

Loiane Massunari

Efeito de flavonoides sobre microrganismos de
interesse endodôntico e sua influência na expressão
de marcadores de mineralização

Araçatuba - SP

2018

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interesse endodôntico e sua influência na expressão
de marcadores de mineralização

Tese de Doutorado apresentada à Faculdade de Odontologia da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de Araçatuba, como parte dos requisitos para a obtenção de título de Doutor em Ciência Odontológica - Área de Concentração: Endodontia.

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À Deus

Meu criador

Sempre em primeiro lugar

À minha família

Pai, Mãe, Ni, Glau, Jhone, Mia e Márcio

Nada teria sentido sem vocês!!!

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Resumo

Massunari, L. **Efeito de flavonoides sobre microrganismos de interesse endodôntico e sua influência na expressão de marcadores de mineralização.** [Tese] Universidade Estadual Paulista (Unesp), Faculdade de Odontologia de Araçatuba. Araçatuba, 2018.

Resumo

O tratamento endodôntico de dentes permanentes jovens representa um grande desafio clínico devido a presença de paredes dentinárias finas, divergentes ou paralelas e ápice aberto, o que dificulta a desinfecção e a execução dos procedimentos convencionais. Terapias de regeneração endodôntica envolvem o uso de materiais capazes de promover uma desinfecção eficaz sem causar citotoxicidade, além de induzir a diferenciação de células-tronco ou bioestimular células remanescentes do tecido pulpar mesmo após a injúria. Nesse contexto, os flavonoides, polifenóis presentes em frutas e vegetais, poderiam ser agentes interessantes para o tratamento endodôntico de dentes imaturos devido a sua amplitude terapêutica. Dessa forma, o objetivo do presente trabalho foi avaliar o efeito antimicrobiano, citotóxico e indutor de mineralização de flavonoides com finalidade de aplicação endodôntica. Este trabalho de tese foi dividido em três capítulos. O capítulo 1 avaliou a toxicidade dos flavonoides taxifolina, crisina, pinocembrina e galangina sobre fibroblastos pelo ensaio de MTT, a atividade antimicrobiana pela determinação da concentração inibitória e bactericida mínima, e a ação antibiofilme do flavonoide com melhor efeito antimicrobiano, por meio de ensaios em placas de poliestireno e em dentina radicular bovina por meio da análise por microscopia confocal. Os resultados mostraram que o flavonoide taxifolina não foi tóxico para os fibroblastos em nenhuma das concentrações analisadas, enquanto que os flavonoides crisina, pinocembrina e galangina apresentaram efeitos citotóxicos. Crisina, pinocembrina e galangina não apresentaram efeito antimicrobiano frente *E. faecalis* and *S. mutans* nas concentrações testadas. A taxifolina foi capaz de inibir todas as bactérias testadas, eliminar biofilmes de *E. faecalis* e *S. mutans* em placas de poliestireno e reduzir significativamente o biofilme de *E. faecalis* em túbulos dentinários. O capítulo 2 avaliou a citotoxicidade do flavonoide taxifolina e o seu potencial sobre a indução de marcadores de mineralização dentinária (produção de fosfatase alcalina - ALP, nódulos de mineralização – NM e expressão dos genes DSPP – sialofosfoproteína dentinária e

DMP-1 – proteína da matriz dentinária - 1) em células semelhantes a odontoblastos MDPC-23, após tratamentos de 24, 72h e contínuo. A taxifolina não apresentou citotoxicidade em nenhum dos três tipos de tratamento analisados. Todas as concentrações do tratamento de 24h e as concentrações de 10 e 5 μ M do tratamento de 72h aumentaram a atividade de ALP. A formação de NM aumentou com os tratamentos de taxifolina à 10 μ M em ambos os tratamentos de 24 e 72h, e à 5 μ M no tratamento de 24h. A expressão de DMP-1 aumentou com o tratamento de taxifolina em ambos os tratamentos de 24 e 72h, enquanto que a de DSPP aumentou apenas com o tratamento de 72h na concentração de 5 μ M. O capítulo 3 avaliou a citotoxicidade da taxifolina, e o seu potencial sobre a indução de marcadores de mineralização óssea (ALP, NM e expressão dos genes ALP e colágeno 1 - Col-1) em células semelhantes a osteoblastos Saos-2, após tratamentos de 24, 72h e contínuo. Os resultados mostraram que os tratamentos com taxifolina nas concentrações de 10, 5 e 1 μ M não foram citotóxicos em nenhum dos períodos analisados. O tratamento de 72h da taxifolina à 10 μ M foi capaz de aumentar a atividade de ALP e a formação de NM, além de aumentar a expressão de Col-1 após 13 dias. O tratamento de 24h da taxifolina na concentração de 10 μ M aumentou a expressão de ALP após 6 dias. Conclui-se que a taxifolina é um flavonoide com potencial uso para o tratamento endodôntico de dentes permanentes jovens, devido à sua ação antimicrobiana/antibiofilme, baixa citotoxicidade e capacidade de estimular a mineralização em odontoblastos e osteoblastos.

Palavras-chave: Flavonoides, testes de sensibilidade microbiana, dentinogênese, osteogênese, técnicas de cultura de células.

Abstract

Massunari, L. **Effect of flavonoids on endodontic microorganisms and their influence on the expression of mineralization markers.** [Thesis] São Paulo State University (Unesp), School of Dentistry, Araçatuba, 2018.

Abstract

The endodontic treatment of young permanent teeth represents a great clinical challenge due to the presence of thin, divergent or parallel dentin walls and the open apex that makes it difficult to disinfect and perform conventional endodontic procedures. Endodontic regeneration therapies involve the use of materials capable of promoting effective disinfection without causing cytotoxicity, in addition to inducing differentiation of stem cells or biostimulating remaining pulp tissue cells even after injury. In this context, the flavonoids, polyphenols present in fruits and vegetables, could be interesting agents for the endodontic treatment of immature teeth due to the wide therapeutic use. Thus, the objective of the present study was to evaluate the antimicrobial, cytotoxic effects and capacity of mineralization induction of flavonoids for endodontic application. This thesis was divided into three chapters. The chapter 1 evaluated the toxicity of taxifolin, chrysin, pinocembrin and galangin flavonoids on fibroblasts by the MTT method, antimicrobial activity by determining the minimum inhibitory and bactericidal concentrations and analyzed the antibiofilm action of the flavonoid with the best antimicrobial effect, by means of the biofilm assays in polystyrene plates and in bovine root dentin and confocal microscopy analysis. The results showed that the flavonoid taxifolin was not toxic on fibroblasts in any tested concentration, while chrysin, pinocembrin and galangin flavonoids showed cytotoxic effects. Chrysin, pinocembrin and galangin showed no antimicrobial effect against *E. faecalis* and *S. mutans* in any tested concentrations. Taxifolin was able to inhibit all tested bacteria, to eliminate *E. faecalis* and *S. mutans* biofilms on polystyrene plates and significantly reduce *E. faecalis* biofilms from the dentin tubules. The chapter 2 evaluated the cytotoxicity of taxifolin and its potential on the induction of dentin mineralization markers (alkaline phosphatase production - ALP, mineralization nodules - MN and expression of genes DSPP - dentin sialophosphoprotein and DMP-1 - dentin matrix protein - 1) on odontoblast-like cells MDPC-23, after treatments of 24, 72h and continuous. Taxifolin did not present cytotoxicity at any of the three types of

treatments analyzed. All concentrations of 24h-treatment and 10 and 5 μ M of 72h-treatment increased ALP activity. NM formation increased with taxifolin treatments at 10 μ M in both 24 e 72h treatments, and at 5 μ M in the 24h-treatment. Expression of DMP-1 increased with taxifolin in both 24 e 72h-treatments, whereas DSPP expression increased only with 72h-treatment at 5 μ M. The chapter 3 evaluated the cytotoxicity of taxifolin and its potential on the induction of bone mineralization markers (ALP, NM and expression of ALP and collagen 1 -Col-1 genes) on Saos-2 osteoblast-like cells, after treatments of 24, 72h and continuous. The results showed that taxifolin treatments at 10, 5 and 1 μ M were not cytotoxic in any of the periods analyzed. The 72h-treatment of taxifolin at 10 μ M was able to increase ALP activity and NM formation, in addition to increasing Col-1 expression after 13 days. The 24h-treatment of taxifolin at 10 μ M increased ALP expression after 6 days. It is concluded that taxifolin is a flavonoid with potential use for endodontic treatment of young permanent teeth due to its antimicrobial/antibiofilm action, low cytotoxicity and ability to stimulate mineralization in odontoblasts and osteoblasts.

Keywords: Flavonoids, microbial sensitivity tests, dentinogenesis, osteogenesis, cell culture techniques.

Lista de Abreviaturas

Lista de abreviaturas

A. israelii – *Actinomyces israelii*

ALP – Fosfatase alcalina (*alkaline phosphatase*)

ANOVA – Análise de variância (*analysis of variance*)

BHI – Infusão cérebro e coração (*brain heart infusion*)

BSA – Albumina do soro bovino (*bovine serum albumin*)

C. albicans – *Candida albicans*

cDNA – Ácido desoxirribonucleico complementar (*complementary deoxyribonucleic acid*)

CFU – Unidades formadoras de colônia (*colony-forming unit*)

CH – Hidróxido de cálcio (*calcium hydroxide*)

CHX – Digluconato de clorexidina (*chlorhexidine digluconate*)

CLSI – Instituto de padrões clínicos e laboratoriais (*Clinical and Laboratory Standard Institute*)

CLSM – Microscopia confocal de varredura a laser (*confocal laser scanning microscopy*)

CO₂ – Gás carbônico (*carbon dioxide*)

Col-1 – Colágeno 1 (*collagen 1*)

Ct – Ciclo limiar (*Threshold cycle*)

CT – Tratamento contínuo (*continuous treatment*)

d – Dias (*days*)

DAP – Pasta biantibiótica (*double antibiotic paste*)

$\Delta\Delta Ct$ – Delta delta ciclo limiar (*delta delta threshold cycle*)

DMEM – Meio de Eagle modificado por Dulbecco (*Dulbecco Modified Eagle's Medium*)

DMP-1 – Proteína da matriz dentinária (*dentin matrix protein – 1*)

DMSO – Dimetilsulfóxido (*dimethyl sulfoxide*)

DPSCs – Células-tronco da polpa dentária (*Dental pulp stem cells*)

DSPP – Sialofosfoproteína dentinária (*dentin sialophosphoprotein*)

E. coli – *Escherichia coli*

E. faecalis – *Enterococcus faecalis*

EGCG – Epigallocatequina galato (*Epigallocatechin gallate*)

FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (*São Paulo Research Foundation*)

FBS – Soro fetal bovino (*Fetal bovine serum*)

FIOCRUZ – Fundação Oswaldo Cruz (*Oswaldo Cruz Foundation*)

FOA – Faculdade de Odontologia de Araçatuba (*School of Dentistry, Araçatuba*)

g – Aceleração da gravidade (*Gravitational acceleration*)

g/mL – Gramas por mililitros (*Grams per milliliters*)

GAPDH – Gliceraldeído-3-fosfato desidrogenase (*Glyceraldehyde-3-phosphate dehydrogenase*)

h – Horas (*Hours*)

HDPCs – Células da polpa dentária humana (*Human dental pulp cells*)

HGF – Fibroblastos gengivais humanos (*Human gingival fibroblasts*)

IL- 1 – Interleucina 1 (*Interleukin 1*)

IL-17 – Interleucina 17 (*Interleukin 17*)

IL-1 β – Interleucina 1 beta (*Interleukin 1 beta*)

IL-6 – Interleucina 6 (*Interleukin 6*)

ISO – Organização internacional de normalização (*International Organization for Standardization*)

L929 – Linhagem celular de fibroblastos de camundongo (*Murine fibroblast cell line*)

LDH – Lactato desidrogenase (*Lactate dehydrogenase*)

Log – Logaritmo (*Logarithm*)

MBC – Concentração bactericida mínima (*Minimum bactericidal concentration*)

MDPC-23 – Células da papila dentária de camundongo (*Mouse dental papilla cells*)

mg/mL – Miligramas por mililitros (*Milligrams per milliliters*)

MIC – Concentração inibitória mínima (*Minimum inhibitory concentration*)

min – Minutos (*Minutes*)

mm – Milímetros (*Millimeters*)

mM – Milimolar (*Millimolar*)

mmol/L – Milimol por litro (*Milimol per liter*)

mRNA – Ácido ribonucleico mensageiro (*Messenger ribonucleic acid*)

MSA – Ágar mitis salivarius (*Mitis salivarius agar*)

MTA – Agregado de trióxido mineral (*Mineral trioxide aggregate*)

MTT – Brometo de 3- (4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

n – Tamanho da amostra (*Sample size*)

NF- κ B - Fator nuclear Kappa beta (*Nuclear factor kappa beta*)

nm – Nanômetros (*Nanometers*)

NM – Nódulos de mineralização (*Mineralization nodule*)

$^{\circ}$ C - Grau Celsius (*Degrees Celsius*)

OD – Densidade óptica (*Optical density*)

OH – Radical hidroxila (*Hydroxyl radical*)

PDSCs – Células-tronco do ligamento periodontal (*Human periodontal ligament stem cells*)

qPCR – Reação da polimerase em cadeia (*Quantitative Polymerase Chain Reaction*)

RANK - Receptor ativador do fator nuclear Kappa beta (*Receptor activator of nuclear factor-kappa beta*)

RANKL - Ligante do receptor ativador do fator nuclear Kappa beta (*Receptor activator of nuclear factor-kappa beta ligand*)

RE – Regeneração endodôntica (*Regenerative Endodontics*)

RET – Terapias de regeneração endodôntica (*Regenerative endodontic therapies*)

RNA – Ácido ribonucleic (*Ribonucleic acid*)

S. aureus – *Staphylococcus aureus*

S. mutans – *Streptococcus mutans*

Saos-2- Linhagem de células semelhantes a osteoblastos humanos (*Human osteoblast-like cell line*)

SD = Desvio padrão (*Standard deviation*)

SHED – Células-tronco de dentes decíduos exfoliados humanos (*Stem cells from human exfoliated deciduous teeth*)

TAP – Pasta triantibiótica (*Triple antibiotic paste*)

TNF- α – Fator de necrose tumoral alfa (*Tumor necrosis factor alpha*)

U/L – Unidades por litro (*Units per liter*)

U/mL – Unidades por mililitros (*Units per milliliters*)

UNESP – Universidade Estadual Paulista (*São Paulo State University*)

VPT – Terapia de vitalidade pulpar (*Vital pulp therapy*)

µg/mL – Microgramas por mililitros (*Micrograms per milliliters*)

µL – Microlitros (*Microliters*)

µm – Micrometros (*Micrometers*)

µM – Micromolar (*Micromolar*)

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Introdução Geral

Introdução geral

Após a erupção do dente permanente na cavidade bucal ainda são necessários aproximadamente 3 anos para o desenvolvimento completo das raízes e o fechamento dos respectivos ápices radiculares (Bhasker 1991). Desta forma, durante esta fase, a ocorrência de traumatismos dentários ou lesões de cárie que comprometam a vitalidade do tecido pulpar, podem interromper a formação da raiz, resultando na presença de paredes dentinárias finas, divergentes ou paralelas e ápice aberto, o que dificultará a execução dos procedimentos endodônticos convencionais, vindo a comprometer o resultado do tratamento a longo prazo (Rafter 2005, Iglesias-Linares *et al.* 2013). Além disso, nesses casos de dentes permanentes jovens, além da necessidade de um tratamento que promova a descontaminação do sistema de canais radiculares, é desejável que esse permita a continuação do desenvolvimento radicular, bem como a recuperação dos tecidos periapicais quando já estiverem comprometidos (Hargreaves *et al.* 2013).

Durante muitos anos, o tratamento de dentes permanentes com rizogênese incompleta baseou-se na vitalidade do tecido pulpar afetado. Assim, para dentes que apresentam vitalidade pulpar tem se indicado procedimentos, como a pulpotomia, que consiste na remoção da polpa infectada e manutenção da polpa radicular, estimulando o desenvolvimento fisiológico contínuo e o término da formação radicular pelo processo de apicogênese. Os casos de dentes que apresentam vitalidade pulpar, porém o tecido pulpar encontra-se inflamado irreversivelmente, bem como os casos de dentes que apresentam necrose pulpar indica-se a 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 2003; Rafter 2005; Soares *et al.* 2008).

Na técnica de apicificação, o hidróxido de cálcio (HC) ainda é o material mais empregado, permitindo que ocorra o selamento biológico pela formação de um tecido duro, semelhante ao cimento. Entretanto, a capacidade indutora de mineralização do

HC é dependente da manutenção da alcalinidade do mesmo, durante todo o período de tratamento, que varia de 8 – 12 meses (Soares *et al.* 2008). Sendo assim, são necessárias trocas periódicas do material, visando renovar a sua propriedade alcalina. Além da necessidade de múltiplas sessões, o que aumenta o risco de contaminação; a apicificação resulta na formação de paredes dentinárias delgadas e desenvolvimento radicular incompleto, tornando o dente suscetível à fratura (Hargreaves *et al.* 2013).

A técnica de apicificação também pode ser empregada por meio da colocação de uma barreira apical artificial - constituída por materiais à base de agregado de trióxido mineral (MTA) - que permite a obturação do canal radicular logo após o protocolo de descontaminação (American Association of Endodontists, 2003). O MTA tem sido indicado principalmente em casos onde há a impossibilidade de acompanhamento a longo-prazo, e tem se mostrado capaz de induzir a formação da barreira apical, semelhantemente ao HC (Shabahang *et al.* 1999). Além da redução no número de sessões durante o tratamento (Damle *et al.* 2012, Marí-Beffa *et al.* 2017), o emprego do MTA também tem sido associado a um menor número de fraturas (Jeeruphan *et al.* 2012). Entretanto, a técnica de apicificação, seja com HC ou plug de MTA, não permite o desenvolvimento radicular completo, tanto em espessura quanto em comprimento (Jeeruphan *et al.* 2012), além de não restabelecer funcionalmente o tecido pulpar (Hargreaves *et al.* 2013).

Recentemente, terapias biológicas denominadas de regeneração endodôntica (RE), que visam recuperar as funções fisiológicas de estruturas do complexo dentino-pulpar e permitir a formação radicular completa estão sendo estudadas (Hargreaves *et al.* 2013, Marí-Beffa *et al.* 2017). Banchs e Trope (2004) trouxeram o conceito de RE pela técnica de revascularização, por meio da indução do sangramento apical após o emprego de uma pasta triantibiótica, observando então o desenvolvimento contínuo da raiz, evidenciado não só pelo aumento do seu comprimento, mas também pelo espessamento das paredes do canal radicular. Embora a técnica de revascularização tenha se mostrado eficaz na continuação da formação radicular (Petrino *et al.* 2010) alguns estudos avaliaram o tecido neoformado no espaço do canal radicular e observaram que o mesmo corresponde a um tecido conjuntivo semelhante ao ligamento periodontal, e a um tecido semelhante ao cimento ou cimento - ósseo

(Becerra *et al.* 2014, Torabinejad *et al.* 2015). Dessa forma, do ponto de vista clínico, a regeneração do tecido perdido não pode ser alcançada, visto que não há o restabelecimento da vascularização, inervação e deposição de dentina como no tecido pulpar normal. Técnicas da bioengenharia têm sido propostas visando a formação de um tecido semelhante à polpa, bem como a diferenciação odontoblástica, por meio do uso de “*scaffolds*” - estruturas tridimensionais que permitem a adesão, proliferação e diferenciação de células tronco mesenquimais - associado ao emprego de biomateriais ou moléculas de sinalização que estimulem esses processos (Hargreaves *et al.* 2008), como o fator de crescimento fibroblástico básico (bFGF) (Chang *et al.* 2017), fator de crescimento derivado de plaquetas (PDGF) (Cai *et al.* 2016), plasma rico em plaquetas (PRP) (Torabinejad & Faras 2012, Torabinejad *et al.* 2015), fibrina rica em plaquetas (PRF) (Subash *et al.* 2016), entre outros.

A RE em dentes permanentes jovens apresenta um prognóstico favorável devido à ampla comunicação entre o canal radicular e os tecidos periapicais. Essa vascularização tem se mostrado capaz de manter a viabilidade celular mesmo após danos aos tecidos pulpar e/ou periapicais (Tsukiboshi *et al.* 2017). Células-tronco são células progenitoras que apresentam ampla capacidade de auto-renovação e diferenciação (Gronthos *et al.* 2002, Huang *et al.* 2006, Limjeerajarus *et al.* 2014, Maglione *et al.* 2017), podendo ser classificadas em totipotentes - apresentam a capacidade de originar qualquer tipo celular, inclusive tecidos extraembrionários como a placenta; pluripotentes – originam todas as células de um organismo, exceto de tecidos extraembrionários; e multipotentes – originam linhagens específicas de células (Rodriguez-Lozano *et al.* 2011). Células-tronco mesenquimais apresentam grande potencial em procedimentos de RE, uma vez que estão presentes na própria polpa dentária - DPSCs (Gronthos *et al.* 2002), dentes decíduos – SHED (Miura *et al.* 2003), ligamento periodontal - PDLSCs (Jo *et al.* 2007) e papila apical - SCAP (Huang *et al.* 2008). Mesmo na presença de processo inflamatório, células da polpa dentária e da região periapical, apresentaram alta expressão de marcadores de células-tronco mesenquimais (Alongi *et al.* 2010, Liao *et al.* 2011).

A busca por tratamentos capazes de induzir a diferenciação de células (Asgary *et al.* 2014, Chen *et al.* 2015, Huang *et al.* 2016), bioestimular células remanescentes

em concentrações não tóxicas (Washington *et al.* 2011, Kuang *et al.* 2016, Chen *et al.* 2015), promover uma desinfecção eficaz sem citotoxicidade (Bottino *et al.* 2013, Palasuk *et al.* 2014, Kamocki *et al.* 2015), ou ainda criar um *scaffold* que permita a proliferação e diferenciação celular, além de oferecer suporte adequado para biomoléculas (Bottino *et al.* 2015, Kuang *et al.* 2016) tem sido alvo de diversos estudos no campo da RE. A análise de marcadores de diferenciação osteogênica após determinado tratamento proposto em células indiferenciadas (d'Aquino *et al.* 2007, Wang *et al.* 2017), ou ainda em células diferenciadas que poderiam ser remanescentes à determinada injúria (Satué *et al.* 2013, Zeng *et al.* 2013, Liu *et al.* 2017) tem sido amplamente realizada com o intuito de analisar a capacidade bioestimulatória de diferentes materiais, visando induzir o processo de reparo. Dentre esses marcadores de mineralização estão a fosfatase alcalina (ALP – enzima responsável pela mineralização da matriz dentinária ou óssea); cuja expressão ocorre em células envolvidas no processo de mineralização, como os odontoblastos e osteoblastos, a proteína da matriz dentinária (DMP-1) e a sialofosfoproteína dentinária (DSPP) que são proteínas não-colagenosas que participam da mineralização da dentina e maturação das fibras colágenas durante o processo de dentinogênese. Essas proteínas permanecem no interior do substrato dentinário, sendo liberadas em resposta às injúrias teciduais, para estimular odontoblastos primários a produzirem dentina terciária reacional, ou ainda para estimular a diferenciação de células pulpares em odontoblastos que irão produzir dentina terciária reparadora (Ferracane *et al.* 2010, de Souza Costa *et al.* 2014).

