO DOS BLOCOS DE DENTINA RADICULAR

Confecção de hemicilindros de dentina radicular bovino

---

1. Raízes de dente bovino (incisivo central), separada da coroa através de disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob-refrigeração (água destilada/deionizada), após o corte, marcação de 1 mm abaixo da junção amelocementária.

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2. Secção da porção radicular utilizando disco diamantado (Extec Diamond Wafer Blade, série 12205, 102mm X 0,3mm X 12,7mm, Extec Corporation, Enfield, CT, USA).

---

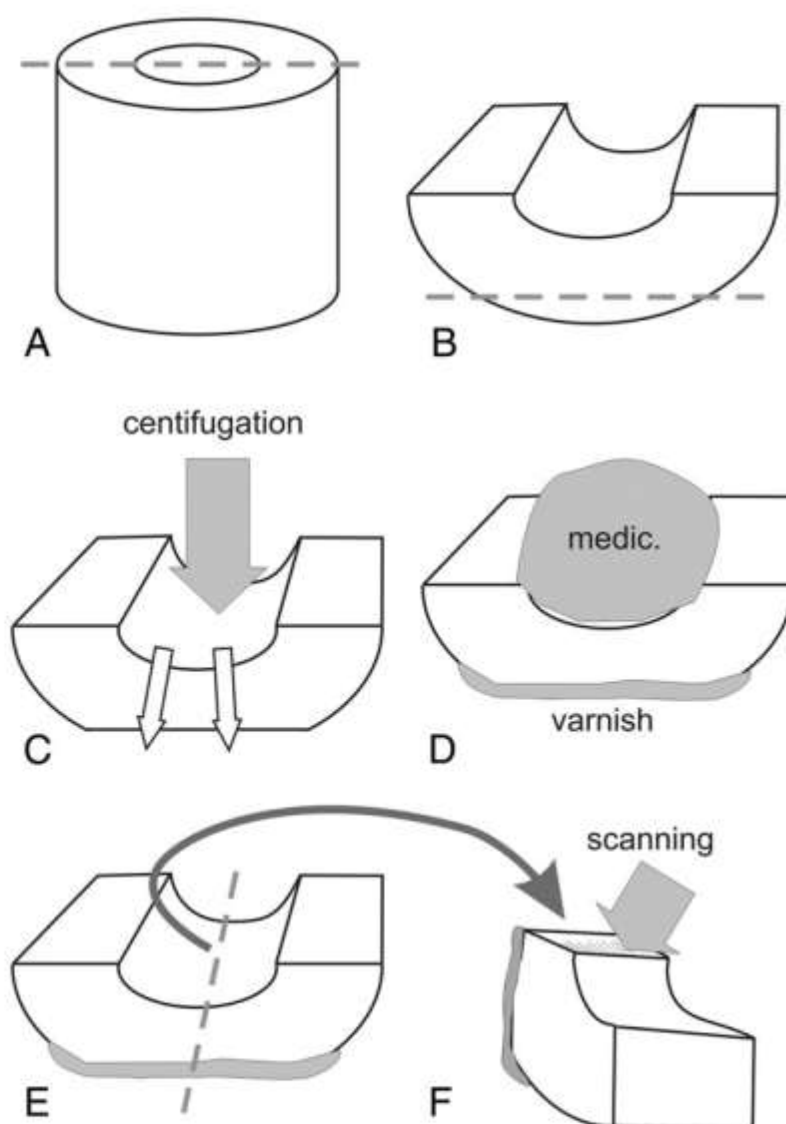
3. Preparo da parede do canal radicular de cada espécime com uma broca largo n° 6.

---

4. Disco da porção radicular fixado no acrílico com auxílio de cera pegajosa.

5. O espécime em formato cilíndrico foi novamente seccionado em duas metades hemicilíndricas com um disco de diamante de 0.6mm (Isomet 5000; BuehlerLtd, LakeBluff, IL).

6. Remoção da camada de *smear layer* de ambos os lados do espécime pela imersão em solução com EDTA 17% em ultrassom por 3 min. e 5 min. com H_2O deionizada. Esterilização em autoclave à 121° C por 15 minutos.



Desenhos esquemáticos da preparação da amostra de acordo com Ma *et al*., (2011)

MICROSCOPIA CONFOCAL

LIVE/DEAD® BacLight™ Bacterial Viability Kit (L13152)

Programa: LAS AF Leica Microsystems

Análise das imagens por Image J

(Concentrações isololadas, combinadas e controle de clorexidina são correspondentes à 10X à Concentração Inibitória Fracionada - FIC)

Biofilme Simples de *E. faecalis*

1. Coletar uma alçada de microrganismos (5 – 10 UFC) para um tubo contendo 5 ml de BHI caldo com glicose 1% ou sacarose 0,5% e incubar por 24 horas em condições favoráveis de crescimento para cada MO testado;
2. Para crescimento da D.O 0.5 após 24 horas de crescimento, coletar uma alíquota de 500 µL da cultura e acrescentar em tubos contendo 5 ml de BHI caldo com glicose, incubar em CO_2 a 37º C;
3. Realizar uma diluição de 1000x em 4,5 ml de BHI caldo (supl.) com glicose.
4. Acrescentar 500 µL do em cada eppendorf contendo a amostra fixada por resina composta com o canal radicular voltado para cima;
5. Realizar centrifugações de 1400 x *g*, 2000 x *g*, 3600 x *g* e 5600 x *g* nessa sequência, duas vezes cada, por 5 minutos. Uma alíquota fresca de bactéria deve ser adicionada entre cada centrifugação e a antiga descartada; Conforme o desenho esquemático acima apresentado (C).
6. Incubar por 14 dias;
7. Realizar trocas do meio de cultura de 48 em 48 horas;
8. Retirar o espécime do eppendorf;
9. Colocar em uma placa de 48 pocos
10. Acrescentar 350 µL aos poços a concentração de 10X o FIC dos antimicrobianos e suas respectivas concentrações isoladas, e água deionizada estéril nos poços contendo os controles de cultura; Conforme o desenho esquemático acima apresentado (D).
11. Incubar por 48 horas;