A desinfecção do sistema de canais radiculares também representa um desafio dentro das terapias de RE, uma vez que esses dentes permanentes jovens apresentam canal radicular e ápice amplos, além de paredes dentinárias delgadas, inviabilizando o preparo biomecânico convencional. A pasta triantibiótica (metronidazol, ciprofloxacina e minociclina) tem se mostrado eficaz em tratamentos de RE para dentes imaturos (Windley *et al.* 2005, Petrino *et al.* 2010); entretanto, foi observada uma significativa redução na viabilidade de células-tronco da papila apical (Ruparel *et al.* 2012) e fibroblastos do ligamento periodontal (Yadlapati *et al.* 2014) após a sua aplicação. Esse efeito citotóxico também foi demonstrado em células da polpa humana, onde a pasta

na concentração de 2,5mg/mL, ou seja bem menor do que aquelas empregadas durante as terapias de RE (aproximadamente 1g/mL), foi capaz de reduzir a proliferação celular (Labban *et al.* 2014). Devido à algumas desvantagens, como a descoloração dentinária (Kim *et al.* 2010), a minociclina – antibiótico semissintético derivado da tetraciclina – foi removida da pasta triantibiótica. A pasta biantibiótica (metronidazol e ciprofloxacina) tem apresentado bons resultados frente à infecção endodôntica (Iwaya *et al.* 2001), porém com maior efeito citotóxico, reduzindo a viabilidade de células pulpares à partir da concentração de 0,5mg/mL (Labban *et al.* 2014). De maneira semelhante, o hidróxido de cálcio também não demonstrou toxicidade em baixas concentrações (até 2,5mg/mL), entretanto a concentração utilizada clinicamente é de aproximadamente 0,7g/mL (Labban *et al.* 2014).

Compostos biativos derivados de plantas como os flavonoides, têm recebido destaque na literatura devido à sua amplitude terapêutica. Os flavonoides são moléculas polifenólicas presentes em frutas e vegetais (Panche *et al.* 2016) que apresentam propriedades antimicrobiana, antioxidante, osteogênica e antiosteoclastogênica (Pietta 2000, Tripoli *et al.* 2007, Sharan *et al.* 2009, Pilsakova *et al.* 2010, Domitrovic 2011). Dentre eles, a taxifolina, um flavonoide tipo catecol, isolado de diversas plantas (da Costa *et al.* 2014, Wang *et al.* 2015, Park *et al.* 2016), e mais recentemente do chá verde (Wang *et al.* 2017), tem apresentado ampla ação terapêutica, entre as quais, atividade antimicrobiana contra *Enterococcus faecalis* resistente a vancomicina (Jeong *et al.* 2009), *Acinetobacter hemolyticus* (Chatzopoulou *et al.* 2010); atividade antifúngica (Kanwal *et al.* 2010); capacidade de estimular o aumento da expressão gênica de osteocalcina, osteoprotegerina e sialoproteína óssea, além de diminuir a expressão de RANKL (marcador de reabsorção óssea) em osteoblastos (Satué *et al.* 2013) e estimular a diferenciação de células-tronco mesenquimais em osteoblastos (Córdoba *et al.* 2015, Wang *et al.* 2017).

Própolis, substância resinosa produzida por abelhas por meio da mistura de suas secreções com produtos obtidos de diversas plantas, tem sido amplamente estudada devido as suas propriedades terapêuticas (Yokoyama *et al.* 2014, Aral *et al.* 2015, Asawahame *et al.* 2015). Dentre essas, a sua atividade antimicrobiana tem sido relatada por alguns autores (Uzel *et al.* 2005, de Luca *et al.* 2014), inclusive frente à

microrganismos de interesse endodôntico, como *Enterococcus faecalis*, *Candida albicans*, *Streptococcus mutans* e *Pseudomonas aeruginosa* (Uzel *et al.* 2005). Dentre os principais compostos isolados da própolis estão os flavonoides galangina, crisina e pinocembrina.

Huh *et al.* (2013) observou que a galangina reduziu as citocinas inflamatórias IL-1 β , TNF- α e IL-17; inibiu a formação de osteoclastos e fatores osteoclastogênicos; além de aumentar a expressão gênica de osteoprotegerina em osteoblastos. Liu *et al.* (2017) também observaram um aumento na expressão de marcadores de diferenciação osteogênica, como colágeno, fosfatase alcalina (ALP), osteocalcina e osteopontina em células derivadas de osteosarcoma após o tratamento com galangina. Esse flavonoide mostrou ainda atividade inibitória contra *S. aureus* e *Enterococcus* (Li *et al.* 2012), apresentando como possível mecanismo de ação um dano direto à membrana citoplasmática ou indireto à parede celular, por provocar a perda de íons potássio (Cushnie e Lamb 2005). O flavonoide crisina também demonstrou atividade contra bactérias Gram positivas e Gram negativas, incluindo *S. aureus* e *E. coli* com concentrações inibitórias mínimas (CIM) que variaram de 50 à 6,25 $\mu\text{g/mL}$ (Liu *et al.* 2010) e promoveu aumento da expressão de marcadores osteogênicos, como colágeno, osteocalcina e osteopontina em osteoblastos (Zeng *et al.* 2013). A pinocembrina apresentou atividade antimicrobiana contra *C. albicans* e *S. aureus*, com CIMs de 6.25 e 12.5 $\mu\text{g/ml}$ respectivamente (Katerere *et al.* 2012), além de atenuar a resposta inflamatória induzida por lipopolissacarídeos (Giri *et al.* 2016). Assim, os flavonoides apresentam diversas propriedades desejáveis nas terapias de regeneração endodôntica devido às suas múltiplas funções biológicas.

Proposição

Proposição

O objetivo geral do presente trabalho foi avaliar o efeito antimicrobiano, citotóxico e indutor de mineralização de flavonoides com finalidade de aplicação endodôntica, tanto em procedimentos de vitalidade pulpar quanto na regeneração endodôntica.

Os objetivos específicos foram:

- Avaliar o efeito citotóxico dos flavonoides taxifolina, crisina, galangina e pinocembrina em cultura de fibroblastos (L-929);
- Avaliar a atividade antimicrobiana dos flavonoides frente *Streptococcus mutans*, *Enterococcus faecalis* e *Actinomyces israelii*;
- Avaliar o efeito antibiofilme do flavonoide taxifolina em biofilme formado em placas de poliestireno e túbulos dentinários;
- Avaliar o efeito citotóxico de diferentes tratamentos com a taxifolina em células semelhantes a odontoblastos (MDPC-23) e a osteoblastos (Saos-2);
- Avaliar a atividade da fosfatase alcalina e produção de nódulos de mineralização após os tratamentos com a taxifolina nas células MDPC-23 e Saos-2;
- Avaliar a expressão gênica de DSPP e DMP-1 nas células MDPC-23 após 24 e 72h de tratamento com a taxifolina;
- Avaliar a expressão gênica de ALP e Col-1 nas células Saos-2 após 24 e 72h de tratamento com a taxifolina.

Capítulo 1

Cytotoxic, antibacterial and anti-biofilm activities of flavonoids intended for endodontic purposes*

Abstract

The rhizogenesis process can be interrupted after dental pulp injury. The aim of this study was to evaluate the toxicity on fibroblasts, antibacterial and anti-biofilm of flavonoids. MTT assays were conducted to analyze taxifolin, chrysin, pinocembrin, and galangin treatments on fibroblasts viability. Minimal inhibitory and bactericidal concentrations were determined for the same flavonoids against *Enterococcus faecalis*, *Actinomyces israelii*, and *Streptococcus mutans*. Biofilm assays were performed only with taxifolin in polystyrene plates and inside dentin tubules. The results showed that taxifolin had no toxic effect on fibroblasts, while the other flavonoids showed cytotoxic effects. Taxifolin was the unique flavonoid able to inhibit all tested bacteria growth through the microdilution broth method, besides to eliminate *E. faecalis* and *S. mutans* biofilms in polystyrene plates, and reduce *E. faecalis* biofilm on dentin tubules. Taxifolin presents potential use in permanent teeth for endodontic purposes, due to its antimicrobial/anti-biofilm effects and low toxicity on fibroblasts.

Keywords: Flavonoids, Cell Culture Techniques, Microbial Sensitivity Tests, Biofilms.

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

Introduction

Dental caries or trauma in immature permanent teeth can interrupt the normal apical closure process due to pulp tissue injury. The exposed pulp allows root canal contamination by bacteria from the oral cavity or those found in deep dentinal caries, such as *Streptococcus mutans* and *Actinomyces israelii* (van Houte et al. 1996; Munson et al. 2004; Rôças et al. 2015), which are able to invade the pulp via dentinal tubules and trigger an endodontic infection. Primary endodontic infection is polymicrobial in nature, probably due to oxygen variation and nutrient availability in root canal thirds. Studies have reported a prevalence of facultative anaerobic bacteria in primary endodontic infections (Gomes et al. 2006; Sedgley et al. 2006; Ruvierre et al. 2007; Ledezma-Rasillo et al. 2010) and similarity in the microbial composition between permanent young teeth and completely formed teeth (Baumotte et al. 2011). Due to a wide root canal, open apex, and thin dentinal walls, chemo-mechanical root canal preparation is limited and therefore disinfection occurs mainly by intracanal medicament.

For a long time the treatment of choice for young permanent teeth was apexification with calcium hydroxide (CH). Despite the success attributed to this technique in virtue of formation of an apical hard tissue barrier (Rafter et al. 2005), the multiple sessions and long-term treatment frequently increases the potential for recontamination, the formation of fragile dentin walls, and subsequent susceptibility to fractures (Hargreaves et al. 2013). Another technique that has been used is artificial apical barrier through the application of mineral trioxide aggregate (MTA) as a plug in the tooth apical third. One advantage of the MTA technique is the reduction in clinical appointments (Marí-Beffa et al. 2017). Inevitably, both these techniques lead to loss of vital pulp tissues including odontoblasts, fibroblasts, stem cells, and the Hertwig epithelial root sheath need for complete root development (Chueh et al. 2006). Studies have appointed higher levels of mesenchymal stem cell markers in dental pulp stem cells even in inflamed pulps or in periapical progenitor cells from inflamed periapical tissue collected during endodontic surgical procedures (Alongi et al. 2010; Liao et al. 2011).

Regenerative endodontic therapies (RET) have been considered as bioengineering therapies as part of the search for new materials that allow the synthesis of a new pulp-like tissue able to restore lost functions (Mohammad et al. 2015; Marí-Beffa et al. 2017). RET are possible in any permanent teeth, however immature permanent teeth present apical enlargement that provides communication between root canal and periapical tissues providing a large blood supply, carrying cellular and molecular components of the immune system (Tsukiboshi et al. 2017). Immature teeth also have been presented a large number of undifferentiated mesenchymal cells and odontoblasts than adult teeth (de Souza Costa et al. 2014), likely due the recruitment of these cells during physiological tooth wear, carious process and cavity preparation in adult teeth.

The material most commonly used based on these techniques is the triple antibiotic paste (TAP - metronidazole, minocycline, and ciprofloxacin) for microbial decontamination followed by the induction of revascularization by blood clot formation (Petrino et al. 2010). However, the use of TAP has generated some discussion regarding bacterial resistance, crown discoloration, and possible allergic reaction (Cohenca et al. 2010; Kim et al. 2010; Akcay et al. 2014). Others antimicrobials commonly used in RET, such as CH and double antibiotic paste (DAP – metronidazole and ciprofloxacin) also had show drawbacks related to their cytotoxic effect. Safest non-toxic concentrations of TAP, DAP and CH were lower than the intracanal concentrations that have been advocated in RET (Labban et al. 2014).

Plants are a wide source of bioactive compounds, which present several biological activities (Dimech et al. 2013; Coronado-Aceves et al. 2016; Wang et al. 2017). Among these natural compounds, flavonoids are an important class known for their therapeutic use. They are plant secondary metabolites, mainly pigments that color flowers, fruits, and seeds, constituted by a polyphenolic structure (Panche et al. 2016). Taxifolin is a catechol-type flavonoid isolated from *Hymenaea courbaril* (da Costa et al. 2014) *Morus laevigata* (Wang et al. 2015), *Hovenia dulcis* (Park et al. 2016), and green tea (Wang et al. 2017) among others. Studies have reported the antimicrobial potential (Chatzopoulou et al. 2010; Kanwal et al. 2010; Jeong et al. 2009) of taxifolin and its ability to stimulate osteogenic differentiation (Satué et al.

2013; Wang et al. 2017). Other flavonoids frequently isolated from propolis, such as pinocembrin, galangin, and chrysin have been related to antimicrobial activity of this resinous substance produced by bees (Uzel et al. 2005; Suleman et al. 2015). Among them, galangin has also inhibited osteoclastogenic factors (Hu et al. 2013) and both galangin and chrysin enhanced the expression of osteoblast differentiation markers and mineralization (Liu et al. 2017; Zeng et al. 2013).

Based on the fact that the commonly used antimicrobial agents have presented cell toxicity in the concentrations advocated for endodontic regeneration (Yadlapati et al. 2014; Labban et al. 2014), the aim of this study was to evaluate the antimicrobial activity and toxicity of taxifolin, chrysin, pinocembrin, and galangin on fibroblasts, besides to analyze the anti-biofilm effect of the flavonoid with the best antimicrobial effect, intended to be used for endodontic purposes. The null hypotheses tested were: (1) flavonoids present cytotoxic effect on L929 fibroblasts, (2) flavonoids do not have antibacterial activity, and (3) taxifolin does not have anti-biofilm effect.

Materials and Methods

Cell viability assays

Flavonoids

Taxifolin (#78666), chrysin (#C80105), pinocembrin (#P5239), and galangin (#282200) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution was prepared in Dimethyl Sulfoxide (DMSO - Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C for each flavonoid. Solution with the final concentration of 0.5% DMSO was included as the control group in the experiments. Chlorhexidine digluconate (CHX, Sigma Aldrich, St. Louis, MO, USA) was used as a positive control.

Cell Lines

Mouse fibroblasts (L-929) were grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, Gaithersburg, MD) streptomycin (50 g/mL), and 1% antibiotic/antimycotic cocktail (300 U/mL penicillin, 300 mg/mL streptomycin, 5 mg/mL amphotericin B, and L-glutamine 0.3 g/L)

(GIBCO BRL, Gaithersburg, MD) under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% CO₂).

MTT assay

The cells were plated at a density of 1×10^5 cells/well in 96-well plates and incubated for 24 hours in a humidified air atmosphere of 5% at 37°C to allow cell attachment. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cell viability according to Takamiya et al. (2016). Briefly, after the cell attachment, the flavonoid groups (600, 300, 150, 75, 37.5, 18.75, and 9.37 µg/mL) were added to the cells. The controls were cultured in medium without flavonoid treatment. At 24h after addition, the treatments were removed and the MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells and the plates were incubated at 37°C for 4h protected from light. This tetrazolium salt is metabolically reduced by viable cells to yield a purple insoluble formazan crystal. Next, the MTT solution was discarded and 200 µL of isopropyl alcohol was added to each well to dissolve the crystals under continuous agitation for 30 min. The solution was transferred to a 96-well plate to measure the optical density (OD) at 570nm in a spectrophotometer (Shimadzu MultSpec-1501; Shimadzu Corporation, Tokyo, Japan). The experiments were performed in triplicate.

Antimicrobial assay

Microbial conditions

The following microbial strains used in the present study were kindly provided by the Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, RJ, BR): *Streptococcus mutans* (ATCC 25175), *Enterococcus faecalis* (ATCC 51299), and *Actinomyces israelii* (ATCC 12102). Stock microbial suspensions were inoculated in Brain Heart Infusion Agar (BHI, Difco Laboratories, Detroit, MI, USA) for *E. faecalis* and *A. israelii* or Mitis Salivarius Agar base (MSA, Difco Laboratories) with 0.2 U mg ml⁻¹ bacitracin (Sigma-Aldrich) for *S. mutans*. Representative colonies of these strains were grown overnight in BHI broth (Difco Laboratories) and incubated at 37°C for 24 hours in 5% CO₂ (Incubator Ultra Safe, HF212-UV). All microorganisms were incubated in these

atmospheric conditions to simulate the low oxygen concentration inside root canals. Growth curve assays were performed for each microorganism in order to determine the optical density at the mid-log phase [approximately 0.5 ($1-5 \times 10^8$ CFU/mL)] to be used in the following experiments. The absorbance was measured using a microplate reader (Eon Microplate Spectrophotometer, BioTek Instruments, Winooski, Vermont, USA) to assess the cell growth.

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

MIC and MBC were determined through the microdilution broth method, in 96-well microtiter plates, following the criteria previously described by Clinical and Laboratory Standard Institute M7-A9 (CLSI, 2012). The final concentration of bacterial suspension in the wells was $1-5 \times 10^5$ CFU/mL. Initially, the flavonoid stock solutions were serially diluted in water and after that, correctly adjusted microbial suspensions were inoculated in each well. The flavonoid concentrations tested ranged from 2 – 0.0002 mg/mL. The plates were incubated at 37°C for 24 hours in a 5% CO₂ atmosphere. Afterwards, 15 µL of 0.01% resazurin (R7017 Sigma-Aldrich – St. Louis, Missouri, USA) was applied in each well and incubated for 4 hours to promote oxidation-reduction and determine the cell viability through visual detection of the color change. Posteriorly the wells corresponding to MIC (the last blue well) and at least three previous wells were homogenized, six times diluted and plated on Mueller-Hinton Agar (Difco Laboratories) for *E. faecalis* and *A. israelii* or Mitis Salivarius Agar base (Difco Laboratories) with 0.2 U mg ml⁻¹ bacitracin (Sigma-Aldrich) for *S. mutans* to determine the MBC. The plates were incubated at 37°C for 24 hours in a 5% CO₂ atmosphere. The colonies were counted and the number of viable bacteria determined in CFU/mL. The MBC was considered when the flavonoid treatment killed more than 99% of the tested microbial culture. Chlorhexidine digluconate (CHX) was used as a positive control. The negative control was the culture without antimicrobial agents. Assays were repeated three times for each microorganism, in three independent experiments.

Anti-biofilm assays

Biofilm formation in polystyrene microplates

Taxifolin was selected for biofilm assays as it presented the best results in the previous assays: MTT, MIC, and MBC. Biofilm assays were conducted according to Massunari et al. (2017) with *E. faecalis*, *S. mutans*, and *A. israelii*. Twenty microliters of each microorganism suspension (approximately $1-5 \times 10^6$ CFU/mL) were inoculated in sterile U-shaped bottom polystyrene 96-well microplates containing 180 μ L of BHI supplemented with 0.5% glucose. The plates were incubated at 37°C in a 5% CO₂ atmosphere. After 48 hours, the culture medium was removed and the wells were washed with sterile saline for subsequent addition of 200 μ L of previously diluted taxifolin per well. The concentration of taxifolin was 10 times higher than the MBC concentration (10x MBC) obtained for each microorganism. The microplates were incubated again in the same conditions for 24 hours. All cultures in the wells were diluted six times and plated in brain heart infusion agar and incubated for 24 hours. After this period, the colony forming units (CFU)/mL were determined. Chlorhexidine digluconate (10x MBC) was used as a positive control as well as biofilm in medium without antimicrobial agents as a negative control. Assays were repeated three times for each microorganism, in three independent experiments.

Biofilm formation in dentin tubules of bovine roots

Biofilm assays for confocal laser scanning microscopy (CLSM) analysis were conducted with *E. faecalis*, using taxifolin and CHX, according to the method proposed by Ma et al. (2011) with some modifications. Briefly, dentin blocks (Ethics Committee on the Use of Animals – FOA/UNESP, Protocol 01194-2017) with a length of 4 mm were obtained from bovine roots (n = 3/group), sectioned horizontally 1 mm below the cement-enamel junction. After enlargement of the root canals with Gates Glidden drill #6 (1.5 mm diameter) (Dentsply, Tulsa, USA), each cylindrical dentin block was fractured into two semi cylindrical halves and ground using 600-grit silicon carbide paper until reaching the final size of 3 × 3 × 2 mm. The blocks were cleaned in an ultrasonic bath initially using 17% EDTA solution for 3 minutes and then distilled water for 5 minutes. After autoclaved, the blocks were fixed in a microtube with a resin composite (3M ESPE, USA) and light cured for 40 seconds. Five hundred microliters of

E. faecalis suspension at 10^7 CFU/mL in BHI broth were added to each microtube and sequentially harvested at 1400, 2000, 3600, and 5600 g, twice each, for 5 minutes. A fresh suspension of bacteria was inserted between every centrifugation and the final solution was discarded. Dentin blocks were incubated individually in 48-well plates in BHI broth for 15 days, replacing the culture medium every 72h. After this period, the blocks were washed twice with sterile saline and transferred, under aseptic conditions, to a new plate and exposed to taxifolin (at 10x MBC) and CHX (100 and 1000x MBC) for 2h under agitation and a further 46h in static conditions. After that, the dentin blocks were washed again twice, cut into transverse slices of 1mm thickness, and polished with 1200 grit sandpaper disks.

After new washing with sterile water, the dentin blocks were stained with 100 μ L of fluorescent LIVE/DEAD BacLight Bacterial Viability stain (L13152, Molecular Probes, Eugene, OR) containing SYTO9 and propidium iodide, according to the manufacturer's instructions. The excitation/emission wavelengths were 480/500 nm for SYTO 9 and 490/635nm for propidium iodide. Two additional untreated specimens were stained using the same protocol as the negative controls. The mounted specimens were observed using a 63x NA 1.4 oil immersion lens. CLSM images were acquired using the software LAS AF (Leica Mic-systems). The Z stack was obtained from the top until the bottom of the biofilms. Using a 0.5 μ m interval stack between each frame, 4 randomly selected areas of each dentin specimen were made for each sample. Each 2D (two-dimension) image was obtained by the max projection of the Z stack. The ratio of red fluorescence to green-and-red fluorescence indicated the proportion of dead cells for each antimicrobial agent, measured using Image J software (Rasband, W.S., Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997-2016).

Statistical analysis

The results were presented as means \pm standard deviation (SD) and analyzed using SPSS version 17.0 software (SPSS, IL, USA). Data from bacterial recovery from biofilm assays were converted in logarithmic scale \log_{10} (CFU+1) and calculated the percentage (%) of bacterial growth compared to control groups. Values were normally distributed as verified by Kolmogorov-Smirnov test. Analysis of variance (ANOVA)

followed by Bonferroni tests were performed for all results, considering $p < 0.05$ as significant.

Results

Cell viability

Taxifolin showed no toxic effect on L-929 fibroblast cells at any tested concentration. Chrysin demonstrated low cytotoxic effect over $150 \mu\text{g mL}^{-1}$. Pinocembrin was cytotoxic from 600 to $150 \mu\text{g mL}^{-1}$. Galangin was the most cytotoxic flavonoid reducing its toxicity only below $37.5 \mu\text{g mL}^{-1}$. Chlorhexidine digluconate was cytotoxic from 600 to $18.75 \mu\text{g mL}^{-1}$ (Figure 1).

Antimicrobial activity

MIC and MBC values for flavonoids ranged between 0.03 and 1 mg mL^{-1} (Table 1). All flavonoids tested demonstrated antibacterial activity against *A. israelii*, except chrysin that had no effect against any of the bacteria on tested concentrations. Only taxifolin was able to inhibit all tested bacteria growth through the microdilution broth method. *S. mutans* and *E. faecalis* growth was not affected by pinocembrin, galangin, or chrysin (Table 1). The control chlorhexidine presented the lowest values of MIC and MBC for all tested bacteria (Table 1).

Anti-biofilm effect in polystyrene plates

Taxifolin at 10x MBC concentration was able to completely eliminate *E. faecalis* and *S. mutans* biofilms, and reduce *A. israelii* biofilm. Chlorhexidine digluconate in the same conditions (10x MBC) was able to eliminate only *S. mutans* biofilm and reduce *A. israelii* biofilm, but did not present an effect against *E. faecalis* biofilm (Figure 2).

Anti-biofilm effect in dentin tubules of bovine roots

Representative CLSM images of *E. faecalis* biofilms formed in bovine root dentin specimens are presented in Figure 3A-D. Both taxifolin and CHX showed higher quantification of dead cells (red points) when compared to the control (culture medium). Taxifolin at 10x MBC strongly reduced *E. faecalis* (99.74% dead cells). Similar

reduction (93.73% dead cells) was observed for CHX at 1000x MBC and a lower reduction (64.61% dead cells) for CHX at 100x MBC (Figure 4).

Discussion

The aims of this study were to evaluate the cytotoxicity and antibacterial activity of four flavonoids, as well to analyze the anti-biofilm effect of the flavonoid which was highlighted in previous assays. The null hypothesis 1 was partially accepted, since taxifolin was the unique tested flavonoid without cytotoxicity on L929. The hypothesis 2 also was partially accepted, once only chrysin did not have antibacterial activity; and the hypothesis 3 was rejected because taxifolin showed anti-biofilm effect.

Among the flavonoids tested, taxifolin did not affect viability of fibroblast cells up to $600 \mu\text{g mL}^{-1}$. Although taxifolin caused 15% of reduction on L929 cell viability at $600 \mu\text{g mL}^{-1}$, this effect is considered low, since ISO 10993-5:2009 recommendations characterized a reduction of from 30% in cell viability as a cytotoxic effect. Gómez-Florit et al. (2014) also evaluated the effect of chrysin, taxifolin and galangin on human primary gingival fibroblasts (HGF cells) and observed that 100-200 μM chrysin, 500 μM galangin and doses higher than 10 μM taxifolin produced a significant release of lactate dehydrogenase (LDH) activity compared to controls. In this same study, the highest concentration of taxifolin (500 μM – around $150 \mu\text{g mL}^{-1}$) caused low toxicity (20%) on fibroblasts; different from the present study, which showed minimal toxic effect of taxifolin at $600 \mu\text{g mL}^{-1}$. Likely this difference is related to different cell types used in the studies. Primary cells gradually reduce their proliferation rate *in vitro*, shortening of telomeres, coming in cellular senescence (Milyavsky et al. 2003); while immortalized cells, such as L929, presents the elongation of telomeres increasing the stability of chromosomes (Maqsood et al. 2013).

Chlorhexidine is a broad-spectrum antiseptic, used as endodontic irrigant or root canal dressing (Gomes et al. 2006, Rôças et al. 2016), however have been presented cytotoxicity against eukaryotic cells (Hidalgo & Domingues 2001; Faria et al. 2007). The present study showed cytotoxic effect of the CLX up to $20 \mu\text{g mL}^{-1}$ on L929. Similarly to ours, Faria et al. (2007) observed CHX at 0,002% ($20 \mu\text{g mL}^{-1}$) caused a

significant increase in the percentage of necrotic cell (34%). Faria et al. (2009) showed CHX causes endoplasmic reticulum overexpression in L929 due accumulation of proteins, resulting in cell death by necrosis or apoptosis, depending on the CHX concentration. According the authors CLX at high concentration (from 0,002%) might retard the periapical healing, due cell necrosis, a passive form of cell death that results from disruption of cell membranes and release of cell components to the extracellular matrix, triggering an inflammatory reaction.

Another important characteristic of a biomaterial indicated for endodontic purposes is the antimicrobial activity, mainly against deep caries-related bacteria, such like *S. mutans* and *A. israelii*, and persistent endodontic infection such as *E. faecalis*. These bacteria were selected in the present study due their close relationship with the endodontic polymicrobial infection. Tsukiboshi et al. (2017) showed the presence of apical periodontitis in radicular vital pulp, characterizing a partial pulp necrosis in immature permanent teeth. According the authors this clinical condition can be due large foraminal opening which carries cellular and molecular components of the immune system, delaying the necrosis procces. Therefore, early colonizers such as *S. mutans* could play a role at the beginning of apical periodontitis formation. *A. israelii* is a common facultative anaerobe isolated from the root canal and periapical tissues due its capacity to form biofilm and break into soft tissue (Xia & Baumgartner 2003). The high production of extracellular polymers can result in a well-organized biofilm with difficult elimination, even after both taxifolin and chlorhexidine treatments at high concentrations, as showed in the Figure 2. *E. faecalis* was included in this study due its presence in persistent endodontic infections, surviving at alkaline pH by proton pump (Evans et al. 2002), and invading dentin tubules forming biofilm into them, as observed in Figure 3.

Taxifolin was able to inhibit all tested bacteria, while other flavonoids demonstrated activity against *A. israelii*, excepted for chrysin that had no effect against any of the tested bacteria. Jeong et al. (2009) also found important taxifolin activity against *E. faecalis* and vancomycin-resistant *E. faecalis* showing MIC values ranging from 128 to 512 $\mu\text{g mL}^{-1}$. In the current study, other flavonoids did not inhibit *E. faecalis* and *S. mutans* growth at the tested concentrations. Studies evaluating the

isolated effect of these flavonoids on oral bacteria were not found. On the other hand, Suleman et al. (2015) observed a noteworthy antimicrobial activity of propolis samples from Brazil and South Africa against important pathogens such as *E. faecalis*, *S. aureus*, and *C. albicans* and attributed this property to flavonoid content composed mainly of pinocembrin, galangin, and chrysin, possibly through the synergic effect between them. Antimicrobial activity of flavonoids has been related to their structure, mainly their B ring. The hydroxy groups of taxifolin were able to bind to site of β -Ketoacyl carrier protein synthase (KAS III) of *E. faecalis*, by hydrogen-bonding networks, showing as a candidate inhibitor of this protein. KAS III is an enzyme responsible for catalyze condensation reaction in type II fatty acid synthesis pathway, that is essential for bacterial survival (Jeong et al. 2009).

In the present study, taxifolin was selected for biofilm assays based on its previous antibacterial and non-cytotoxic effects. This is the first study to evaluate the anti-biofilm effect of taxifolin. Biofilm assays were performed at a 10x MBC concentration for taxifolin as the architecture of biofilms provides advantages to bacteria, such as more resistance regarding environmental stresses, including antimicrobial agent effects. In the present study, taxifolin and CHX at 10x MBC were able to eliminate *S. mutans* biofilm. Lee et al. (2016) also showed that CHX treatment reduced bacterial viability in mature *S. mutans* biofilms. CHX antimicrobial activity is related to its cationic nature, which allows it to bond strongly to anionic sites on cell membranes and walls (Jensen, 1977). *A. israelii* biofilm presented a remarkable reduction after CHX and taxifolin treatment, but elimination was not observed. Barnard et al. (1996) suggested that this difficulty in *A. israelii* elimination is due to the genotypic tolerance of the species. Only taxifolin at 10x MBC eliminated *E. faecalis* in both biofilm assays. *E. faecalis* has demonstrated high resistance to endodontic medicaments (Eswar et al. 2013), and the ability to form biofilms both in treated and untreated root canals (Gomes et al. 2006, Vidana et al. 2016). The absence of a CHX effect on *E. faecalis* biofilm is associated with the concentration chosen for the biofilm assays in polystyrene plates (10x MBC). In dentin tubules, CHX at 100x MBC was effective in reducing *E. faecalis* biofilm and practically eliminated biofilm at 1000x

MBC. In other studies, CHX was also effective against *E. faecalis* biofilm at high concentrations (0.2-2% or 2-20 mg mL⁻¹) (Eswar et al. 2013, Komiyama et al. 2016).

This study was performed with fibroblast cells due its wide application, being present in both the pulp and periapical tissues. Primary these cells are known as structural elements, constructing an important structure to support tissue integrity and repair. Moreover, fibroblasts can behave as immunoregulatory cell, producing chemokines that initiate the recruitment of bone-marrow-derived cells, after activated by several bacterial products, such as LPS (Smith et al 1997). Further studies are need to test cytotoxic effect of taxifolin on undifferentiated cells, besides their capacity to promote pulp regeneration and stimulate the formation of mineralized tissue when proposed as pulp-capping agent.

Chrysin, pinocembrin and galangin showed cytotoxic effects at any tested concentrations besides not displaying antibacterial activity against *S. mutans* and *E. faecalis*. Taxifolin promotes a potential antimicrobial/anti-biofilm effect against all tested bacteria, without toxicity on fibroblasts, could be indicated for endodontic purposes, such as vital pulp and regenerative endodontic therapies.

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Disclosure statement

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Figures and Tables

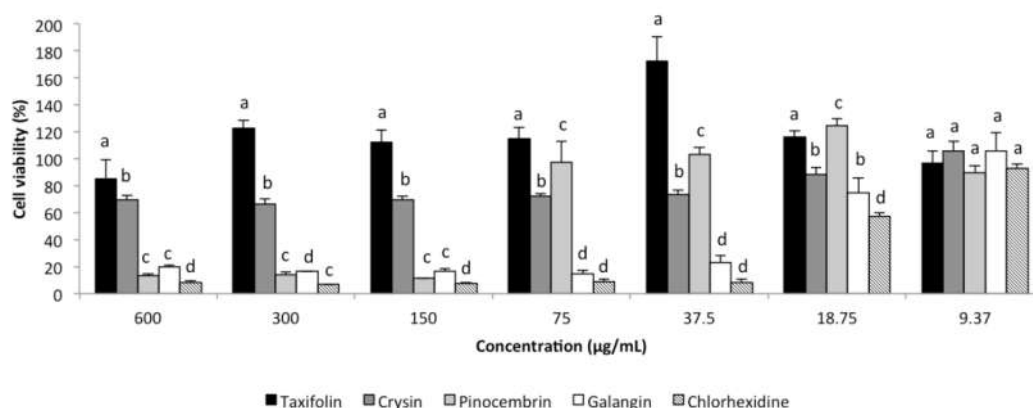


Figure 1. Mean (bars) of the percentage of cell viability of fibroblasts from L-929 line after exposure to different concentrations of flavonoids, using MTT assays.

^aDifferent lower case letters show statistical differences between the groups, considering each concentration separately, according to ANOVA and Bonferroni tests, considering $p < 0.05$.

Table 1. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of flavonoids.

Flavonoid	Bacteria	MIC (mg/mL)	MBC (mg/mL)
Taxifolin	<i>S. mutans</i>	0.5	1
	<i>E. faecalis</i>	0.25	1
	<i>A. israelii</i>	0.25	0.5
Chrysin	<i>S. mutans</i>	>2	>2
	<i>E. faecalis</i>	>2	>2
	<i>A. israelii</i>	>2	>2
Pinocembrin	<i>S. mutans</i>	>2	>2
	<i>E. faecalis</i>	>2	>2
	<i>A. israelii</i>	0.03	0.03
Galangin	<i>S. mutans</i>	>2	>2
	<i>E. faecalis</i>	>2	>2
	<i>A. israelii</i>	0.03	0,03
Chlorhexidine Digluconate	<i>S. mutans</i>	0.0002	0.002
	<i>E. faecalis</i>	0.004	0.004
	<i>A. israelii</i>	0.002	0.002

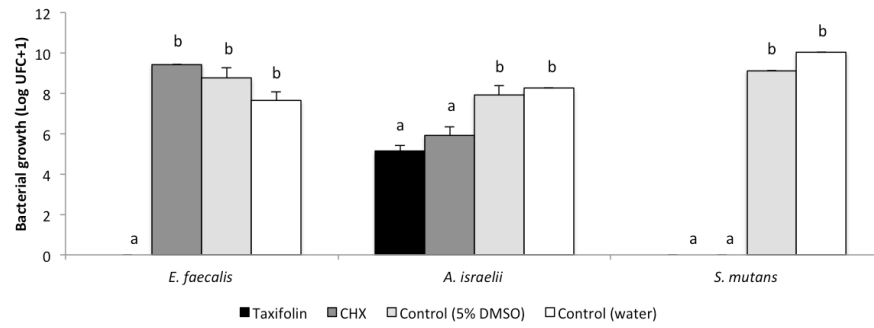


Figure 2. Bacterial recovery (Log CFU+1) after 24h of taxifolin and chlorhexidine digluconate (CHX) treatments (10x MBC) on 48h-growth biofilm.

^aDifferent lower case letters show statistical differences between the groups, considering each strain separately, according to ANOVA e Bonferroni tests, considering $p < 0.05$.

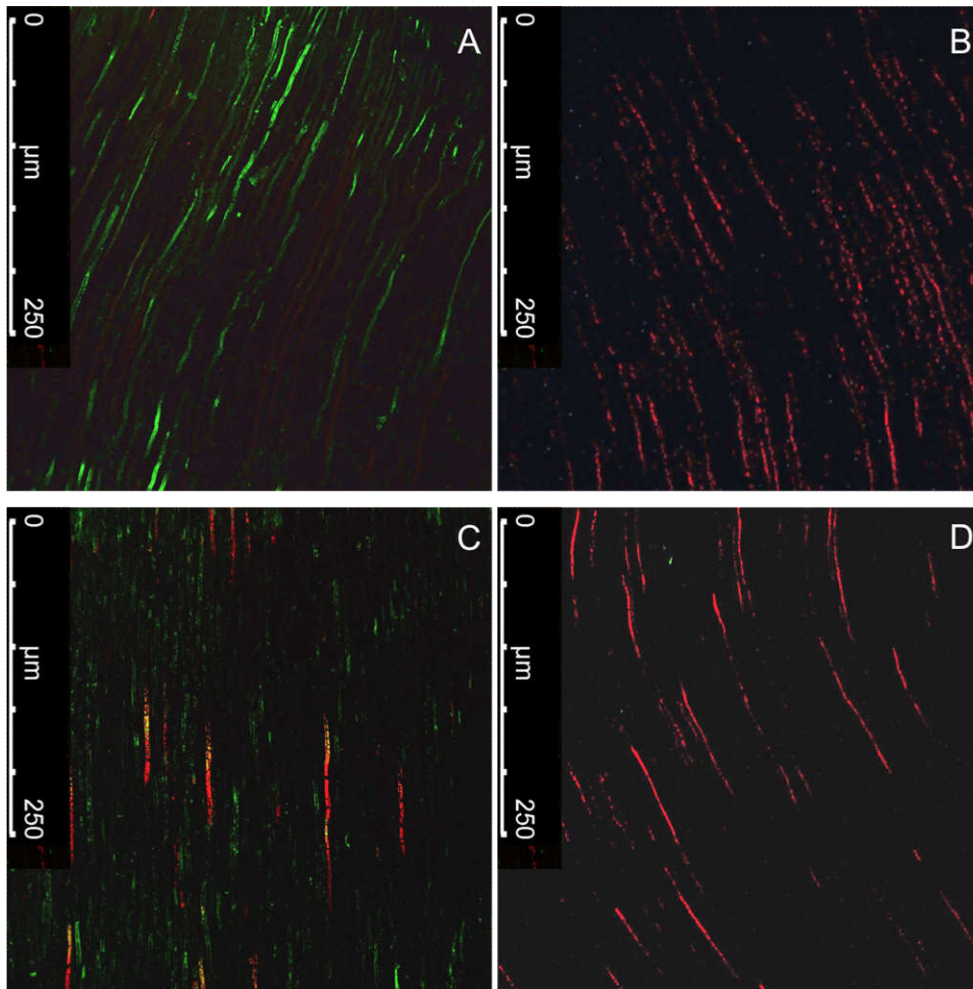


Figure 3. Representative CLSM images (63x) of *E. faecalis* biofilms inside dentin tubules after 48h of the treatments. (A) control – culture medium; (B) taxifolin 10x MBC; (C) CHX 100x MBC; (D) CHX 1000x MBC.

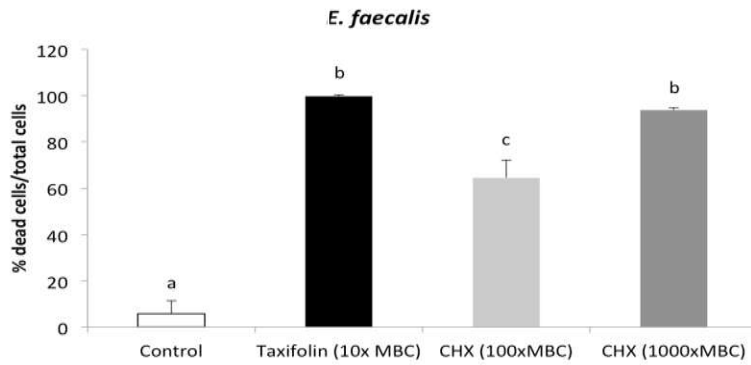


Figure 4. Means (bars-standard deviation) of the percentage of dead cells obtained after CLSM analysis of *E. faecalis* biofilm.

Different lower case letters show statistical differences between the groups, considering each strain separately, according to ANOVA e Bonferroni tests, considering $p < 0.05$.

Capítulo 2

Cytotoxicity and potential of taxifolin, a catechol-type flavonoid, to induct mineralization markers in odontoblast-like cells*

Abstract

The aim of this study was to evaluate the effects of treatment with taxifolin flavonoid on the viability of odontoblast-like cells and expression of mineralization markers. MDPC-23 was exposed to different concentrations of taxifolin treatment (10, 5, and 1 μ M) for different periods (24h, 72h, and continuous treatment). Cell viability, alkaline phosphatase (ALP) activity, mineralization nodule formation, and expression of DMP-1 and DSPP were determined. For all periods, taxifolin treatment was not cytotoxic to cells in the concentrations tested, according to ANOVA and Tukey tests. Taxifolin at the concentrations 10 to 1 μ M for 24h and 10 to 5 μ M for 72h stimulated ALP activity of cells. The percentage of mineralization nodule formation at 13d increased after 24h of treatment with taxifolin at 10 and 5 μ M, and 72h of treatment at 10 μ M. Continuous treatment did not stimulate ALP activity or mineralization by cells. The most elevated DMP-1 mRNA levels were observed on day 13 after 72h of treatment with 10 μ M of taxifolin. Groups exposed to taxifolin at 5 μ M for 72h presented elevated DSPP mRNA levels on days 6 and 13 without statistical difference between them, according to Kruskal-Wallis and Mann-Whitney tests. In conclusion, taxifolin treatments of 24 and 72h were more effective than continuous treatment, demonstrating that a lower dose of taxifolin over short periods of time has a biostimulatory effect on MDPC-23 cells. Our results suggest that taxifolin could be used as a biomaterial to stimulate remaining primary odontoblasts and odontoblast-like cells to produce a mineralized tissue barrier in vital pulp procedures.

Running title: Taxifolin biostimulate MDPC-23

Keywords: Cell Culture Techniques, Flavonoids, Odontoblasts, Pulpotomy.

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

Introduction

Immature permanent teeth with pulp exposed by caries, trauma, or restorative procedures require different treatments depending on the pulpal status, size of exposure, and microbial contamination (Bortolluzi *et al.* 2008). Vital pulp therapy (VPT) procedures have been extensively studied aiming at maintaining the vitality of radicular pulp and thus allowing dentin formation and completed root development (Lima *et al.* 2011, Keswani *et al.* 2014, Tsukiboshi *et al.* 2017). VPT includes pulp capping and pulpotomy procedures and their success depends mainly on the capacity of healing of the pulp tissue.

Pulpotomy is a procedure based on amputation of the infected and inflamed coronal pulp and treatment of remaining vital radicular pulp tissue with medicaments (American Academy of Pediatric Dentistry 2014). When there is little pulp exposed, direct pulp capping is the treatment of choice (Keswani *et al.* 2014, Taha & Khazali 2017). Different from pulpotomy, pulp-capping material is placed in contact with the exposed pulp tissue, without amputation, however, both procedures allow the formation of mineralized tissue on the exposed area and apexogenesis - the continued physiological development and formation of the root apex. VPT procedures have been indicated for young permanent teeth without clinical or radiographic signs of irreversible pulpitis or necrosis (American Academy of Pediatric Dentistry 2014).

Odontoblasts are the main link between the dentin and pulp, whose primordial functions are synthesis and deposition of dentin matrix. These specialized pulp cells are the first line of defense of the dentin-pulp complex, and are also therefore the first cells injured by harmful effects (de Souza Costa *et al.* 2014). A complicating factor in VPT is the difficulty of predicting the degree of pulpal damage. Generally, low intensity injuries induce primary odontoblasts to produce reactionary dentin; however the repair of severe injuries involves the recruitment of undifferentiated mesenchymal cells from pulp due to the death of the primary odontoblasts. These stem cells differentiate into odontoblast-like cells and start to produce reparative dentin (de Souza Costa *et al.* 2014).

Calcium hydroxide (CH) and mineral trioxide aggregate (MTA) are conventional endodontic materials frequently used in VPT (Keswani *et al.* 2014, Taha & Khazali 2017,

Tsukiboshi *et al.* 2017). In partial pulpotomy, MTA has presented a higher success rate than CH (Taha & Khazali 2017). However, both materials demonstrate drawbacks. Tunnel defects in dentin bridges and microleakage have been observed in pulpotomy with CH which, over time, can lead to infection/necrosis of pulp (Schuurs *et al.* 2000). Studies have pointed out difficulties with the handling and insertion of MTA due to its grainy consistency and the possibility of break down due to a long setting time and prolonged maturation phase (Kogan *et al.* 2006), allied to high cost and tooth discoloration (Belobrov & Parasho 2011).

Bioactive molecules have been suggested as VPT materials in several studies (Bortolluzi *et al.* 2008, Parolia *et al.* 2010, Li *et al.* 2011, Lima *et al.* 2011, Kim *et al.* 2013, Keswani *et al.* 2014, Daltoé *et al.* 2016, Balata *et al.* 2017). According to Tziafas *et al.* (2000), an ideal pulp-capping agent should present effective adhesion to dentin, antimicrobial effect, and promote dentinogenesis, stimulating the formation of dentin bridge, indicative of a favorable prognosis after VPT (Kim *et al.* 2013). Alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), and dentin matrix protein 1 (DMP1) are mineralization markers and have been used to assess the biostimulating action of materials (Li *et al.* 2011, Kim *et al.* 2013, Daltoé *et al.* 2016, Wang *et al.* 2017)

Flavonoids are secondary plant metabolites constituted by a polyphenolic structure (Panche *et al.* 2016). Taxifolin is a catechol-type flavonoid isolated from green tea (Wang *et al.* 2017), which has presented antimicrobial activity (Jeong *et al.* 2009) as well as stimulating osteoblast differentiation in the mouse osteoblastic cell line (Satué *et al.* 2013) and in human bone marrow mesenchymal stem cells (Wang *et al.* 2017). The biostimulatory effect of taxifolin on odontoblast cells has not yet been studied. Therefore, the present study aimed to evaluate the effects of taxifolin treatment on the viability of odontoblast-like cells and expression of mineralization markers. The null hypotheses tested were: (1) taxifolin present cytotoxic effect on MDPC-23 cells, and (2) taxifolin treatments would not stimulate odontoblast-like cells to increase the expression of mineralization markers.

Materials and Methods

Materials

Culture medium, antibiotics, and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Taxifolin (#78666, Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, MO, USA) and the stock solution stored at -20° C.

Cell Culture and study design

Immortalized odontoblast-like (MDPC-23) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA), penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2 mmol/L) (GIBCO, Grand Island, NY, USA). Cells were seeded (2.5×10^3 cells/well) in 96-well plates and incubated at 37°C under a 5% CO₂ and 95% air atmosphere (Thermo Plate, Fisher Scientific, Pittsburgh, PA, USA) for 24 h. After incubation, taxifolin (T) treatments were performed as follows: 24h treatment – cells were exposed once to the flavonoid treatment; 72h treatment – cells were exposed three times; continuous treatment – cells were exposed daily up to 13 days. All treatments were assayed with three taxifolin concentrations: 10, 5, and 1µM (T10, T5, and T1). After 24 or 72h of taxifolin treatment, the DMEM was replaced every 24h until completion of the experimental period (6 or 13 days). The negative control was DMEM without flavonoid, and the control group was DMSO 10µM (Table 1).

Cell Viability Analysis

Methylthiazol tetrazolium assay was performed to determine cell viability 24h, 72h, 6d, and 13d after beginning treatments. MTT assay is based on the succinate dehydrogenase enzyme produced by mitochondria, which reduces the MTT salt metabolically, converting it into formazan crystals. The treatments or DMEM were aspirated, then MTT 5mg/mL (Sigma-Aldrich, Saint Louis, MO, USA) was applied into wells and the plate was incubated at 37° C under a 5% CO₂ and 95% air atmosphere for 4 h. Thereafter, the MTT solution was aspirated and replaced by acidified isopropanol solution to dissolve the formazan crystals. Cell viability was determined by absorbance in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader; Biotek Instruments, Winooski, VT, USA) at 570 nm. This assay was performed in duplicate.