12. Após incubar, retirar a solução dos poços e lavar com solução salina 0,9%

13. Seccionar os espécimes ao meio utilizando disco diamantado (Extec Diamond Wafer Blade, série 12205,102mm X 0,3mm X 12,7mm, Extec Corporation, Enfield, CT, USA). Conforme o desenho esquemático acima apresentado (E e F).

14. Aplicar 150 µL da solução do Kit Live/Dead (contendo SYTO 9 e Iodo propídeo) em cada poço (solução confeccionada de acordo com as instruções do fabricante);

15. Incubar por 30 minutos no escuro;

16. Com auxílio de pinças, retirar os espécimes de dentina dos poços e secar levemente com lenço de papel macio;

17. Montar o bloco de dentina sobre a lamínula do microscópio (Leica TCS SP5, Microsystems GmbH) utilizando o óleo do Kit, certificando-se que a parte que se quer analisar esteja para baixo;

18. Comprimentos de onda de excitação e emissão: 480/500 nm para SYTO 9 e 490/635 nm para Iodo propídeo;

19. A fluorescência é captada pelo equipamento Leica TCS SP5, Microsystems GmbH;

20. Objetiva de 63X (lente que deve ser usada com óleo de imersão);

21. Software LAS AF Leica Microsystems com resolução de 1024 por 1024 pixels por imagem;

OBS. Devem ser selecionadas mais de uma área para captação das imagens, sendo uma delas utilizada para realizar as imagens tridimensionais através da função Z step size;

22. A quantificação das bactérias vivas e mortas é realizada pelo programa image J 1,48.

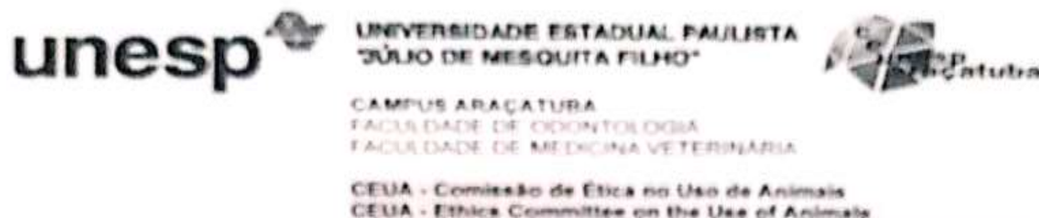
ANEXO I

ENSAIO DE MTT (HGF-1) – Análise de Viabilidade celular (24 HORAS)

1. Realizar plantio das células (Fibroblastos gengivais HGF-1) semeadas em meio DMEM com 10% de soro fetal bovino em placas de 96 poços (100µL → 3 X 10^4 por poço);
2. Incubar por 48 horas à 37ºC com 5% CO2 e 95% de ar;
3. Aspirar o meio;
4. Aplicar Pré-Tratamento com a mistura de DMEM com as concentrações dos antimicrobianos teste sobre as células, mantendo três poços para cada concentração que se quer analisar, inclusive para o controle sem antimicrobiano (meio DMEM puro) por 2 horas e aplicar 50 µg/mL de LPS (*A. actinomycetemcomitans*) – um grupo recebe o LPS e outo não.
5. Incubar por 24 horas para análise da viabilidade celular.
6. Após incubação, aspirar o meio e acrescentar a 100 µL solução de MTT (10 µL da solução de MTT em uma concentração de 5mg/mL em PBS 1X e 90 µL de DMEM)
7. Incubar por 4 horas;
8. Aspirar a solução de MTT;
9. Acrescentar 100 µL em cada poço de solução de Isopropanol acidulada;
10. Agitar por 30 minutos;
11. Homogeneizar e transferir 100 µL para uma nova placa de 96 poços;
12. Leitura da absorbância em 570 nm em espectrofotômetro.

ANEXO J

COMITÊ DE ÉTICA (Capítulo 1)



CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Combinações de flavonoides em sistemas de hidrogéis termossensiveis como propostas de medicação endodôntica para dentes permanentes jovens"**, Processo FOA nº 01194-2017, sob responsabilidade de Cristiane Duque apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 12 de Dezembro de 2017.

VALIDADE DESTE CERTIFICADO: 15 de Dezembro de 2019

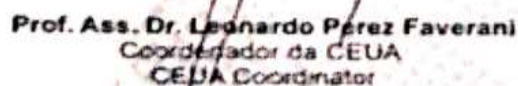
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 15 de Janeiro de 2020

CERTIFICATE

We certify that the study entitled **"Flavonoids combinations in thermosensitive hidrogels as proposals of endodontic medications for young permanent teeth"**, Protocol FOA nº 01194-2017, under the supervision of Cristiane Duque presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on December 12, 2017.

VALIDITY OF THIS CERTIFICATE: December 15, 2019

DATE OF SUBMISSION OF THE FINAL REPORT: January 15, 2020



Prof. Ass. Dr. Leonardo Pérez Faverani
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br