Alkaline Phosphatase Assays

Total Protein Production

The quantification of total protein was performed according to Huck *et al.* (2017) with some modifications. On the sixth day, the treatments were aspirated and 200µL of sodium lauryl sulfate 0.1% (Sodium dodecyl sulfate, Sigma-Aldrich, Saint Louis, MO, USA) previously dissolved in deionized water were added to each well to lyse the cells. After 40 min at room temperature, the solution was homogenized and 100µL were separated for ALP activity assay. Next, 100µL of Lowry reagent (Sigma-Aldrich, Saint Louis, MO, USA) were added to the lysed cells and incubated for 20 min at room temperature. Posteriorly, 50µL of Folin (Folin-Ciocalteu's phenol reagent, Sigma-Aldrich, Saint Louis, MO, USA), previously diluted in deionized water at a ratio of 1:3, were applied to each well and incubated for 30 min. After this period, all the samples were read in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader; Biotek Instruments, Winooski, VT, USA) to determine absorbance at 655 nm. A standard curve containing 32, 64, 96, 128, and 160 µg mL⁻¹ of bovine albumin was determined to measure total protein of each sample (BSA, Sigma-Aldrich, Saint Louis, MO, USA).

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was evaluated following the protocol of the Alkaline Phosphatase Kit-Test Endpoint (Labtest Diagnostics SA, Lagoa Santa, MG, Brazil) based on dephosphorylation of thymolphthalein by ALP. Six days after beginning the experiments, one tube containing 50µL of substrate (thymolphthalein monophosphate 22 mmol/L – Reagent 1) and 500µL of buffer solution (300 mmol/L, pH 10.1 – Reagent 2) were prepared for each well. Then, 50µL of cell lysis solution (prepared in the previous assay) were added to the respective tubes and incubated for 20 min at 37° C. Posteriorly, 2 mL of color reagent (Sodium carbonate 94 mmol/L and sodium hydroxide 250 mmol/L – Reagent 3) were placed in each tube and homogenized. The absorbance of the solution was determined in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT, USA), at 590 nm. In order to measure the ALP activity a standard curve containing 0.43, 1.3, 2.6, 5.2, 6.92, and 8.65 U/L of alkaline phosphatase (Standard 45 U/L – Reagent 4) was used. The absorbance values of ALP were normalized by values of total protein.

Mineralization Nodule Formation

Alizarin red stain was used to evaluate the mineral nodule formation on the final day of treatment (day 13). This assay is based on quantification of the mineralized matrix produced by odontoblast-like cells. The treatments were aspirated and the cells fixed in 70% cold ethanol for 2h. Subsequently, the ethanol was aspirated and each well received 100 μ L of alizarin red (40nm; pH 4.2; Sigma-Aldrich, Saint Louis, MO, USA), after which the samples were incubated under shaking for 20 min. The wells were rinsed twice with deionized water and incubated at room temperature to dry for 24h. Next, mineral nodules of each group were identified and photographed by a light microscope (Olympus BX51; Olympus, Miami, USA) equipped with a digital camera (Olympus C5060; Olympus, Miami, USA). Finally, 150 μ L of cetylpyridinium chloride solution (Sigma-Aldrich) were added to each well and maintained under shaking for 15min to dissolve the nodules. For quantitative analysis, the mineral nodule formation was measured by absorbance in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT, USA), at 570 nm.

DSPP and DMP-1 gene expression

Based on the results of previous assays, Real Time quantitative PCR (qPCR) analysis was performed only with the highest concentrations (10 and 5 μ M) of the 24 and 72h treatments. On days 6 and 13, the treatments were aspirated and total RNA extracted following the protocol of the RNAqueous Kit (Ambion Inc., Austin, TX, USA). RNA quantification was performed through absorbance with 1 μ L of total RNA in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT, USA). The cDNA synthesis was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's protocol. The qPCR was performed by TaqMan chemistry (Applied Biosystems, Carlsbad, CA, USA) and pre-designed and optimized primer and probe sets (Gene expression assays, Applied Biosystems, Carlsbad, CA, USA) for quantification of gene expression for dentin matrix protein-1 (DMP-1, Rn01450122_m1), dentin sialophosphoprotein (DSPP, Rn02132391_s1) and β -actin (Hs01060665_m1) which was used as the endogenous control. The CT value corresponding to the number of cycles required for the fluorescence signal to reach the threshold of detection was determined for each sample. The data were analyzed as

relative changes in comparison to unstimulated controls by the DDCT method using the thermocycler software (StepOne Plus, Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

Experiments were performed on two independent days using four wells per group. Data from cell viability, ALP activity, and mineralization nodule formation were normally distributed as verified by Kolmogorov-Smirnov test, presented as mean \pm SD and analyzed using ANOVA and Tukey tests. Data from the qPCR were presented in box whisker plots and analyzed using the Kruskal-Wallis and Mann-Whitney tests. SPSS 11.0 software (SPSS Inc, Chicago IL, USA) was used to run the statistical analysis, considering $p < 0.05$.

Results

Cell Viability Analysis

Figure 1 presents the percentage of MDPC-23 cell viability after exposure to different concentrations of taxifolin for 24 or 72h, immediately after the pre-treatments. At 24 and 72h, there was no statistical difference between the flavonoid groups (T10, T5, and T1) and control (DMSO group), showing that taxifolin was not cytotoxic to cells in the concentrations tested up to 72h. The same was observed for days 6 and 13 (Figure 2), considering the three types of treatment: 24h, 72h, and continuous treatment (CT). On day 13, only the CT at 10 μ M differed from the control, however, cell growth was higher than 80%.

ALP activity

Taxifolin at the concentrations 10 to 1 μ M for 24h and 10 to 5 μ M for 72h stimulated ALP activity of MDPC-23 cells when compared to the DMSO group. ALP activity from cells under continuous treatment with taxifolin at 10 to 1 μ M and treated with 1 μ M for 72h did not differ from the DMSO group (Figure 3).

Mineralization Nodule Formation

The mineralization ability of MDPC-23 cells submitted to taxifolin treatments is represented in Figure 4. The percentage of mineralization nodule formation on day 13, determined by alizarin red staining, increased after the 24h-treatment with taxifolin at 10 and 5 μ M and 72h-treatment at 10 μ M. Independent of taxifolin concentration, continuous treatment did not stimulate mineralization by cells. Representative images

of alizarin red staining show an increase in mineral nodule formation after the 24 and 72h treatments with taxifolin from 5 (Figures 5-B and 5-C) to 10 μ M (Figures 5-E and 5-F) and lower mineral deposition after continuous treatment with taxifolin at 5 and 10 μ M (Figures 5-D and 5-G), compared to the control (Figure 5-A - DMSO at 10 μ M).

DMP-1 and DSPP gene expression

The most elevated DMP-1 mRNA levels were observed on day 13 after 72h of treatment with 10 μ M of taxifolin (T10-72h), increasing more than 3 times compared to the control (C). Taxifolin at 5 μ M for 24h almost doubled the DMP-1 mRNA levels compared to the control (C) on days 6 and 13. There was a tendency to increase in the DMP-1 gene expression after 72h of treatment with taxifolin at 5 μ M for both time points, but without statistical difference compared to the control. Groups exposed to taxifolin at 5 μ M for 72h presented elevated DSPP mRNA levels on days 6 and 13, without statistical difference between them, but different from the control group.

Discussion

Recent reports have appointed the flavonoid taxifolin as an osteoblast differentiation inductor due to its ability to increase the expression of osteogenic differentiation markers, such as Osteocalcin (Satué *et al.* 2013, Wang *et al.* 2017), Osteopontin, Runx2, and Osterix (Wang *et al.* 2017), besides increasing ALP activity and mineral nodule formation (Wang *et al.* 2017). However, no study was found evaluating the effects of taxifolin on odontoblast-like cells. Both null hypotheses were rejected, since taxifolin had no cytotoxic effect on MDPC-23, and the taxifolin treatments stimulated odontoblast-like cells to increase the ALP activity, mineral nodule formation, and DMP-1/DSPP expression.

The highest tested concentration (10 μ M) displayed a reduction in MDPC-23 metabolism only when applied in a continuous manner and at the later time (13d); however, cell growth was higher than 80%. According to ISO 10993-12:2012 recommendations, reductions of up to 30% of cell viability do not characterize a cytotoxic effect. The cytotoxicity of taxifolin was previously evaluated by Satué *et al.* (2013) and Wang *et al.* (2017) after 48 h of treatment and the authors reported that concentrations of taxifolin up to 200 μ M for mouse osteoblastic cell line and 100 μ M for human bone marrow mesenchymal stem cells were not cytotoxic. The cytotoxic

concentration of taxifolin differed between these studies can be due to the different cell types tested, since primary cells are more sensitive than immortalized cell lines. Primary cells gradually reduce their proliferation rate in vitro, shortening of telomeres, coming in cellular senescence (Milyavsky *et al.* 2003); while immortalized cells, such as MDPC-23, presents the elongation of telomeres increasing the stability of chromosomes (Maqsood *et al.* 2013). Similarly to the present study, Lee *et al.* (2016) tested a treatment with the flavonoid baicalein over a long period and conducted the MTT assay at 14 days, concluding that 1, 5, and 10 μ M were not cytotoxic concentrations for human dental pulp cells, besides presenting a biostimulating action, promoting odontoblastic differentiation and angiogenesis.

Matrix vesicles are structures derived from the plasma membrane of mineral forming cells such as odontoblasts, and they are like initial sites of mineral formation (Golub 2009). Alkaline phosphatase is an enzyme present on the external surface of these vesicles, responsible for dentine matrix biomineralization by pyrophosphate – a calcification inhibitor - hydrolysis to inorganic phosphate – a mineralization promoter – to form hydroxyapatite (Golub 2009). Therefore, ALP activity can be used as an early stage marker of mineralization. Our results showed no effect on ALP activity of continuous treatment with taxifolin in any tested concentrations, since these groups did not differ from the control. On the other hand, the 24 and 72h-treatments with taxifolin demonstrated a significant increase in ALP activity, except the T1-72h group. A significant increase in ALP activity was observed by Wang *et al.* (2017) for human bone marrow mesenchymal stem cells after 7 days of treatment with taxifolin, with the culture media replaced every 3-4 days, similar to our 72h-treatment. Different from the results of the present study, the authors noted an increase in ALP activity in a dose-dependent manner, likely to be due to the large gap between the concentrations.

The mensuration of mineralized nodule formation in odontoblast-like cells culture allows to assess the cellular phenotype in the presence of biomolecules (Tang & Saito 2017). Alizarin red-staining reacts with calcium cation to form a chelate (Wang *et al.* 2006), and has been used to detect mineralized matrix deposition at the late stage of proposed treatments (Wang *et al.* 2006, Kim *et al.* 2013). Similarly to ALP assays, the best results were observed for the 24 and 72h-treatments, mainly at the highest concentrations. High taxifolin concentrations were also able to promote

calcium mineral deposits on primary human bone mesenchymal stem cells under osteogenic differentiation media (Wang *et al.* 2017). In our study, we decided to use lower taxifolin concentrations; since MDPC-23 are differentiated cells and, unlike stem cells, do not need to be induced for osteo/odontoblastic differentiation.

DMP-1 and DSPP are non-collagenous matrix proteins associated with the dentinogenesis process, participating in the dentin mineralization process and collagen fiber maturation (Qin *et al.* 2007). After dentinogenesis, these bioactive proteins remain in dentin substrate and are released when a harmful process occurs. Once released, DMP-1 and DSPP are able to induce the synthesis, deposition, and mineralization of dentin matrix by primary odontoblasts, besides participating in the differentiation process of dental pulp cells into odontoblasts (de Souza Costa *et al.* 2014). In the current study, DMP-1 expression was increased in a time-dependent manner (6 to 13d). The highest concentration over a longer exposure time (T10-72 group on day 13 of evaluation) presented the best result. This treatment stimulated an increase in DMP-1 expression of more than 3 times compared to the control, showing that DMP-1 expression acts as a marker in the late-stage of the mineralization process. High levels of DMP-1 mRNA expression were observed on human dental pulp cells (HDPCs) by Lee *et al.* (2016) after 7 and 14 days of treatment with the flavonoid baicalein at 10 μ M. In the present study, an increase in DMP-1 expression was observed on day 6 only in T5-24h group. One possible explanation is that these authors used osteogenic medium during the treatment, which may have stimulated earlier DMP-1 expression by HDPCs.

DSPP gene encodes two proteins: DSP and DPP, both participate of calcium binding to previously produced collagenous matrix, initiating the formation of hydroxyapatite crystals within collagen fibers (Goldberg *et al.* 2011). DSPP expression was up regulated in MDPC-23 treated with T5-72h group at both time points, 6 and 13 days. According Suzuki *et al.* (2009) DSP and DPP have distinct roles in dentin mineralization, while DSP showed to regulate the beginning of dentin mineralization, DPP being involved in the maturation of mineralized dentin. Similarly to our study, epicatechin, another catechol type flavonoid isolated from green tea, increased DSPP gene expression and ALP activity on day 7 and mineralization nodule formation in a later period (Lim *et al.* 2016). On the contrary, DMP-1 expression was not affected by

the presence of epicathecin. DSPP and DMP-1 have overlapping, but distinct functions in mineralized tissues, considering not only extracellular proteins but also cell signaling molecules. Thus, different levels of DSPP and DMP-1 expression affected bone/dentin formation and the mineralization levels of these tissues (Suzuki *et al.* 2012). Several studies have investigated a variety of flavonoids as inductors of osteogenic (Satué *et al.* 2013, Zhang *et al.* 2016, Wang *et al.* 2017) and dental pulp cell differentiation (Lee *et al.* 2016, Lim *et al.* 2016) however; this is the first study evaluating the effects of taxifolin on odontoblast-like cells.

Taxifolin continuous treatment showed no biostimulatory effect on MDPC-23, since did not stimulate to increase of mineralization markers in this study. According to Middleton *et al.* (2000), flavonoids are able to affect the activity of many mammalian enzymes in a dose-dependent manner. Quercetin – a flavonoid with similar chemical structure to taxifolin (Kumar & Pandey 2013) act as a competitive inhibitor of ATP binding to the enzyme, resulting in inhibition of Ca²⁺ATPases (Sheweel *et al.* 1981) and consequently cell metabolism reduction, as noted in T10-CT after 13 days on MTT assay.

Taxifolin is a catechol-type flavonoid, presenting o-dihydroxy (3', 4'-diOH, i.e., catechol) on its B ring (Àmic *et al.* 2007). Since activities of the flavonoids are structure dependent, hydroxyl radical (OH) has mediated antioxidant effects (Kumar & Pandey 2013), besides it seems play a role on hard tissue formation (Matsui *et al.* 2007, Matsui *et al.* 2009). OH are able to alkalinize the medium, causing a rupture in the integrity of the bacterial membrane, besides increasing ALP activity on odontoblastic cells (Lee *et al.* 2006) and to mediating the hard tissue formation by human dental pulp cells (Matsui *et al.* 2007). This flavonoid had showed poor solubility, which difficult to be absorbed and metabolized by the body, leading to a low bioavailability (Zu *et al.* 2012). Since solubility of drugs is related to the surface area of materials, when particle size of drugs is reduced, the contact surface is greater, improving the solubility rate of the material (Zu *et al.* 2012). Therefore, a possible vehicle for taxifolin delivery should allows the release of ions, through new approaches to increase the flavonoid solubility, such as the particle size reduction or to be incorporated in solid dispersion formulations (Shikov *et al.* 2009).

In addition to OH role, taxifolin presents chelating sites (Kumar & Pandey 2013) that could to act as nucleation sites, stimulating Ca^{2+} and PO_4^{3-} binding in biomineralization process. Interactions between phospholipids, proteins, Ca^{2+} and PO_4^{3-} arrange a molecular architecture which nucleates apatite crystallization *in vivo* (Golub *et al.* 2009). P-glycoprotein and multidrug resistance protein 2 are transporter proteins that play an important role in the absorption and distribution of many flavonoids (Brand *et al.* 2006). Wang *et al.* (2009) showed an increase in P-glycoprotein expression after taxifolin treatment in Caco-2 cells, besides noting that the inhibition of multidrug resistance protein 2 was able to decrease the efflux ratio of taxifolin in these cells, but not inhibit the transport of taxifolin completely, suggesting that there may be another transporter for taxifolin. Once in the intracellular environment, taxifolin can scavenge reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) through donation of hydrogen and an electron. Stabilization of ROS reduces cellular injuries, since it inhibits both lipid peroxidation (Kumar & Pandey 2013), and NF- $\kappa\beta$ activation by H_2O_2 (Gloire *et al.* 2006).

Taxifolin was able to biostimulate odontoblast-like cells, can be used as pulp-capping material to promote dentinogenesis, mainly in immature permanent teeth cases. Tsukiboshi *et al.* (2017) showed the presence of apical periodontitis in radicular vital pulp, characterizing a partial pulp necrosis in immature teeth. According the authors this clinical condition can be due large foraminal opening which carries cellular and molecular components of the immune system, delaying the necrosis process. In addition, studies testing vehicles for taxifolin delivery with adequate physical and mechanical properties are necessary for its application as a pulp capping or pulpotomy agent.

Conclusion

Taxifolin 24 and 72h-treatments were more effective than continuous treatment, showing that lower doses of taxifolin over short periods of time have a biostimulatory effect on MDPC-23 cells. Our results suggest that taxifolin could be used as a biomaterial to stimulate remaining primary odontoblasts and odontoblast-like cells to produce a mineralized tissue barrier in VPT procedures.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Figures and Tables

Table 1. Description of groups chosen for the present study, according to the type of treatment and concentration (μM).

Group	Compound/Concentration (μM)	Treatment
T10-24h	Taxifolin 10 μM	24h-treatment
T5-24h	Taxifolin 5 μM	24h-treatment
T1-24h	Taxifolin 1 μM	24h-treatment
T10-72h	Taxifolin 10 μM	72h-treatment
T5-72h	Taxifolin 5 μM	72h-treatment
T1-72h	Taxifolin 1 μM	72h-treatment
T10-CT	Taxifolin 10 μM	Continuous treatment
T5-CT	Taxifolin 5 μM	Continuous treatment
T1-CT	Taxifolin 1 μM	Continuous treatment
DMSO-10	DMSO 10 μM	Continuous treatment

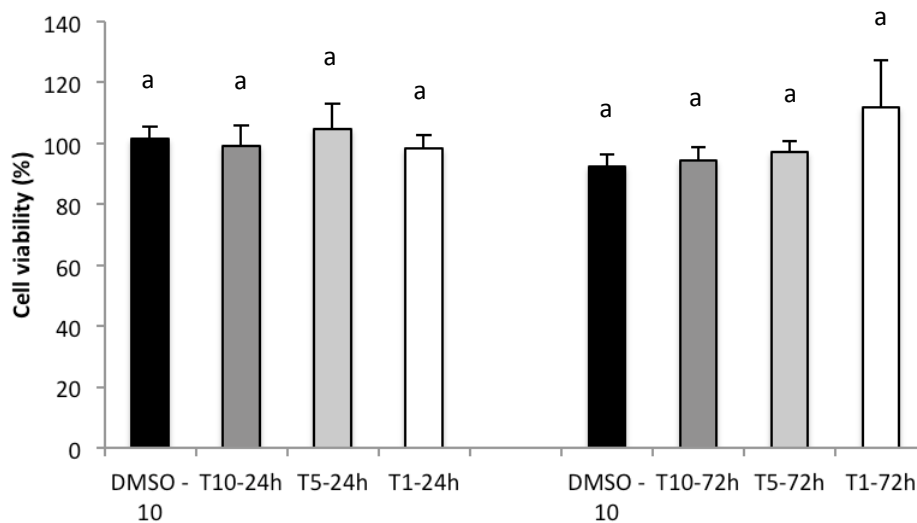


Figure 1. Cell viability immediately after 24 and 72 h of treatments with different concentrations of taxifolin, using MTT assays.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

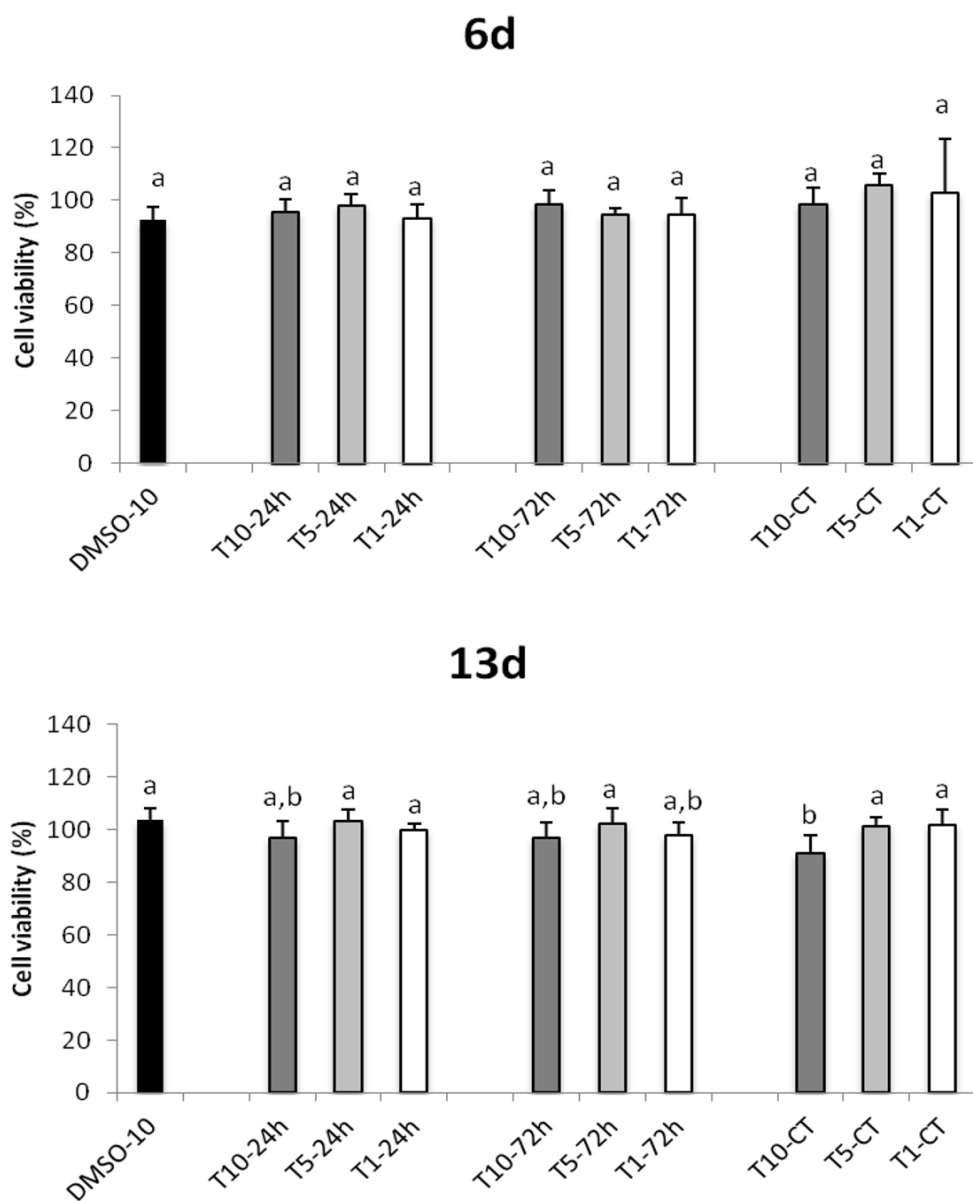


Figure 2. Cell viability of cells after 6 and 13 days of the three forms of treatment (24 h, 72 h, and continuous treatment) with taxifolin, using MTT assays.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

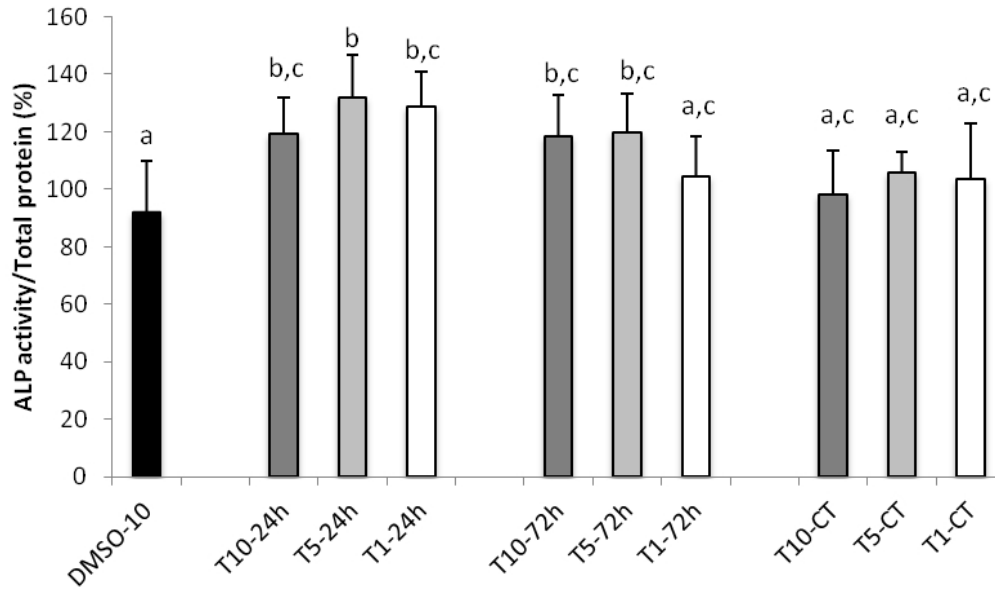


Figure 3. Alkaline phosphatase (ALP) activity of cells after 6 days of the three forms of treatment with taxifolin.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

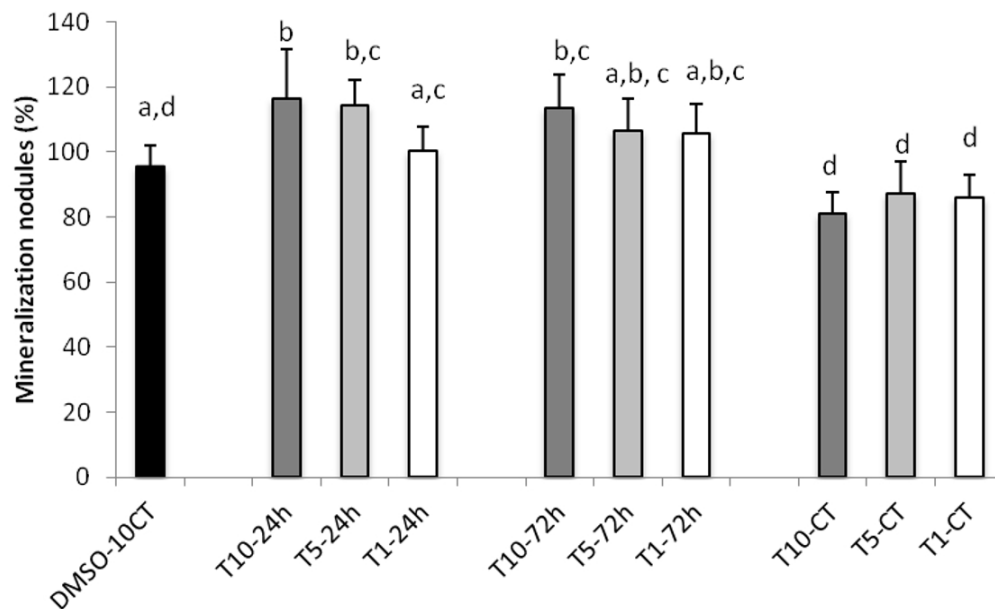


Figure 4. Mineralization ability of cells after 13 days of the three forms of treatment with taxifolin, using alizarin red staining.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

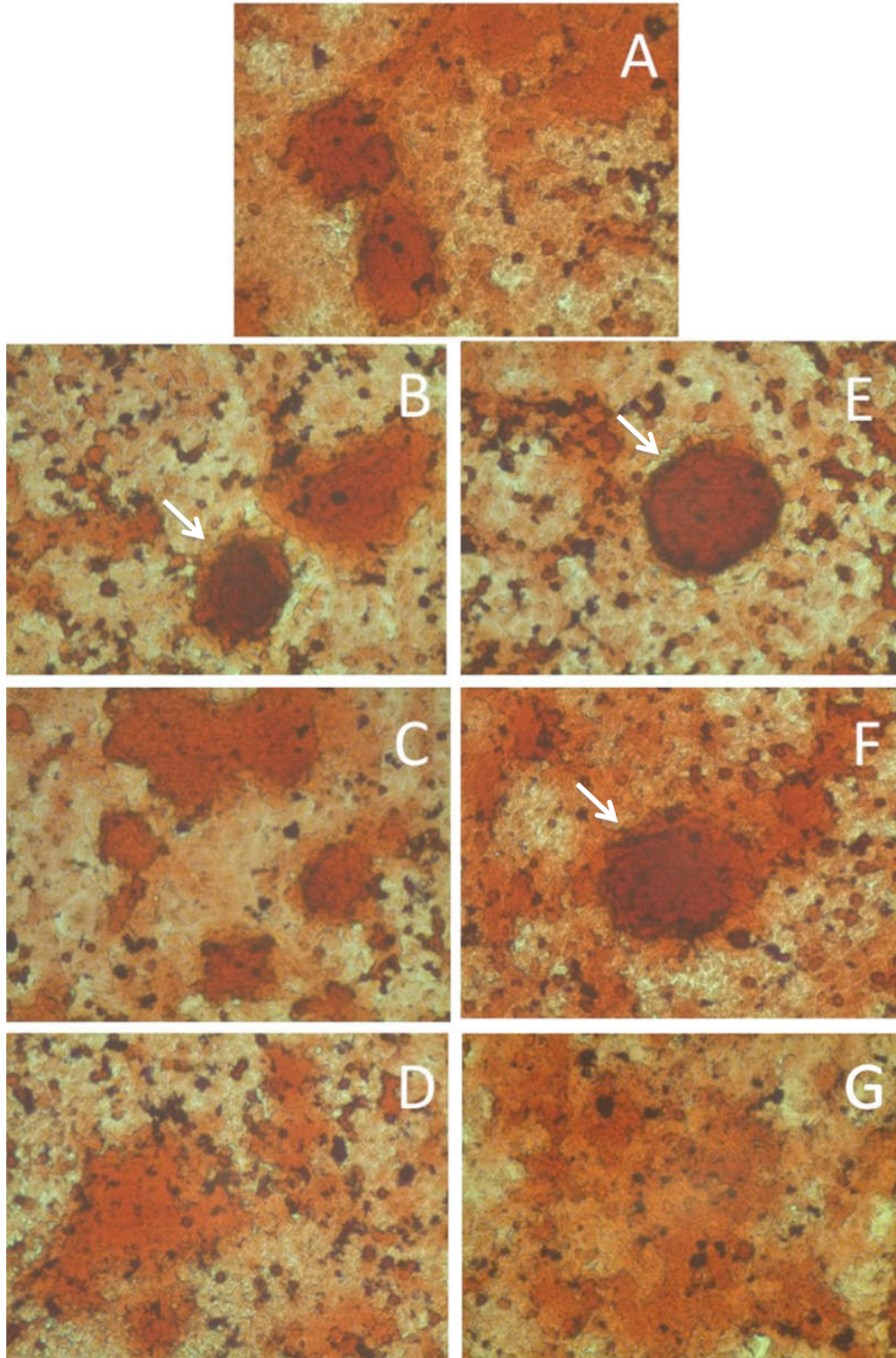


Figure 5. Representative images of alizarin red staining showing mineralization ability of MDPC-23 cells after 13 days of taxifolin treatment. A: Control group; B: T5-24h; C: T5-72h; D: T5-CT; E: T10-24h; F: T10-72h and G: T10-CT. White arrows show mineralization nodules.

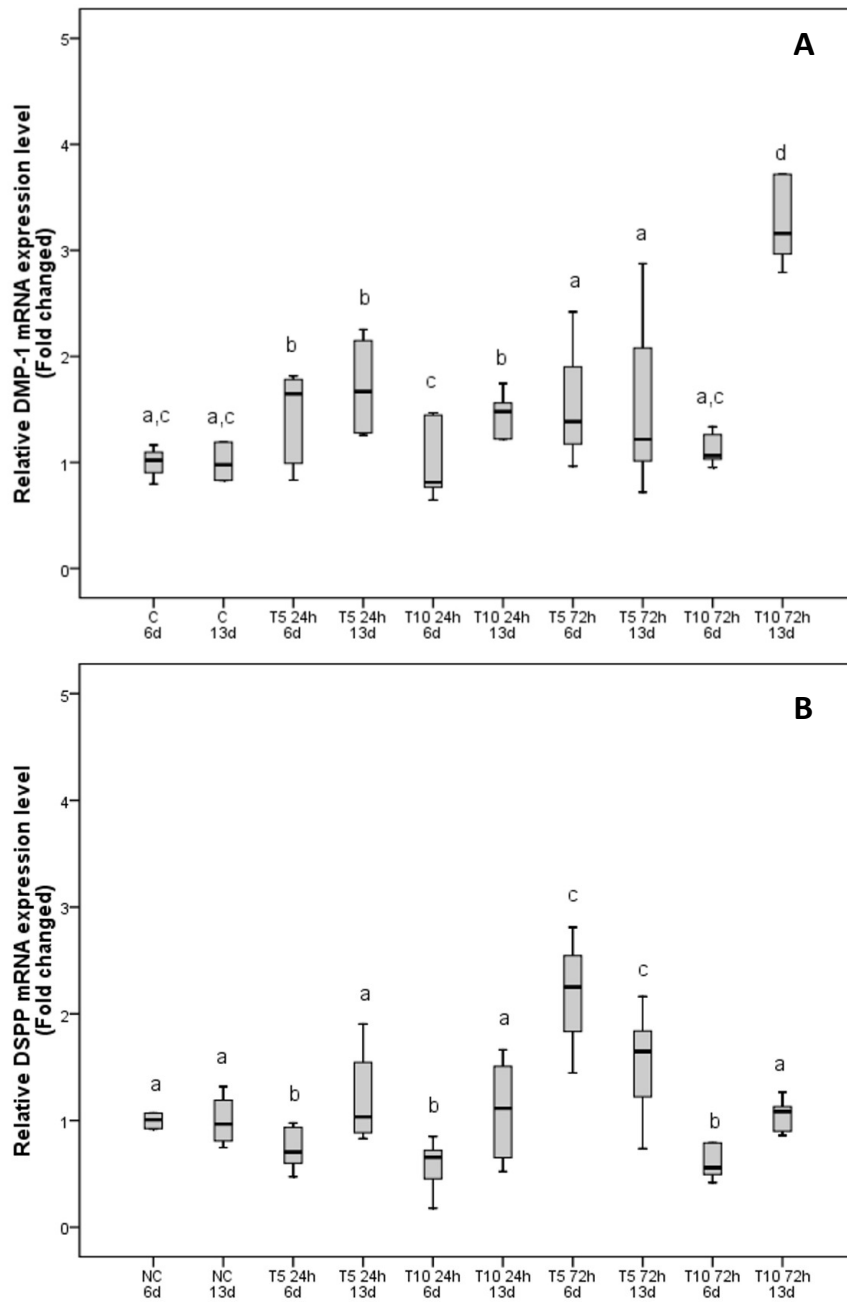


Figure 6. Expression of dentin matrix protein - 1 (A) and dentin sialophosphoprotein (B) from MDPC-23 cells after 6 and 13 days of treatment with taxifolin. Total RNAs were extracted from MDPC-23, the relative mRNA expression of mineralization marker genes were analyzed by quantitative real-time PCR.

^a Different lower case letters show statistical difference between the groups, according to Kruskal-Wallis and Mann-Whitney tests.

Capítulo 3

Effect of Taxifolin on viability and mineralization markers of osteoblast-like cells*

Abstract

Seeking endodontic materials able to induce periapical healing without causing cytotoxicity, the present study aimed to evaluate the effects of flavonoid taxifolin on the viability of osteoblast-like cells and expression of mineralization markers. Cultures of Saos-2 osteoblast-like cells were exposed to three different concentrations of taxifolin treatments (10, 5, and 1 μ M) for different periods (24h, 72h, and continuous treatment). Cell viability by MTT assay, alkaline phosphatase (ALP) activity, deposition of mineralization nodules by alizarin red stain, and expression of ALP and Col-1 by qPCR were determined. Data were analyzed statistically, considering $p < 0.05$. The MTT results showed that taxifolin was not cytotoxic to cells in the concentrations tested. Taxifolin at 10 μ M for 24h or 72h stimulated ALP activity of Saos-2 cells when compared to the DMSO group. Taxifolin at 10 μ M for 72h increased mineralization nodule deposition by cells. Continuous treatment with taxifolin was not effective in stimulating cell mineralization. ALP gene expression increased with taxifolin treatments, however only 24h of taxifolin treatment on day 6 differed from the control. Both 24 and 72h taxifolin treatments increased Col-1 mRNA levels on day 6. When cells were exposed to taxifolin for 72h, there was a significant increase in Col-1 gene expression on day 13. In conclusion, taxifolin 24 and 72h-treatments were more effective than continuous treatment in inducing mineralization markers of osteoblast-like cells and could be a potential medication for stimulating the healing process during treatment of bone lesions, such as apical periodontitis.

Keywords: Flavonoids, Cell Culture Techniques, Osteoblasts, Collagen, Periapical Periodontitis.

Highlights

- Osteoblast-like cells were biostimulated by the flavonoid taxifolin.
- Taxifolin in low concentrations demonstrated a positive effect on mineralization markers.
- Taxifolin has potential use for inducing bone healing.

*The manuscript is according to the guide for authors of *Archives of oral biology*.

Introduction

Non-treated endodontic infection leads to an inflammatory response caused by the direct damaging effect of bacterial products as well as synthesis of local pro-inflammatory mediators by host cells. The break of bone homeostasis and up-regulation of resorption factors cause bone loss and formation of periapical lesions (Siqueira & Roças, 2007). Immune inflammatory response activates an intracellular signaling cascade, involving production of inflammatory cytokines, such as TNF- α , IL-1, and IL-6, which are related to inflammatory cell migration and osteoclastogenesis (Bahuguna et al., 2016; Krum et al., 2010). Several factors are required for the occurrence of periapical healing such as elimination of pulp infection, control of inflammatory responses, and induction of mineralization (Holland et al., 2016).

Osteoblasts are the major cellular component of periapical tissues and jointly with osteoclasts and osteocytes mediate bone formation and reabsorption (Holland et al., 2016; Karygianni et al., 2012; Seibel, 2005). Markers of bone formation are produced by active osteoblasts or derived from procollagen metabolism, and reflect different aspects of osteoblast differentiation and bone remodeling (Hlaing & Compston, 2014). Markers of bone metabolism provide dynamic information about the turnover of osseous tissue. Alkaline phosphatase is an ectoenzyme anchored to the outer surface of mineral forming cells such as osteoblasts, able to cleave pyrophosphate to produce inorganic phosphate and free calcium that participate in the osteoid tissue formation and mineralization (Seibel, 2005). Type 1 collagen is an abundant structural protein and the most prevalent component of the extracellular matrix (Shoulders & Raines 2009). Osteoblasts synthesize procollagen which is enzymatically cleaved in tropocollagen after secretion into extracellular space (Seibel, 2005). Tropocollagen is individual collagen triple helices that form a complex of fibers and networks in tissue, bone and basement membranes (Shoulders & Raines 2009).

Some authors have explored the osteogenic potential of natural compounds such as flavonoids for application in the treatment of osteoporosis, a bone metabolism disorder (Satué et al., 2013; Wang et al., 2017). Flavonoids are a large group of phenolic compounds presenting a benzo- γ -pyrone structure, commonly synthesized by a variety of plants in response to microbial infections. Several beneficial effects of

flavonoids have been reported including antimicrobial, anti-inflammatory, and antioxidant effects (Kumar & Pandey, 2013). The properties of flavonoids are related to their structure. Their basic structure is a flavan nucleus consisting of two benzene rings combined by an oxygen-containing pyran ring (Amić et al., 2007). Lee et al. (2009) showed that flavonoid epigallocatechin gallate (EGCG) decreased Cyr61 synthesis – a protein involved in inflammatory bone loss - in the human osteoblast MG63 cell line, besides attenuating bone resorption associated with apical periodontitis in a rat model. Taxifolin, a catechol type flavonoid isolated from green tea (Wang et al., 2017), has been reported to present antibacterial activity (Jeong et al., 2009), promote osteogenic differentiation of human bone marrow mesenchymal stem cells (Wang et al., 2017) and MC3T3-E1 osteoblastic cells (Satué et al., 2013), induce mineralization (Córdoba et al., 2015; Satué et al., 2013; Wang et al., 2017), and have an anti-osteoclastogenic effect (Satué et al., 2013).

Stimulation of periapical healing by means of osteoblast differentiation and bone remineralization is required not only for permanent teeth with complete root formation but also for young permanent teeth. Even in immature teeth clinically diagnosed with partial pulp necrosis and apical periodontitis, new treatment approaches have been studied aiming at preserving residual pulp cells to allow root maturation and resident periapical cells to repair bone lesions (Chueh et al., 2006; Tsukihoshi et al., 2017). According these studies this clinical condition can be due large foraminal opening which carries cellular and molecular components of the immune system, delaying the necrosis process.

Seeking endodontic materials able to induce periapical healing without causing cytotoxicity, the present study aimed to evaluate the effects of taxifolin treatments on the viability of Saos-2 human osteoblast-like cells and expression of mineralization markers. The null hypotheses tested were: (1) taxifolin present cytotoxic effect on Saos-2 cells, and (2) taxifolin treatments would not stimulate osteoblast-like cells to increase the expression of mineralization markers.

Materials and Methods

Materials

Culture medium, antibiotics, and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Taxifolin (#78666, Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, MO, USA) and the stock solution stored at -20° C.

Cell Culture and study design

Immortalized culture (Saos-2 human osteoblast-like cells) was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA), penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2 mmol/L) (GIBCO, Grand Island, NY, USA). Cells were seeded (2.5×10^3 cells/well) in 96-well plates and incubated at 37°C under a 5% CO₂ and 95% air atmosphere (Thermo Plate, Fisher Scientific, Pittsburgh, PA, USA) for 24h. After incubation, taxifolin (T) treatments were performed as follows: 24h pre-treatment – cells were exposed once to the flavonoid treatment; 72h pre-treatment – cells were exposed three times; continuous treatment – cells were exposed daily up to 13 days. Both pre and continuous treatments were assayed with three taxifolin concentrations: 10, 5, and 1µM (T10, T5, and T1). After 24 or 72h of taxifolin treatment, the DMEM was replaced every 24h until completion of the experimental period (6 or 13 days). The negative control was DMEM without flavonoid, and the control group was DMSO 10 µM (Table 1).

Cell Viability Analysis

Methylthiazol tetrazolium assay was performed to determine cell viability 24h, 72h, 6d, and 13d after beginning treatments. MTT assay is based on the succinate dehydrogenase enzyme produced by mitochondria, which reduces the MTT salt metabolically, converting it into formazan crystals. The treatments or DMEM were aspirated, then MTT 5mg/mL (Sigma-Aldrich, Saint Louis, MO, USA) was applied into wells and the plate was incubated at 37° C under a 5% CO₂ and 95% air atmosphere for 4 h. Thereafter, the MTT solution was aspirated and replaced by acidified isopropanol solution to dissolve the formazan crystals. Cell viability was determined by absorbance

in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader; Biotek Instruments, Winooski, VT, USA) at 570 nm. This assay was performed in duplicate.

Alkaline Phosphatase Assays

Total Protein Production

The quantification of total protein was performed according to Huck *et al.* (2017) with some modifications. On the sixth day, the treatments were aspirated and 200 μ L of sodium lauryl sulfate 0.1% (Sodium dodecyl sulfate, Sigma-Aldrich, Saint Louis, MO, USA) previously dissolved in deionized water were added to each well to lyse the cells. After 40 min at room temperature, the solution was homogenized and 100 μ L were separated for ALP activity assay. Next, 100 μ L of Lowry reagent (Sigma-Aldrich, Saint Louis, MO, USA) were added to the lysed cells and incubated for 20 min at room temperature. Posteriorly, 50 μ L of Folin (Folin-Ciocalteu's phenol reagent, Sigma-Aldrich, Saint Louis, MO, USA), previously diluted in deionized water at a ratio of 1:3, were applied to each well and incubated for 30 min. After this period, all the samples were read in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader; Biotek Instruments, Winooski, VT, USA) to determine absorbance at 655 nm. A standard curve containing 32, 64, 96, 128, and 160 μ g/mL of bovine albumin was determined to measure total protein of each sample (BSA, Sigma-Aldrich, Saint Louis, MO, USA).

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was evaluated following the protocol of the Alkaline Phosphatase Kit-Test Endpoint (Labtest Diagnostics SA, Lagoa Santa, MG, Brazil) based on dephosphorylation of thymolphthalein by ALP. Six days after beginning the experiments, one tube containing 50 μ L of substrate (thymolphthalein monophosphate 22 mmol/L – Reagent 1) and 500 μ L of buffer solution (300 mmol/L, pH 10.1 – Reagent 2) were prepared for each well. Then, 50 μ L of cell lysis solution (prepared in the previous assay) were added to the respective tubes and incubated for 20 min at 37 $^{\circ}$ C. Posteriorly, 2 mL of color reagent (Sodium carbonate 94 mmol/L and sodium hydroxide 250 mmol/L – Reagent 3) were placed in each tube and homogenized. The absorbance of the solution was determined in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT, USA), at 590 nm. In order to measure the ALP activity a standard curve containing 0.43,

1.3, 2.6, 5.2, 6.92, and 8.65 U/L of alkaline phosphatase (Standard 45 U/L – Reagent 4) was used. The absorbance values of ALP were normalized by values of total protein.

Mineralization Nodule Formation

Alizarin red stain was used to evaluate the mineral nodule formation on the final day of treatment (day 13). This assay is based on quantification of the mineralized matrix produced by osteoblast-like cells. The treatments were aspirated and the cells fixed in 70% cold ethanol for 2 h. Subsequently, the ethanol was aspirated and each well received 100 μ L of alizarin red (40 nm; pH 4.2; Sigma-Aldrich, Saint Louis, MO, USA), after which the samples were incubated under shaking for 20 min. The wells were rinsed twice with deionized water and incubated at room temperature to dry for 24h. Next, mineral nodules of each group were identified and photographed by a light microscope (Olympus BX51; Olympus, Miami, USA) equipped with a digital camera (Olympus C5060; Olympus, Miami, USA). Finally, 150 μ L of cetylpyridinium chloride solution (Sigma-Aldrich) were added to each well and maintained under shaking for 15 min to dissolve the nodules. For quantitative analysis, the mineral nodule formation was measured by absorbance in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT, USA), at 570 nm.

ALP and Col-1 gene expression

Based on the results of previous assays, Real Time quantitative PCR (qPCR) analysis was performed only with the highest concentrations of the pre-treatments (24-72h). On days 6 and 13, the treatments were aspirated and total RNA was extracted following the protocol of the RNAqueous Kit (Ambion Inc., Austin, TX, USA). RNA quantification was performed through absorbance with 1 μ L of total RNA in a spectrophotometer (Synergy H1 Hybrid multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT, USA). The cDNA synthesis was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's protocol. The qPCR was performed by SYBR[®] Green reagents (Applied Biosystems, Foster City, CA, USA) and specific primers and probe sets were designed for alkaline phosphatase (ALP), Collagen-1 (Col-1), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) analyses (Table 2). Data were analyzed by Step One Plus Software (Applied Biosystems) with relative quantification of each mRNA according to GAPDH as the constitutive gene. Two independent

experiments were performed in triplicate for each group.

Statistical analysis

Experiments were performed on two independent days using four wells per group. Data from cell viability, ALP activity, and mineralization nodule formation were normally distributed as verified by Kolmogorov-Smirnov test, presented as mean±SD and analyzed using ANOVA and Tukey tests. Data from the qPCR were presented in box whisker plots and analyzed using the Kruskal-Wallis and Mann-Whitney tests. SPSS 17.0 software (SPSS Inc, Chicago IL, USA) was used to run the statistical analysis, considering $p < 0.05$.

Results

Figure 1 presents the percentage of Saos-2 cell viability immediately after exposure to different concentrations of taxifolin. There were no statistical differences between the concentrations; independent of the period of treatment (24h or 72h), showing that taxifolin was not cytotoxic to cells in the concentrations tested. The same was observed for 6 and 13d (Figure 2) considering three types of treatment: 24h, 72h, and continuous treatment (CT). For both time points, cell viability of groups treated continually with 10 μ M of taxifolin decreased, however, cell growth was higher than 70% at day 6 and 80% at day 13.

Taxifolin at 10 μ M for 24h or 72h stimulated ALP activity of Saos-2 cells when compared to the DMSO group. ALP activity from cells under other types of treatments did not differ from the DMSO group (Figure 3).

Figure 4 shows the mineralization ability of Saos-2 cells after 13 days of the three forms of treatment with taxifolin (24h, 72h, and CT), determined by Alizarin red staining, taxifolin at 10 μ M for 72h increased mineralization nodules deposition by cells. Continuous treatment with taxifolin was not effective in stimulating cell mineralization in any concentration tested. Representative images of alizarin red staining show an increase in mineral nodule formation after 24 and 72h-treatments with taxifolin at 10 μ M (Figures 5-B and 5-C) and lower mineral deposition after continuous treatment with taxifolin at 10 μ M (Figure 5D), compared to the control (Figure 5-A - DMSO at 10 μ M).

ALP gene expression increased with taxifolin treatments, however only T10-24h on day 6 presented statistical difference from the control group (Figure 6-A). When cells were exposed to taxifolin for 72h, there was a significant increase in Col-1 gene expression on day 13 (Figure 6). On day 6, both 24h and 72h taxifolin treatments also increased Col-1 mRNA levels when compared to the control (Figure 6-B).

Discussion

This study showed that treatment with 10 μ M for 24h or 72h was not cytotoxic and stimulated ALP activity, mineral nodule deposition, and ALP/Col-1 gene expression, rejecting both the null hypotheses. The MTT results demonstrated that taxifolin at 1, 5, and 10 μ M did not lead to cytotoxic effects after 24 h, 72 h, 6 d, and 13 d of treatment compared to the DMSO group. DMSO 10 μ M was used as the control group, since all tested taxifolin concentrations were diluted in this solvent. Only continuous treatment with the highest taxifolin concentration was able to cause a significant decrease in Saos-2 metabolism at the final evaluation point (day 13), however this reduction is not considered a cytotoxic effect, according to ISO 10993-12:2012 recommendations. Studies evaluating the stimulatory potential of taxifolin on osteoblastic differentiation used higher concentrations (Satué et al., 2013; Wang et al., 2017) than those used in the present study. As we aimed to biostimulate the immortalized osteoblast cell line, not induce differentiation, low concentrations were able to enhance mineralization markers expression over time, testing late set points such as 6 and 13 days.

Immortalized cell lines have been used due their availability of unlimited number of cells, ease of culture and greater phenotypical stability than primary cells, allowing repeatability of results in experiments (Czekanska et al., 2013). The present study was performed with Saos-2 - a cell line derived from the primary osteosarcoma. Saos-2 showed a higher proliferation rate than primary human osteoblast cells, besides similar ALP activity, mineralization potential and gene regulation to primary osteoblasts (Czekanska et al., 2013), can be used as a model of osteoblast behavior to test biomaterials.

Both 24 and 72h-treatments with taxifolin 10 μ M were able to enhance ALP activity, an enzyme commonly used as an early stage marker of mineralization. Lee et

al. (2016) tested the same concentrations as the present study and observed that 5 and 10 μM of the flavonoid baicalein increased ALP activity in human dental pulp cells (hDPC cells) at all time points. These differences can be attributed to the characteristics of flavonoids and cell type used in both studies. Moreover, the hDPCs were incubated in osteogenic medium, which could stimulate the increase in ALP activity at 5 and 1 μM flavonoid concentrations. Similarly to ALP assay, the 24 and 72h-treatments with taxifolin at 10 μM demonstrated better results for mineral nodule formation. However only the T10-72h group presented a significant increase in mineralized matrix deposition compared to the control group. Wang et al. 2017 tested higher concentrations of taxifolin in primary human bone mesenchymal stem cell culture and noted an increase in mineralization nodules in a dose-dependent manner. Our data corroborate with these findings for both 24 and 72h-treatments.

The results of ALP activity and mineral nodule formation assays were better in 24 and 72h-treatments than those found for continuous treatment, showing that low concentrations of taxifolin applied for short periods of time can more effectively stimulate Saos-2 osteoblast-like cell activity than longer periods. Taxifolin stimulation seems to reach a threshold at 72h, and increases in time of exposure exceeding this limit of biostimulation on Saos-2, demonstrate no more positive effects. According to Middleton et al. (2000), flavonoids are able to affect the activity of many mammalian enzymes in a dose-dependent manner. Quercetin – a flavonoid with similar chemical structure to taxifolin (Kumar & Pandey, 2013) act as a competitive inhibitor of ATP binding to the enzyme, resulting in inhibition of Ca^{2+} ATPases (Sheweel et al., 1981) and consequently cell metabolism reduction, as noted in T10-CT after 13 days on MTT assay.

ALP gene expression increased with taxifolin treatments, however only 24h of taxifolin treatment on day 6 differed from the control. Similarly to our results, Lee et al. (2016) showed an increase in ALP gene expression after 7 d of baicalein treatment at 10 μM . Alkaline phosphatase is an enzyme coupled to the cell surface of osteoblasts, which assists in the formation of extracellular matrix competent for mineralization at the early stage of osteogenesis (Córdoba et al., 2015). Considering Col-1, both 24 and 72 h of taxifolin treatment presented increased gene expression at 6 d. Collagen is the main organic component of bone extracellular matrix, promoting a binding site for

mineral components also at the early stages of mineralization (Córdoba et al., 2015). Here in, when cells were exposed to taxifolin for 72h, there was a significant increase in Col-1 gene expression on day 13. A similar effect was observed by Córdoba et al. (2015) when mesenchymal stem cells cultured on bioactive surface taxifolin coating increased Col-1 gene expression after 14d. The ALP function is to create a source of inorganic phosphate for hydroxyapatite formation, cleaving pyrophosphate and releasing calcium and inorganic phosphate. The products of this cleavage are able to act as signaling molecules and stimulate Col-1 and ALP gene expression (Beck, 2003, Pujari-Palmer et al., 2016). In the present study ALP activity was not performed at late stage, but alizarin red staining showed an increased of mineral nodule formation on the 13 d of T10-72h group, this way it is assumed that there were inorganic phosphate and free calcium participating of this mineralization process.

Catechol-type flavonoids, such as taxifolin, present the o-dihydroxy (3',4'-diOH, i.e., catechol) structure in the B ring, and have an important role in metal chelating and scavenging of free radicals, conferring high antioxidant properties (Amić et al., 2007). Taxifolin presents chelating sites (Kumar & Pandey, 2013) that could to act as nucleation sites, stimulating Ca^{2+} and PO_4^{3-} binding in biomineralization process. Interactions between phospholipids, proteins, Ca^{2+} and PO_4^{3-} arrange a molecular architecture which nucleates apatite crystallization *in vivo* (Golub et al., 2009).

Wang et al. (2009) showed an increase in P-glycoprotein – a transporter protein - expression after taxifolin treatment in Caco-2 cells, besides noting that the inhibition of an another transporter protein (multidrug resistance protein 2) was able to decrease the efflux ratio of taxifolin in these cells, but not inhibit the transport of taxifolin completely, suggesting that there may be another transporter for taxifolin. Once in the intracellular environment, taxifolin can scavenge reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) through donation of hydrogen and an electron. Stabilization of ROS reduces cellular injuries, since it inhibits both lipid peroxidation (Kumar & Pandey, 2013), and NF- κ B activation by H_2O_2 (Gloire et al., 2006), since the classical activation of NF- κ B signaling pathway is induced by inflammatory cytokines, such as TNF- α (Devin et al., 2000), however its activation also is possible by ROS, such as H_2O_2 (Gloire et al., 2006).

According to Wang et al. (2017), the activation of NF- κ B signaling pathway suppresses osteoblasts function and inhibits osteogenic effect of taxifolin. NF- κ B signaling pathway activation can promote the induction of osteoclast differentiation genes, prolonged survival of osteoclasts, increased bone resorption (Krum et al., 2010), decreased osteogenic differentiation, and bone formation (Wang et al., 2017). Binding between RANK receptor - expressed in osteoclast progenitor cells - and RANKL - a cytokine expressed in osteoblasts - can be blocked by osteoprotegerin when it links to RANKL, regulating osteoclast formation and consequently decreasing lesion progression. Therefore, treatments able to increase the osteoprotegerin level or inhibit the NF- κ B pathway are potential therapies to prevent bone degradation (Krum et al., 2010).

A suitable approach that promotes the damaged tissue repair should completely fill the bone defect and can be introduced by minimally invasive procedures (Maglione et al., 2017). The injectable scaffolds would allow the filling of defect besides to carry taxifolin incorporated in formulations that increase the flavonoid solubility (Shikov et al., 2009), since it had showed poor solubility (Zu et al., 2012). Therefore, further studies are necessary to explore potential signaling pathways involved in the mineralization stimulation effect of taxifolin, as well as assessing possible vehicles for the application of taxifolin for apical periodontitis treatment.

Conclusion

In conclusion, taxifolin 24 and 72h-treatments were effective in inducing mineralization markers in osteoblast-like cells and could be a potential medication for stimulating the healing process during treatment of bone lesions, such as apical periodontitis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Figures and Tables

Table 1. Description of groups chosen for this study

Group	Compound/Concentration	Treatment
T10-24h	Taxifolin 10 μ M	24h-treatment
T5-24h	Taxifolin 5 μ M	24h -treatment
T1-24h	Taxifolin 1 μ M	24h -treatment
T10-72h	Taxifolin 10 μ M	72h -treatment
T5-72h	Taxifolin 5 μ M	72h -treatment
T1-72h	Taxifolin 1 μ M	72h -treatment
T10-CT	Taxifolin 10 μ M	Continuous treatment
T5-CT	Taxifolin 5 μ M	Continuous treatment
T1-CT	Taxifolin 1 μ M	Continuous treatment
DMSO-10	DMSO 10 μ M	Continuous treatment

Table 2. Nucleotides sequence of primers used for each selected gene.

Gene	Sequence
ALP	Forward 5'-GACAAGAAGCCCTTCACTGC-3'
	Reverse 5'-AGACTGCGCCTGGTAGTTGT-3'
Col-1	Forward 5'-CAGCCGCTTACCTACAGC-3'
	Reverse 5'-TTTTGTATTCAATCACTGTCTTGCC-3'
GAPD H	Forward 5'-CTCTGCTCCTCCTGTTTCGAC-3'
	Reverse 5'-TTAAAAGCAGCCCTGGTGAC-3'

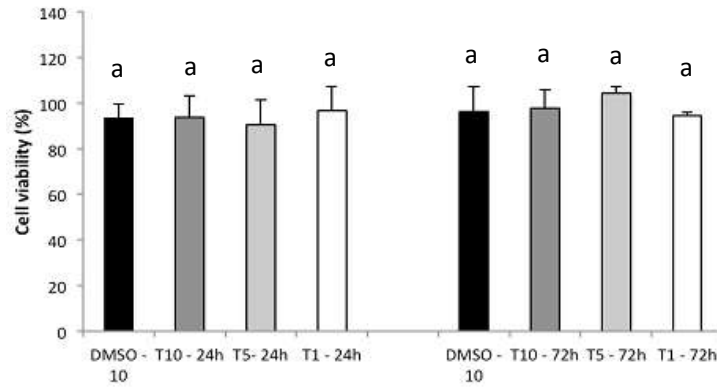


Figure 1. Cell viability immediately after 24 and 72h of treatments with different concentrations of taxifolin, using MTT assays.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

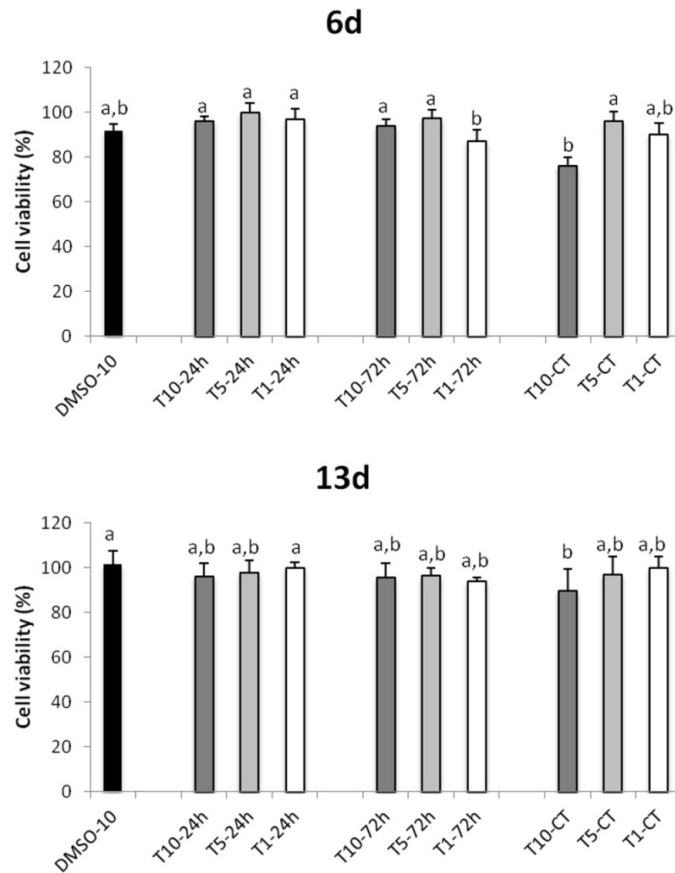


Figure 2. Cell viability of cells after 6 and 13 days of the three forms of treatment (24h, 72h and continuous treatment) with taxifolin, using MTT assays.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

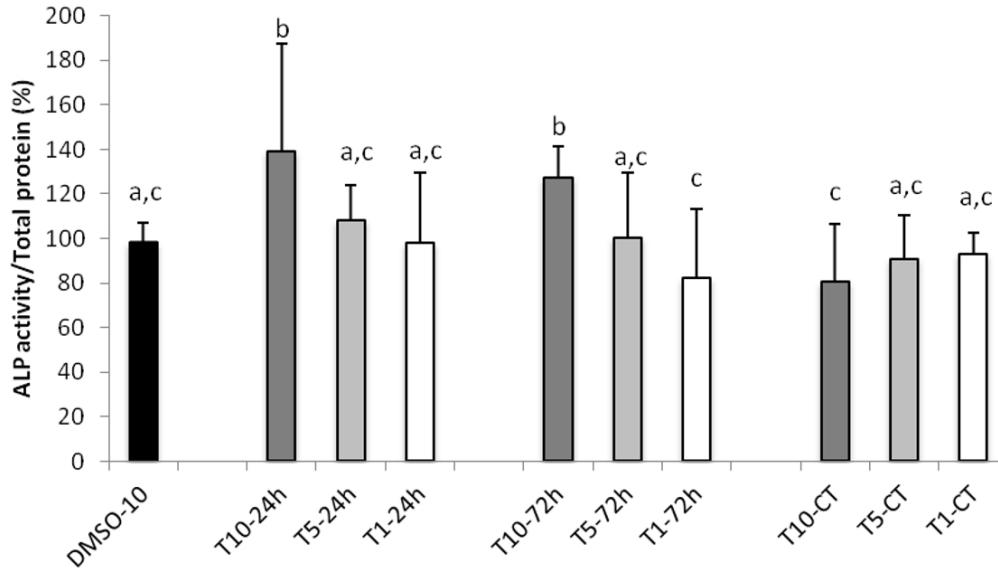


Figure 3. Alkaline phosphatase (ALP) activity of cells after 6 days of the three forms of treatment with taxifolin.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

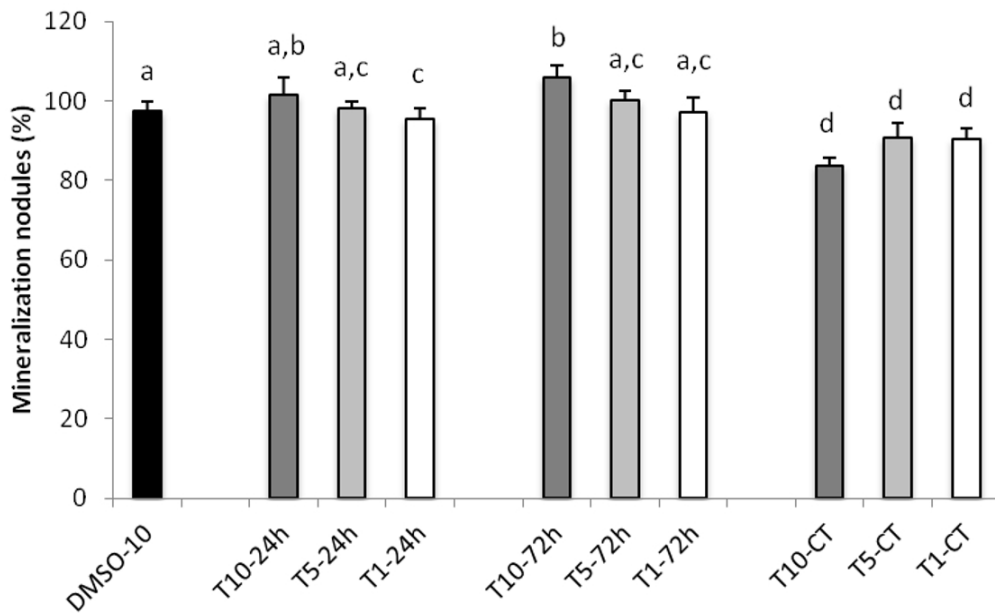


Figure 4. Mineralization ability of cells after 13 days of the three forms of treatment with taxifolin, using alizarin red staining.

^a Different lower case letters show statistical difference between the groups, considering each form of treatment separately, according to ANOVA and Tukey tests.

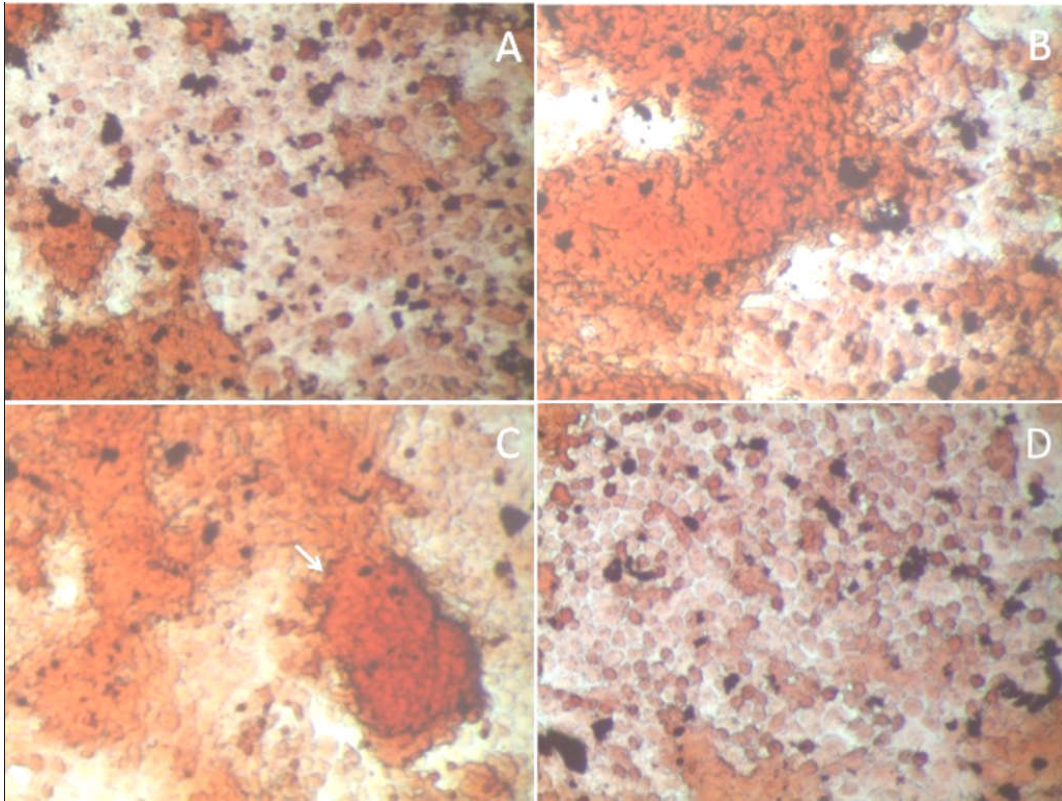


Figure 5. Representative images of alizarin red staining showing mineralization ability of Saos-2 cells after 13 days of taxifolin treatments. A: Control group, B: T10-24, C: T10-72 and D: T10-CT. White arrows show mineralization nodules.

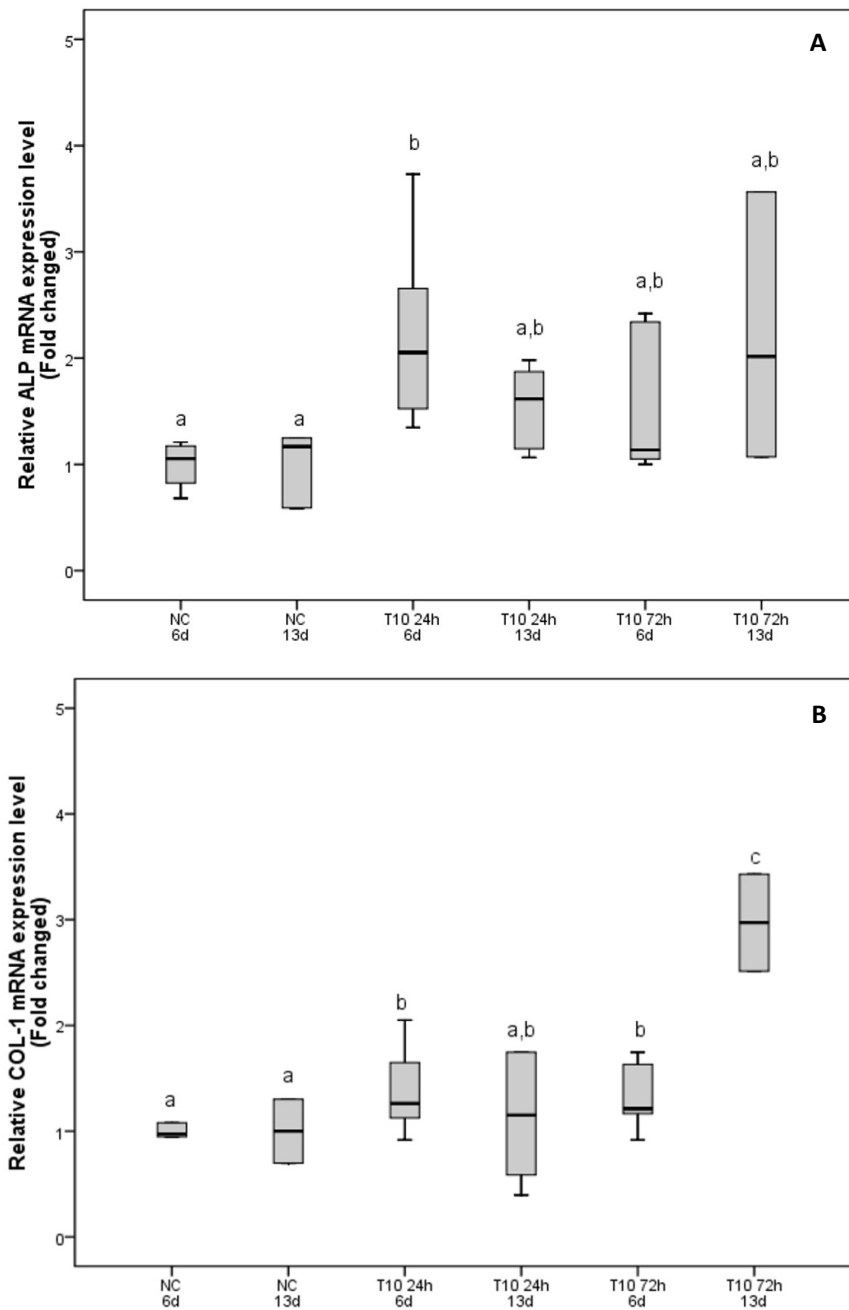


Figure 6. Expression of alkaline phosphatase (A) and collagen-1 (B) from Saos-2 cells after 6 and 13 days of treatment with taxifolin. Total RNAs were extracted from Saos-2, the relative mRNA expression of mineralization marker genes were analyzed by quantitative real-time PCR.

^a Different lower case letters show statistical difference between the groups, according to Kruskal-Wallis and Mann-Whitney tests.

Conclusão

Conclusão

Com base nos objetivos propostos, concluiu-se que:

- O flavonoide taxifolina não apresentou citotoxicidade, mesmo em altas concentrações; enquanto crisina, pinocembrina e galangina apresentaram efeito citotóxico em cultura de fibroblastos.

- Taxifolina apresentou efeito antimicrobiano frente *S. mutans*, *E. faecalis* e *A. israelii*;

- Taxifolina apresentou efeito antibiofilme frente *S. mutans*, *E. faecalis* e *A. israelii*;

- Taxifolina não apresentou efeito citotóxico em nenhuma das concentrações testadas em ambas as culturas, MDPC-23 e Saos-2;

- Os tratamentos de 24 e 72h foram mais eficazes que o tratamento contínuo de taxifolina, estimulando as células MDPC-23 e Saos-2, a aumentar a atividade de ALP e produção de nódulos de mineralização;

- Baixas concentrações de taxifolina, aplicadas em curto período de tempo, também foi capaz de aumentar a expressão de DSPP e DMP-1 em células MDPC-23;

- Os tratamentos de 24 e 72h na concentração de 10 μ M de taxifolina aumentaram a expressão ALP e Col-1 em células Saos-2;

- A taxifolina é um flavonoide com potencial uso para o tratamento endodôntico de dentes permanentes jovens, devido à sua ação antimicrobiana/antibiofilme, baixa citotoxicidade e capacidade de estimular a mineralização em odontoblastos e osteoblastos.

- Estudos futuros devem ser realizados com o objetivo de determinar um veículo adequado, em relação à liberação da taxifolina durante o tratamento, avaliar o seu efeito em células indiferenciadas, além de analisar os mecanismos envolvidos na ação bioestimulatória desse flavonoide.

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Anexos

Anexo A

Guidelines for Publishing Papers in the Biofouling

Manuscript preparation

On the title page, please give the word count of your paper, as follows:

1. Text
2. References
3. Figures
4. Tables

1. Text

1. Include the Abstract, Introduction, Materials and methods, Results, Discussion, Acknowledgements, table titles and all figure captions. Do not include the title page, author list and affiliations, any words that form part of a table or figure, the reference list, and supplemental material, as these are excluded from the word count.

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3, 4 Give the word number (or word equivalents), but do not include in the total.

Manuscripts should normally be no more than 8,000 words and should not contain more than 10 figures (excluding supplemental material). However, this can be reviewed on a case-by-case basis in consultation with the editor if necessary.

Authors who would like to submit mini-reviews or reviews should discuss them with the editor-in-chief or an associate editor beforehand. Mini-reviews should not exceed 7,000 words and reviews should be no longer than 9,000 words (excluding references). They should provide an original critical appraisal of the subject area which adds to the existing body of knowledge and indicates directions for further exploration.

- Manuscripts are accepted only in English. Either American or British English spelling may be used, but must be consistent within an article. Please use single quotation marks, except where 'a quotation is "within" a quotation'.

- For clarity, authors are requested to use the simple past tense for stating what was done, either by others or by themselves, including the procedures, observations, and data of the study being reported. The Materials and Methods and Results sections should be written exclusively in the past tense. Present tense is correct for statements of fact and when reporting your own general conclusions.

- Papers should be written in the third person using the passive voice. Please avoid the use of first and second person pronouns ('I', 'we', 'our', 'you', 'your').

- Non-English speaking authors should have their manuscripts checked for correct use of English before submission.

- For further information on language editing and translation services and correctly preparing a manuscript for submission please visit the Taylor & Francis Author Services website

- Manuscripts should be compiled in the following order: title page; abstract; keywords; main text; acknowledgments; references; tables(s) with caption(s) (on individual pages); figure(s) with caption(s) (as a list); supplemental material (as appropriate).

- Abstracts of 100-150 words are required for all manuscripts submitted.

- Each manuscript should have up to 6 keywords.
- In the Materials & Methods section, full details must be given of all the materials used, such that the work could be repeated exactly by other investigators. Where specific products are named, the manufacturer's name and location (city and country) must be included.
- Search engine optimization (SEO) is a means of making articles more visible. Please consult our guidance here.
- Section headings should be concise. Level 1 : Bold, Lower case; Level 2: Bold, italic ; Level 3: Non-bold, italic; Level 4: Italic followed by a dot, then lead straight on into text.
- The first mention in the text of the Latin name(s) of species used in an investigation should include the full generic and specific name(s), together with the authority. Thereafter, the generic name(s) may be abbreviated to the initial capital letter. All Latin binomials should be italicised (but not in italicised subheadings), but NOT the names of phylla, classes or orders.
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For multiple agency grants
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- Poster presentations and conference papers cannot be cited unless they are documented in published proceedings accessible to everyone. Therefore please ignore the conference paper and conference poster section of the reference guide. The name of the publisher and the place of publication and the page numbers of the article must be clearly stated in all cases. Where a reference is only available online, please include the doi or url.

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- Description of the Journal's article style
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- No more than 5 text citations are permitted in support of any statement made.
- The reference list should be arranged alphabetically and chronologically.
- Papers submitted to, but not accepted by, a named journal may not be cited.
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- It is in the author's interest to provide the highest quality figure format possible. Please be sure that all imported scanned material is scanned at the appropriate resolution: 1200 dpi for line art, 600 dpi for grayscale and 300 dpi for colour and halftones (photographs).
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- Table and figure captions must be saved separately, as part of the file containing the complete text of the manuscript, and numbered correspondingly.
- The file name for a graphic should be descriptive of the graphic, e.g. Figure1, Figure2a.
- All microscope images must show a clear, well-defined scale bar, with its value.

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- Authors must ensure that research reported in submitted manuscripts has been conducted in an ethical and responsible manner, in full compliance with all relevant codes of experimentation and legislation. All manuscripts which report in vivo experiments or clinical trials on humans or animals must include a written Statement in the Methods section that such work was conducted with the formal approval of the local human subject or animal care committees, and that clinical trials have been registered as legislation requires.

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Anexo B

Guidelines for Publishing Papers in the International Endodontic Journal

Author Guidelines

Content of Author Guidelines: 1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance

Useful Websites: Submission Site, Articles published in International Endodontic Journal, Author Services, Wiley's Ethical Guidelines, Guidelines for Figures

The journal to which you are submitting your manuscript employs a plagiarism detection system. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published works.

1. GENERAL

International Endodontic Journal publishes original scientific articles, reviews, clinical articles and case reports in the field of Endodontology; the branch of dental sciences dealing with health, injuries to and diseases of the pulp and periradicular region, and their relationship with systemic well-being and health. Original scientific articles are published in the areas of biomedical science, applied materials science, bioengineering, epidemiology and social science relevant to endodontic disease and its management, and to the restoration of root-treated teeth. In addition, review articles, reports of clinical cases, book reviews, summaries and abstracts of scientific meetings and news items are accepted.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in International Endodontic Journal. Authors are encouraged to visit Wiley Author Services for further information on the preparation and submission of articles and figures.

2. ETHICAL GUIDELINES

International Endodontic Journal adheres to the below ethical guidelines for publication and research.

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Authors submitting a paper do so on the understanding that the manuscript has been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal.

International Endodontic Journal adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE, authorship criteria should be based on 1) substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3.

Acknowledgements: Under acknowledgements please specify contributors to the article other than the authors accredited. Please also include specifications of the source of funding for the

study and any potential conflict of interests if appropriate. Please find more information on the conflict of interest form in section 2.6.

2.2. Ethical Approvals

Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study. The authors MUST upload a copy of the ethical approval letter when submitting their manuscript. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

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The International Endodontic Journal asks that authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following public clinical trials registries: www.clinicaltrials.gov, <https://www.clinicaltrialsregister.eu/>, <http://isrctn.org/>. Other primary registries if named in the WHO network will also be considered acceptable. The clinical trial registration number and name of the trial register should be included in the Acknowledgements at the submission stage.

2.3.1 Randomised control clinical trials

Randomised control clinical trials should be reported using the guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.

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Submitting authors of epidemiological human observations studies are required to review and submit a 'strengthening the reporting of observational studies in Epidemiology' (STROBE) checklist and statement. Compliance with this should be detailed in the materials and methods section. (www.strobe-statement.org)

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Systematic reviews should be reported using the PRISMA guidelines available at <http://prisma-statement.org/>. A PRISMA checklist and flow diagram (as a Figure) should also be included in the submission material.

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Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

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3.1 MANUSCRIPT SUBMISSION PROCEDURE

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3.2. Getting Started

- Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online Submission Site: <http://mc.manuscriptcentral.com/iej>
- Log-in, or if you are a new user, click on 'register here'.
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 - After clicking on 'register here', enter your name and e-mail information and click 'Next'. Your e-mail information is very important.
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Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the abstract, main text, references, tables, and figure legends, but no embedded figures or Title page. The Title page should be uploaded as a separate file. In the main text, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for the individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

3.5. Blinded Review

Manuscript that do not conform to the general aims and scope of the journal will be returned immediately without review. All other manuscripts will be reviewed by experts in the field (generally two referees). International Endodontic Journal aims to forward referees' comments and to inform the corresponding author of the result of the review process. Manuscripts will be considered for fast-track publication under special circumstances after consultation with the Editor.

International Endodontic Journal uses double blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper and the name(s) of the author(s) will not be disclosed to the reviewers.

To allow double blinded review, please submit (upload) your main manuscript and title page as separate files.

Please upload:

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- Figure files under the file designation 'figures'
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All documents uploaded under the file designation 'title page' will not be viewable in the html and pdf format you are asked to review in the end of the submission process. The files viewable in the html and pdf format are the files available to the reviewer in the review process.

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You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

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To submit a revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision'. Please remember to delete any old files uploaded when you upload your revised manuscript.

4. MANUSCRIPT TYPES ACCEPTED

Original Scientific Articles: must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original Scientific Articles must conform to the highest international standards in the field.

Review Articles: are accepted for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy. Reviews should generally include a clearly defined search strategy and take a broad view of the field rather than merely summarizing the authors' own previous work. Extensive or unbalanced citation of the authors' own publications is discouraged.

Mini Review Articles: are accepted to address current evidence on well-defined clinical, research or methodological topics. All are refereed by experts in the field who are asked to comment on timeliness, general interest, balanced treatment of controversies, and scientific rigor. A clear research question, search strategy and balanced synthesis of the evidence is expected. Manuscripts are limited in terms of word-length and number of figures.

Clinical Articles: are suited to describe significant improvements in clinical practice such as the report of a novel technique, a breakthrough in technology or practical approaches to recognised clinical challenges. They should conform to the highest scientific and clinical practice standards.

Case Reports: illustrating unusual and clinically relevant observations are acceptable but they must be of sufficiently high quality to be considered worthy of publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered. Illustrative material must be of the highest quality and healing outcomes, if appropriate, should be demonstrated.

Supporting Information: International Endodontic Journal encourages submission of adjuncts to printed papers via the supporting information website (see submission of supporting

information below). It is encouraged that authors wishing to describe novel procedures or illustrate cases more fully with figures and/or video may wish to utilise this facility.

Letters to the Editor: are also acceptable.

Meeting Reports: are also acceptable.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Language: The language of publication is English. It is preferred that manuscript is professionally edited. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication

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Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

5.2. Structure

All manuscripts submitted to International Endodontic Journal should include Title Page, Abstract, Main Text, References and Acknowledgements, Tables, Figures and Figure Legends as appropriate

Title Page: The title page should bear: (i) Title, which should be concise as well as descriptive; (ii) Initial(s) and last (family) name of each author; (iii) Name and address of department, hospital or institution to which work should be attributed; (iv) Running title (no more than 30 letters and spaces); (v) No more than six keywords (in alphabetical order); (vi) Name, full postal address, telephone, fax number and e-mail address of author responsible for correspondence.

Abstract for Original Scientific Articles should be no more than 250 words giving details of what was done using the following structure:

- **Aim:** Give a clear statement of the main aim of the study and the main hypothesis tested, if any.
- **Methodology:** Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.
- **Results:** Give the main results of the study, including the outcome of any statistical analysis.
- **Conclusions:** State the primary conclusions of the study and their implications. Suggest areas for further research, if appropriate.

Abstract for Review Articles should be non-structured of no more than 250 words giving details of what was done including the literature search strategy.

Abstract for Mini Review Articles should be non-structured of no more than 250 words, including a clear research question, details of the literature search strategy and clear conclusions.

Abstract for Case Reports should be no more than 250 words using the following structure:

- Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.
- Summary: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any.
- Key learning points: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Abstract for Clinical Articles should be no more than 250 words using the following structure:

- Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.
- Methodology: Describe the methods adopted.
- Results: Give the main results of the study.
- Conclusions: State the primary conclusions of the study.

Main Text of Original Scientific Article should include Introduction, Materials and Methods, Results, Discussion and Conclusion

Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.

(i) Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.

(ii) Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if

applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

(iii) Suppliers: Suppliers of materials should be named and their location (Company, town/city, state, country) included.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.

Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion section should progress with a review of the methodology before discussing the results in light of previous work in the field. The Discussion should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

Conclusion: should contain a summary of the findings.

Main Text of Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should be focused to place the subject matter in context and to justify the need for the review. The Review section should be divided into logical sub-sections in order to improve readability and enhance understanding. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The use of tabulated and illustrative material is encouraged. The Conclusion section should reach clear conclusions and/or recommendations on the basis of the evidence presented.

Main Text of Mini Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should briefly introduce the subject matter and justify the need and timeliness of the literature review. The Review section should be divided into logical sub-sections to enhance readability and understanding and may be supported by up to 5 tables and figures. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The Conclusions section should present clear statements/recommendations and suggestions for further work. The manuscript, including references and figure legends should not normally exceed 4000 words.

Main Text of Clinical Reports and Clinical Articles should be divided into Introduction, Report, Discussion and Conclusion,. They should be well illustrated with clinical images, radiographs, diagrams and, where appropriate, supporting tables and graphs. However, all illustrations must be of the highest quality

Acknowledgements: International Endodontic Journal requires that all sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential conflicts of interest noted. Grant or contribution numbers may be acknowledged, and principal grant holders should be listed. Acknowledgments should be brief and should not include thanks to anonymous referees and editors. See also above under Ethical Guidelines.

5.3. References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. The EndNote reference style can be obtained upon request to

the editorial office (iejeditor@cardiff.ac.uk). Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.asp

In the text: single or double authors should be acknowledged together with the year of publication, e.g. (Pitt Ford & Roberts 1990). If more than two authors the first author followed by et al. is sufficient, e.g. (Tobias et al. 1991). If more than 1 paper is cited the references should be in year order and separated by "," e.g. (Pitt Ford & Roberts 1990, Tobias et al. 1991). Reference list: All references should be brought together at the end of the paper in alphabetical order and should be in the following form.

(i) Names and initials of up to six authors. When there are seven or more, list the first three and add et al.

(ii) Year of publication in parentheses

(iii) Full title of paper followed by a full stop (.)

(iv) Title of journal in full (in italics)

(v) Volume number (bold) followed by a comma (,)

(vi) First and last pages

Examples of correct forms of reference follow:

Standard journal article

Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen-expressing cells in experimentally induced pulpitis. *International Endodontic Journal* 24, 8-14.

Corporate author

British Endodontic Society (1983) Guidelines for root canal treatment. *International Endodontic Journal* 16, 192-5.

Journal supplement

Frumin AM, Nussbaum J, Esposito M (1979) Functional asplenia: demonstration of splenic activity by bone marrow scan (Abstract). *Blood* 54 (Suppl. 1), 26a.

Books and other monographs

Personal author(s)

Gutmann J, Harrison JW (1991) *Surgical Endodontics*, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Chapter in a book

Wesselink P (1990) Conventional root-canal therapy III: root filling. In: Harty FJ, ed. *Endodontics in Clinical Practice*, 3rd edn; pp. 186-223. London, UK: Butterworth.

Published proceedings paper

DuPont B (1974) Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. *Proceedings of the Third Annual Meeting of the International Society for Experimental Rematology*; pp. 44-46. Houston, TX, USA: International Society for Experimental Hematology.

Agency publication

Ranofsky AL (1978) *Surgical Operations in Short-Stay Hospitals: United States-1975*. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8

Dissertation or thesis

Saunders EM (1988) *In vitro and in vivo investigations into root-canal obturation using thermally softened gutta-percha techniques (PhD Thesis)*. Dundee, UK: University of Dundee.

URLs

Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text.

Smith A (1999) Select committee report into social care in the community [WWW document].

URL <http://www.dhss.gov.uk/reports/report015285.html>

[accessed on 7 November 2003]

5.4. Tables, Figures and Figure Legends

Tables: Tables should be double-spaced with no vertical rulings, with a single bold ruling beneath the column titles. Units of measurements must be included in the column title.

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5 column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same typeface should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction, symbols should be at least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general, multi-part figures should be arranged as they would appear in the final version. Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

Figures should be on a white background, and should avoid excessive boxing, unnecessary colour, shading and/or decorative effects (e.g. 3-dimensional skyscraper histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. The line spacing should be wide enough to remain clear on reduction to the minimum acceptable printed size.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same typesize as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by a thin space (1 000). Unusual units or abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general, visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc.)

Figure legends: Figure legends should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used; they should not contain any details of methods.

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Further information can be obtained at Wiley Blackwell's guidelines for figures: <http://authorservices.wiley.com/bauthor/illustration.asp>.

Check your electronic artwork before submitting it: <http://authorservices.wiley.com/bauthor/eachecklist.asp>.

5.5. Supporting Information

Publication in electronic formats has created opportunities for adding details or whole sections in the electronic version only. Authors need to work closely with the editors in developing or using such new publication formats.

Supporting information, such as data sets or additional figures or tables, that will not be published in the print edition of the journal, but which will be viewable via the online edition, can be submitted. It should be clearly stated at the time of submission that the supporting information is intended to be made available through the online edition. If the size or format of the supporting information is such that it cannot be accommodated on the journal's website, the author agrees to make the supporting information available free of charge on a permanent Web site, to which links will be set up from the journal's website. The author must advise Wiley Blackwell if the URL of the website where the supporting information is located changes. The content of the supporting information must not be altered after the paper has been accepted for publication.

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Preparation of Supporting Information: Although provision of content through the web in any format is straightforward, supporting information is best provided either in web-ready form or in a form that can be conveniently converted into one of the standard web publishing formats:

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- GIF or JPEG for still graphics. Graphics supplied as EPS or TIFF are also acceptable.
- MPEG or AVI for moving graphics.

Subsequent requests for changes are generally unacceptable, as for printed papers. A charge may be levied for this service.

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6. AFTER ACCEPTANCE

Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor who is responsible for the production of the journal.

6.1. Figures

Hard copies of all figures and tables are required when the manuscript is ready for publication. These will be requested by the Editor when required. Each Figure copy should be marked on the reverse with the figure number and the corresponding author's name.

6.2 Proof Corrections

The corresponding author will receive an email alert containing a link to a web site. A working email address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site. Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following Web site: www.adobe.com/products/acrobat/readstep2.html. This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs. Proofs must be returned to the Production Editor within three days of receipt. As changes to proofs are costly, we ask that you only correct typesetting errors. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately. Other than in exceptional circumstances, all illustrations are retained by the publisher. Please note that the author is responsible for all statements made in his work, including changes made by the copy editor.

6.3 Early Online Publication Prior to Print

International Endodontic Journal is covered by Wiley Blackwell's Early View service. Early View articles are complete full-text articles published online in advance of their publication in a printed issue. Early View articles are complete and final. They have been fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated. Because they are in final form, no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot be cited in the traditional way. They are therefore given a Digital Object Identifier (DOI), which allows the article to be cited and tracked before it is allocated to an issue. After print publication, the DOI remains valid and can continue to be used to cite and access the article.

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6.5 Author Material Archive Policy

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7 Guidelines for reporting of DNA microarray data

The International Endodontic Journal gives authors notice that, with effect from 1st January 2011, submission to the International Endodontic Journal requires the reporting of microarray data to conform to the MIAME guidelines. After this date, submissions will be assessed according to MIAME standards. The complete current guidelines are available at http://www.mged.org/Workgroups/MIAME/miame_2.0.html. Also, manuscripts will be published only after the complete data has been submitted into the public repositories, such as GEO (<http://www.ncbi.nlm.nih.gov/geo/>) or ArrayExpress (http://www.ebi.ac.uk/microarray/submissions_overview.html), in MIAME compliant format, with the data accession number (the identification number of the data set in the database) quoted in the manuscript. Both databases are committed to keeping the data private until the associated manuscript is published, if requested.

Prospective authors are also encouraged to search for previously published microarray data with relevance to their own data, and to report whether such data exists. Furthermore, they are encouraged to use the previously published data for qualitative and/or quantitative comparison with their own data, whenever suitable. To fully acknowledge the original work, an appropriate reference should be given not only to the database in question, but also to the original article in which the data was first published. This open approach will increase the availability and use of these large-scale data sets and improve the reporting and interpretation of the findings, and in increasing the comprehensive understanding of the physiology and pathology of endodontically related tissues and diseases, result eventually in better patient care.

Anexo C

Guidelines for Publishing Papers in the Archives of Oral Biology

Preparation

Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Manuscript Structure

Follow this order when typing manuscripts: Title, Authors, Affiliations, Abstract, Keywords, Main text (Introduction, Materials & Methods, Results, Discussion for an original paper), Acknowledgments, Appendix, References, Figure Captions and then Tables. Do not import the Figures or Tables into your text. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table footnotes) should be identified with superscript Arabic numbers.

Introduction

This should be a succinct statement of the problem investigated within the context of a brief review of the relevant literature. Literature directly relevant to any inferences or argument presented in the Discussion should in general be reserved for that section. The introduction may conclude with the reason for doing the work but should not state what was done nor the findings.

Materials and Methods

Enough detail must be given here so that another worker can repeat the procedures exactly. Where the materials and methods were exactly as in a previous paper, it is not necessary to repeat all the details but sufficient information must be given for the reader to comprehend what was done without having to consult the earlier work.

Authors are requested to make plain that the conditions of animal and human experimentation are as outlined in the "Ethics" and "Studies on Animals" sections above

Results or Findings

These should be given clearly and concisely. Care should be taken to avoid drawing inferences that belong to the Discussion. Data may be presented in various forms such as histograms or tables but, in view of pressure on space, presentation of the same data in more than one form is unacceptable.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

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- Author names and affiliations. Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
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As titles frequently stand alone in indexes, bibliographic journals etc., and indexing of papers is, to an increasing extent, becoming computerized from key words in the titles, it is important that titles should be as concise and informative as possible. Thus the animal species to which the observations refer should always be given and it is desirable to indicate the type of method on which the observations are based, e.g. chemical, bacteriological, electron-microscopic, histochemical, etc. A "running title" of not more than 40 letters and spaces must also be supplied. A keyword index must be supplied for each paper.

Structured abstract

The paper should be prefaced by an abstract aimed at giving the entire paper in miniature. Abstracts should be no longer than 250 words and should be structured as per the guidelines published in the Journal of the American Medical Association (JAMA 1995; 273: 27-34). In brief, the abstract should be divided into the following sections: (1) Objective; (2) Design - if clinical, to

include setting, selection of patients, details on the intervention, outcome measures, etc.; if laboratory research, to include details on methods; (3) Results; (4) Conclusions.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

As Archives of Oral Biology is a journal with a multidisciplinary readership, abbreviations, except those universally understood such as mm, g, min. u.v., w/v and those listed below, should be avoided if possible. Examples of abbreviations which may be used without definition: ADP, AMP, ATP, DEAE-cellulose, DNA, RNA, EDTA, EMG, tris.

Other abbreviations used to improve legibility should be listed as a footnote on the title page. Chemical symbols may be used for elements, groups and simple compounds, but excessive use should be avoided. Abbreviations other than the above should not be used in titles.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Bacterial nomenclature

Organisms should be referred to by their scientific names according to the binomial system. When first mentioned the name should be spelt in full and in italics. Afterwards the genus

should be abbreviated to its initial letter, e.g. '*S. aureus*' not '*Staph. aureus*'. If abbreviation is likely to cause confusion or render the intended meaning unclear, the names of microbes should be spelt in full. Only those names which were included in the Approved List of Bacterial Names, *Int J Syst Bacteriol* 1980; 30: 225-420 and those which have been validly published in the *Int J Syst Bacteriol* since 1 January 1980 have standing in nomenclature. If there is good reason to use a name that does not have standing in nomenclature, the names should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example see *Int J Syst Bacteriol* 1980; 30: 547-556). When the genus alone is used as a noun or adjective, use lower case Roman not italic, e.g. '*organisms were staphylococci*' and '*streptococcal infection*'. If the genus is specifically referred to use italics e.g. '*organisms of the genus Staphylococcus*'. For genus in plural, use lower case roman e.g. '*salmonellae*'; plurals may be anglicized e.g. '*salmonellas*'. For trivial names, use lower case Roman e.g. '*meningococcus*'

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Examples:

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Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. *Journal of Scientific Communications*, 163, 51–59.

Reference to a book:

Strunk, W., Jr., & White, E. B. (2000). *The elements of style*. (4th ed.). New York: Longman, (Chapter 4).

Reference to a chapter in an edited book:

Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), *Introduction to the electronic age* (pp. 281–304). New York: E-Publishing Inc.

Reference to a website:

Cancer Research UK. Cancer statistics reports for the UK. (2003). <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/> Accessed 13 March 2003.

Reference to a dataset:

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Statistical analysis

Authors should ensure that the presentation and statistical testing of data are appropriate and should seek the advice of a statistician if necessary. A number of common errors should be avoided, e.g.:-

- Use of parametric tests when non-parametric tests are required
- Inconsistencies between summary statistics and statistical tests such as giving means and standard deviations for data which were analysed with non-parametric tests.
- Multiple comparisons undertaken with multiple t tests or non-parametric equivalents rather than with analysis of variance (ANOVA) or non-parametric equivalents.
- Post hoc tests being used following an ANOVA which has yielded a non-significant result.
- Incomplete names for tests (e.g. stating "Student's t test" without qualifying it by stating "single sample", "paired" or "independent sample")
- N values being given in a way which obscures how many independent samples there were (e.g. stating simply $n=50$ when 10 samples/measurements were obtained from each of 5 animals/human subjects).
- Stating that $P=0.000$ (a figure which is generated by some computer packages). The correct statement (in this case) is $P<0.0005$.

Anexo D



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"



CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

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

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado "**Combinações de flavonoides em sistemas de hidrogéis termossensíveis 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.

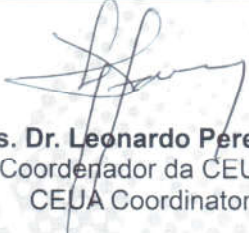
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 15 de Janeiro de 2020.

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 Perez Faverani
Coordenador da CEUA
CEUA Coordinator

Anexo E

1. Protocolo do Ensaio de MTT

- 1- Preparar a solução de MTT
 - 5mg do sal de MTT em 1mL de PBS
 - Filtrar dentro do fluxo laminar
- 2- Diluir a solução de MTT em meio de cultura
 - Aspirar os tratamentos
 - Para a placa de 96 poços: 20µL da solução em 180µL de DMEM sem FBS
- 3- Incubadora 37°C, 5%CO₂ por 4h
 - Proteger da luz
- 4- Aspirar a solução de MTT
- 5- Adicionar 100µL de isopropanol acidificado em cada poço
- 6- Ressuspender e transferir para uma nova placa de 96 poços
- 7- Leitura no espectrofotômetro (570nm)

2. Protocolo de Determinação da Concentração Inibitória Mínima e Concentração Bactericida Mínima

Dia 1: Reativar o microrganismo em BHI agar para *E. faecalis* e *A. israelii* e MSB agar para *S. mutans* (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar a 37°C e 5%CO₂ durante 24h.

Dia 2: Repicar o microrganismo da placa para o respectivo meio de cultura em caldo (5 UFC em 5ml) e incubar nas mesmas condições por 24h.

Dia 3: Diluição dos flavonoides

- Preparar a solução estoque em DMSO
- 5% de DMSO na concentração final de cada poço

Preparar o inóculo de acordo com a curva de crescimento: DO = 0,5

- Centrífuga (4000rpm por 10min)
- Diluir 1000x no meio de cultura Mueller-Hinton (500µl do inóculo em 4,5ml de meio – 3 tubos)
- Ressuspender o inóculo em cada poço; tampar a placa e incubar por 24h.

Dia 4: Pipetar 15µl de solução de Resazurina 0,01% em cada poço e incubar por 4h.

- Plaquear o último poço com coloração azulada (CIM) e, no mínimo, 3 poços anteriores para se determinar a CBM.

Dia 5: Leitura das placas de petri

- Contagem das unidades formadoras de colônias.

3. Protocolo do Ensaio de Biofilme em Placas de Poliestireno

Dia 1: Reativar o microrganismo em BHI agar para *E. faecalis* e *A. israelii* e MSB agar para *S. mutans* (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar á 37°C e 5%CO₂ durante 24h.

Dia 2: Repicar o microrganismo da placa para o respectivo meio de cultura em caldo (5 UFC em 5ml) e incubar.

Dia 3: Formação do biofilme

- Preparar a placa de 96 poços com fundo em U (não tratada)

- Preparar o inóculo de acordo com a curva de crescimento: DO 0,5 para todos os microrganismos teste;

- Adicionar 20µl do inóculo e 180µl de BHI suplementado com 0,5% de glicose; incubar por 48h.

Dia 5: Aspirar o sobrenadante com ponteira estéril, e lavar os poços com 200µl de solução salina 0,9%, remover toda a solução com ponteira estéril;

- Acrescentar 200µl do antimicrobiano na concentração 10 vezes maiores que a CBM pré-determinada;

*Incluir as mesmas concentrações para o controle positivo (Clorexidina)

*Cada concentração deverá ser testada em 3 poços por experimento (triplicata);

- Incubar por 24h.

Dia 6: Com o auxílio da ponta da ponteira remover o biofilme aderido no fundo do poço e ressuspende-lo na própria solução antimicrobiana presente.

-Diluição e plaqueamento de todos os poços.

Dia 7: Leitura das placas de petri (contagem das CFU)

4. Protocolo do Ensaio de Biofilme em túbulos dentinários

- 1-Obtenção e limpeza dos dentes bovinos
- 2-Confecção das amostras
 - a. Cortadeira: obtenção dos cilindros de dentina a partir da secção horizontal 1mm abaixo da junção cimento-esmalte (4mm)
 - b. Brocas Gates-Glidden para padronizar o diâmetro interno (1.5mm)
 - c. Corte vertical para obtenção dos hemicilindros (3x3x2mm)
- 3-Limpeza dos blocos em ultrassom: EDTA 17% (3m) + Água destilada (5m)
- 4-Esterilizar das amostras na autoclave
- 5-Fixar as amostras em tubos eppendorfs com resina composta
- 6-Contaminação das amostras
 - a. 500µL da cultura de *E. faecalis* (10^7 CFU/mL) em caldo BHI
 - b. Centrifugação crescente dos eppendorfs: 1400, 2000, 3600 e 5600g durante 5min (2x)
- 7-Maturação do biofilme
 - a. Transferência das amostras contaminadas para placas de 48 poços contendo caldo BHI
 - b. Trocas de BHI foram realizadas a cada 72h durante 15d
- 8-Tratamentos
 - a. 10x MBC da taxifolina
 - b. 100 e 1000x MBC da clorexidina
 - c. 48h de incubação
- 9-Secção das amostras
- 10- Corante Live-Dead
 - a. 20min, 37°C
 - b. Lavar com água destilada
- 11- Análise por microscopia confocal de varredura a laser
- 12- Quantificação das células mortas/vivas pelo *Image J*.

5. Protocolo do Ensaio de atividade da Fosfatase alcalina – ALP

- 1- Aspirar os tratamentos e adicionar 200µL de SDS (Sigma Ref. L5750) por poço
 - Incubar em temperatura ambiente por 40min
- 2- Preparar os tubos
 - Numerar de acordo com as amostras
 - Organizar em estante
 - Não esquecer da curva padrão (P1-P6) e do branco
- 3- Adicionar 50µL do substrato (Reagente 1 do Kit Labtest Ref 40) nos tubos das amostras e da curva
- 4- Adicionar 500µL de tampão (Reagente 2 do kit) nos tubos
- 5- Preparar a curva padrão

Branco = 550µL de tampão
P1 = 525µL do tampão + 25µL do padrão
P2 = 475µL do tampão + 75µL do padrão
P3 = 400µL do tampão + 150µL do padrão
P4 = 250µL do tampão + 300µL do padrão
P5 = 150µL do tampão + 400µL do padrão
P6 = 50µL do tampão + 500µL do padrão

- 6- Transferir 50µL de cada amostra para o seu respectivo tubo, no banho-maria (37°C)
- 7- Incubar por 20min no banho-maria (37°C)
- 8- Remover a estante do banho e adicionar 2mL do reagente de cor (Reagente 3 do Kit) em cada tubo, inclusive na curva e no branco.
- 9- Leitura no espectrofotômetro (590nm)

6. Protocolo para Determinação da Produção de Proteína Total

- 1- Pegar uma placa nova de 96 poços e transferir 100µL das amostras que sobraram em SDS
- 2- Preparar a curva padrão em uma fileira livre da placa
 - Branco = 100µL de água deionizada
 - P1 = 80µL de água deionizada + 20µL de BSA (Sigma Ref P5619)
 - P2 = 60µL de água deionizada + 40µL de BSA (Sigma Ref P5619)
 - P3 = 40µL de água deionizada + 60µL de BSA (Sigma Ref P5619)
 - P4 = 20µL de água deionizada + 80µL de BSA (Sigma Ref P5619)
 - P5 = 100µL de BSA (Sigma Ref P5619)
- 3- Adicionar 100µL do Reagente de Lowry (Sigma Ref. L3540) em cada poço, inclusive na curva e branco
- 4- Incubar por 20min em temperatura ambiente
- 5- Adicionar 50µL de Folin (Sigma F9252) em cada poço, inclusive na curva e branco
- 6- Incubar por 30min em temperatura ambiente
- 7- Leitura no espectrofotômetro (655nm)

7. Protocolo para Coloração com Alizarin Red

- 1- Aspirar os tratamentos e adicionar 100µL de álcool 70% em cada poço
- 2- Incubar a 4°C por 2h
- 3- Aspirar o álcool
- 4- Adicionar 100µL da solução de Alizarin
 - 1.37g em 100mL de água
 - Ajustar pH para 4.2
- 5- Agitar por 20min (shaker)

- 6- Aspirar a solução e lavar 2x com água deionizada
- 7- Deixar sob a bancada secando por 24h
- 8- Fotomicrografias
- 9- Preparar a solução de Cloreto de Cetilperidíneo 10%
 - 1g (Sigma C9002) para 10mL de PBS
 - Vortexar até ficar transparente
- 10- Adicionar 150µL da solução em cada poço
- 11- Agitar por 15min (shaker)
- 12- Ressuspender e transferir 100µL para uma nova placa
- 13- Leitura no espectrofotômetro (570nm)

8. Protocolo para extração de RNA (kit RNAqueous Ref AM1931)

RNAqueous®-Micro Procedure

A. Preparation of Materials

Add 10.5 mL ethanol to Wash Solution 1 Concentrate

Add 10.5 mL of ACS grade 100% ethanol to the bottle labeled Wash Soln 1 Concentrate. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

Add 22.4 mL 100% ethanol to Wash Solution 2/3 Concentrate

Add 22.4 mL of ACS grade 100% ethanol to the bottle labeled Wash 2/3 Concentrate. Place a check mark in the empty box on the label to indicate that the ethanol has been added.



NOTE

Once ethanol has been added, cap the Wash Solution bottles tightly to prevent evaporation.

As you begin the procedure:

- For each sample, label a Micro Filter Cartridge Assembly and a Micro Elution Tube.
- Warm Wash Solution 2/3 to room temperature.
- Heat an aliquot of Elution Solution in a tightly closed RNase-free tube (not supplied with kit) in a heat block set to 75°C. The volume needed depends on the number of samples processed; each sample is typically eluted in 20 µL, however we recommend heating at least 50 µL of Elution Solution per sample to allow for ease of handling.

B. Sample Preparation and Disruption

1. Sample preparation

Cultured cells ($\leq 5 \times 10^5$ cells):

- Count cells; pellet up to 500,000 cells by centrifugation, and thoroughly remove supernatant by aspiration.
- The cell pellet can be washed with 1X phosphate buffered saline (PBS) prior disruption, but this is not essential. To wash cells in PBS, resuspend in 1 mL PBS, repellet the cells, and thoroughly remove the fluid.
- Proceed immediately to sample disruption (next step).

2. Disrupt sample in at least 100 μ L Lysis Solution

Cultured cells:

Resuspend cell pellet (up to ~500,000 cells) by vortexing vigorously in at least 100 μ L Lysis Solution.

C. RNA Isolation Procedure

1. Add one-half volume of 100% ethanol to the lysate and mix

For a standard prep of 100 μ L of lysate, add 50 μ L of 100% ethanol, and vortex briefly but thoroughly.

If desired, centrifuge the tube briefly to collect the sample at the bottom of tube.

**NOTE**

The basic RNAqueous-Micro procedure does not efficiently recover small RNAs such as tRNA, 5S rRNA, and micro RNA, but can be modified for recovery these species by adding 1.25 volumes of ethanol in step 1, instead of adding 0.5 volumes of ethanol. This modification may result in more variation in RNA yields compared to the standard procedure.

2. Pass the lysate/ethanol mixture through a Micro Filter Cartridge Assembly 150 μ L at a time

- a. Load the lysate/ethanol mixture (up to 150 μ L) onto a Micro Filter Cartridge Assembly and close the cap.
- b. Centrifuge for ~10 sec at maximum speed or until all of the mixture has passed through the filter. Longer centrifugation times may be needed to filter the lysate from tissue samples.

For lysate/ethanol mixtures >150 μ L, load and filter the first 150 μ L, then repeat with additional aliquots until the entire sample has passed through the filter. The Collection Tube has a capacity of ~700 μ L when assembled with a Micro Filter Cartridge; if more than 150 μ L of lysate/ethanol mixture is filtered, empty the Collection Tube before proceeding.

The RNA is now bound to the filter in the Micro Filter Cartridge.

**NOTE**

All centrifugation in the following steps should be done at 13,400–16,500 \times g. This is typically 12,000–13,200 rpm for standard micro-centrifuges.

3. Wash the filter with 180 μ L Wash Solution 1

- a. Open the Micro Filter Cartridge, add 180 μ L of Wash Solution 1 (working solution mixed with ethanol) to the filter and close the cap.
- b. Centrifuge for ~10 sec to pass the solution through the filter.

4. Wash the filter with 2 x 180 μ L Wash Solution 2/3

- a. Open the Micro Filter Cartridge, add 180 μ L of Wash Solution 2/3 (working solution mixed with ethanol) to the filter and close the cap.
- b. Centrifuge for ~10 sec to pass the solution through the filter.
- c. Repeat with a second 180 μ L aliquot of Wash Solution 2/3.

5. Discard the flow-through and centrifuge the filter for 1 min at max speed

- a. Open the Micro Filter Cartridge assembly, remove the filter cartridge from the Collection Tube, and pour out the flow-through.
- b. Replace the Micro Filter Cartridge into the same Collection Tube, close the cap, and centrifuge at maximum speed for 1 min to remove residual fluid and dry the filter.

6. Elute the RNA into a Micro Elution Tube with 2 x 5–10 μ L preheated Elution Solution

- a. Label a Micro Elution Tube (1.5 mL tubes provided with the kit) and transfer the Micro Filter Cartridge into it.
- b. Apply 5–10 μ L of Elution Solution, preheated to 75°C, to the center of the filter. Close the cap and store the assembly for 1 min at room temperature. Centrifuge the assembly for ~30 sec to elute the RNA from the filter.
Tension from the hinge of the Micro Elution Tube can occasionally cause the cap to pop off during the elution spin. To minimize the chance of this happening, bend the cap hinge back and forth several times, then press the cap securely onto the Micro Filter Cartridge.
- c. Repeat with a second 5–10 μ L aliquot of preheated Elution Solution, collecting the eluate in the same Micro Elution Tube.



NOTE

The exact volume of Elution Solution used is not critical and may be increased if desired. In general, ~75–85% of the RNA will be recovered from samples derived from up to ~100,000 cells (or 3 mg tissue) using 2 x 5 μ L of Elution Solution, and \geq 85% will be recovered using 2 x 10 μ L of Elution Solution. A larger volume of Elution Solution may be required for thorough elution of the RNA from larger samples, especially tissue samples >3 mg.

9. Protocolo para Síntese de cDNA (Kit High-Capacity Ref. 4368814)

High-Capacity cDNA Reverse Transcription Kits

Catalog Numbers 4368813, 4368814, 4374966, and 4374967

Pub. No. 4375222 Rev. C

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit uses the random primer scheme for initiating cDNA synthesis. Random primers ensure that the first strand synthesis occurs efficiently with all species of RNA molecules present, including mRNA and rRNA. The kit has been tested extensively and validated against various RNA templates, including G/C-rich and A/U-rich RNA species. The effect of relative mRNA abundance was also examined. An essential requirement for the relative quantitation of cDNA is that the reverse transcriptase reaction generates products in a manner directly dependent on the amount of input RNA template. In all cases, quantitative conversion of mRNA and 18S ribosomal RNA species was observed.

Available kits

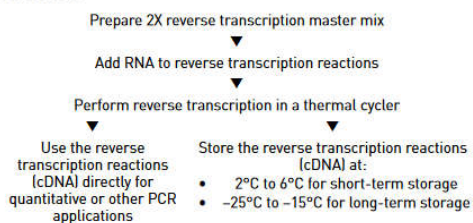
Kit	Cat. No.
High-Capacity cDNA Reverse Transcription Kit, 200 reactions	4368814
High-Capacity cDNA Reverse Transcription Kit, 1000 reactions	4368813
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor, 200 reactions	4374966
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor, 1000 reactions	4374967

Contents and storage

Contents	Cat. Nos. 4368813 and 4374967	Cat. Nos. 4368814 and 4374966	Storage
10X RT Buffer, 1.0 mL	2 tubes	1 tube	-25°C to -15°C
10X RT Random Primers, 1.0 mL	2 tubes	1 tube	
25X dNTP Mix (100 mM)	1 tube, 1.0 mL	1 tube, 0.2 mL	
MultiScribe™ Reverse Transcriptase, 50 U/μL	1 tube, 1.0 mL	2 tubes, 0.1 mL	
RNase Inhibitor, 100 μL ⁽¹⁾	10 tubes	2 tubes	

⁽¹⁾ Included in Cat. Nos. 4374966 and 4374967 only.

Workflow



Reverse transcription reaction guidelines

The kit contains reagents that, when combined, form a 2X reverse transcription (RT) master mix. An equal volume of RNA sample should be added. To avoid RNase contamination, RNase-free reagents and consumables must be used.

Prepare the 2X RT master mix

1. Allow the kit components to thaw on ice.
2. Calculate the volume of components needed to prepare the required number of reactions.

Note: Prepare the RT master mix on ice.

Component	Volume	
	With RNase Inhibitor	Without RNase Inhibitor
10X RT Buffer	2.0 μL	2.0 μL
25X dNTP Mix (100 mM)	0.8 μL	0.8 μL
10X RT Random Primers	2.0 μL	2.0 μL
MultiScribe™ Reverse Transcriptase	1.0 μL	1.0 μL
RNase Inhibitor	1.0 μL	—
Nuclease-free H ₂ O	3.2 μL	4.2 μL
Total per reaction	10.0 μL	10.0 μL

IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

3. Place the 2X RT master mix on ice and mix gently.

Prepare the reverse transcription reactions

1. Pipette 10 μL of 2X RT master mix into each well of a 96-well reaction plate or individual tube.
2. Pipette 10 μL of RNA sample into each well, pipetting up and down two times to mix.
3. Seal the plates or tubes.
4. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

Program the thermal cycling conditions

Program the thermal cycler using the conditions below.

IMPORTANT! These conditions are optimized for use with the High-Capacity cDNA Reverse Transcription Kits.

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	∞

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

10. Protocolo para o Ensaio de Real-time PCR (TaqMan Fast Ref. 4444556)

Prepare the PCR reaction mix

- Combine the following components for the number of reactions required, plus 10% overage.

Component	Volume per reaction		Final concentration
	384-well plate	96- or 48-well plates ^[1]	
TaqMan [®] Fast Advanced Master Mix (2X)	5.0 µL	10.0 µL	1X
TaqMan [®] Assay (20X)	0.5 µL	1.0 µL	1X
Nuclease-Free Water ^[2]	3.5 µL	7.0 µL	—
Total volume per reaction	9.0 µL	18.0 µL	—

^[1] Standard and Fast.

^[2] Adjust the volume of Nuclease-Free Water for a larger volume of cDNA.

- Vortex briefly to mix.
- Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.

Prepare the PCR reaction plate

- Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate.
- Add cDNA template (1 pg to 100 ng in Nuclease-Free Water), or Nuclease-Free Water for NTC, to each well.
 - 1.0 µL for a 384-well plate

- 2.0 µL for 96- and 48-well plates (Standard and Fast)

Note: Be sure to adjust the volume of Nuclease-Free Water in the PCR reaction mix for a larger volume of cDNA.

- Seal the reaction plate with optical adhesive film, then centrifuge briefly to bring the PCR reaction mix to the bottom of the well and eliminate air bubbles.
- Apply a compression pad to the plate, if required by your real-time PCR system.

Real-time PCR system	UNG incubation ^[1]	Polymerase activation ^[2]	PCR (40 cycles)	
	Hold 50°C	Hold 95°C	Denature 95°C	Anneal / extend 60°C
<ul style="list-style-type: none"> QuantStudio[™] 3 and 5 Real-Time PCR Instruments QuantStudio[™] 6 and 7 Flex Real-Time PCR System QuantStudio[™] 12K Flex Real-Time PCR System 7900HT Real-Time PCR Instrument 7900HT Fast Real-Time PCR Instrument ViiA[™] 7 Real-Time PCR System StepOne[™] Real-Time PCR System StepOnePlus[™] Real-Time PCR System 	2 minutes	20 seconds	1 second	20 seconds
<ul style="list-style-type: none"> 7500 Fast Real-Time PCR System 7500 Real-Time PCR System 	2 minutes	20 seconds	3 seconds	30 seconds