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LUCIANE DIAS DE OLIVEIRA

ANTIMICROBIANOS E EXTRATOS NATURAIS PARA USO EXTERNO NA ODONTOLOGIA

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Tese apresentada ao curso de Odontologia do Instituto de Ciência e Tecnologia, UNESP – Univ Estadual Paulista, Campus de São José dos Campos, como parte dos requisitos para obtenção do título de LIVRE DOCENTE nas disciplinas Farmacologia e Terapêutica Clínica, junto ao Departamento de Biociências e Diagnóstico Bucal

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"A minha alma descansa somente em DEUS;
Dele vem a minha salvação.
Somente Ele é a rocha que me salva;
Ele é minha torre segura!"

Salmo 62:1-2

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SUMÁRIO

Oliveira LD. Antimicrobianos e extratos naturais para uso externo na Odontologia. [tese]. São José dos Campos (SP): Instituto de Ciência e Tecnologia, UNESP - Univ Estadual Paulista; 2015.

RESUMO

O objetivo desta tese foi reunir os resultados de estudos que avaliaram os efeitos *in vitro* e *in vivo* de irrigantes endodônticos e medicações intracanais, incluindo própolis e extratos de plantas, sobre micro-organimos, endotoxinas (LPS) e ácido lipoteicóico (LTA) em canais radiculares, bem como a citotoxicidade e atividade antimicrobiana de extratos de plantas contra micro-organismos de interesse para Odontologia. No estudo dos efeitos do hidróxido de cálcio e polimixina B sobre endotoxinas em canais radiculares, observou-se que estas medicações intracanais detoxificaram endotoxinas alterando sua capacidade de estimular a produção de anticorpos por linfócitos B. No próximo estudo, foram avaliados os efeitos *in vitro* de irrigantes endodônticos (NaOCl 2,5% e 5%; clorexidina 2%, hidróxido de cálcio 0,14% e polimixina B) sobre endotoxinas em canais radiculares. Somente hidróxido de cálcio e polimixina B conseguiram neutralizar endotoxinas quando utilizados como irrigantes, sendo que NaOCl e clorexidina não foram efetivos. Quanto à análise da ação de extrato de própolis como irrigante dos canais radiculares sobre *Escherichia coli* e endotoxinas, verificou-se que o extrato de própolis promoveu eliminação completa de *E. coli* e redução significativa de endotoxinas, porém, somente com a utilização de medicações contendo hidróxido de cálcio os níveis de endotoxinas ficaram baixos. O estudo seguinte foi *in vivo*, analisando a eficácia do tratamento endodôntico para redução de endotoxinas em canais radiculares com infecção primária. Observou-se que a combinação de clorexidina com água de cal (hidróxido de cálcio 0,14%) como irrigante foi a mais efetiva na redução de endotoxinas e que a medicação intracanal (pasta de hidróxido de cálcio e clorexidina) neutralizou os efeitos citotóxicos do conteúdo dos canais. Em um estudo mais recente, foi avaliada *in vitro* a ação de medicações intracanais em neutralizar os efeitos do ácido lipoteicóico (LTA) de induzir a produção de citocinas pró-inflamatórias e óxido nítrico em macrófagos. Observou-se que as medicações contendo hidróxido de cálcio foram as mais efetivas na neutralização dos efeitos citotóxicos de LTA, assim como observado nos estudos anteriores com LPS. Com intuito de ampliar a terapia endodôntica, foi avaliada a atividade antimicrobiana *in vitro* de NaOCl 2,5%, clorexidina 2%, extratos naturais (*Aloe vera* e *Zingiber officinale*) e

óleo de *Ricinus comunis* sobre *Candida albicans* e *Enterococcus faecalis* em canais radiculares e verificou-se potencial antimicrobiano do extrato de *Z. officinale* para uso intracanal. Em outro estudo, foi analisada a atividade antimicrobiana e anti-endotoxina do extrato de *Z. officinale* e verificou-se que este extrato é um forte agente antimicrobiano para *C. albicans, E. faecalis* e *E. coli*, porém não neutraliza endotoxinas. Diante da grande diversidade de plantas e seu uso restrito na Odontologia, foi analisada a citotoxicidade e atividade de extratos de plantas (*Equisetum arvense*, *Glycyrrhiza glabra*, *Punica granatum, Stryphnodendron barbatimam e Arctium lappa)* sobre micro-organismos de interesse odontológico (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Candida albicans, Candida tropicalis,* and *Candida glabrata)*, em culturas planctônicas e biofilmes. Todos os extratos foram efetivos sobre os micro-organismos avaliados, em diferentes concentrações, sendo que os extratos de *G. glabra* e *A. lappa* foram os menos citotóxicos para macrófagos e o extrato de *E. arvense* apresentou citotoxicidade superior a 50%. O extrato de *P. americana* foi avaliado sobre biofilme de *C. albicans* e apresentou potencial antifúngico, embora tenha sido citotóxico para macrófagos em altas concentrações. Como conclusão geral, os estudos demostraram que: a) para neutralização de endotoxinas (LPS) e ácido lipoteicóico (LTA) o hidróxido de cálcio é o agente antimicrobiano mais eficaz, possibilitando a neutralização de seus efeitos citotóxicos; b) os extratos de gengibre (*Z. officinale*), barbatimão (*S. barbatiman*), bardana (*A. bappa*), romã (*Punica granatum),* alcaçuz *(G. glabra),* cavalinha (*E. arvense) e* abacateiro *(P. americana)* são importantes agentes antimicrobianos sobre bactérias e leveduras de interesse odontológico, sendo os extratos de bardana e alcaçuz os menos citotóxicos para macrófagos. Com isso, os extratos de plantas apresentam potencial antimicrobiano que deve ser mais explorado e seu uso clínico odontológico mais difundido.

Palavras-chave: Antimicrobianos. Extratos de plantas. Hidróxido de cálcio. Endotoxinas. Ácido lipoteicóico. Odontologia.

Oliveira LD. *Antimicrobials and natural extracts for external use in Dentistry. [thesis]. São José dos Campos (SP): Institute of Science and Technology, UNESP - Univ Estadual Paulista; 2015.*

ABSTRACT

This thesis aimed to collect results from studies that evaluated in vitro and in vivo effects of endodontic irrigants and intracanal medication, such as propolis and plant extracts on microorganisms, endotoxins (LPS) and lipoteichoic acid (LTA) in root canals, as well as cytotoxicity and antimicrobial activity of plant extracts against microorganisms of interest for Dentistry. In the study of the effects of calcium hydroxide and polymyxin B for endotoxins in root canals, it was observed that these intracanal medications neutralized endotoxins by altering its ability to stimulate the production of antibodies by B-lymphocytes. In the next study, it was evaluated in vitro effects of endodontic irrigants (2.5% and 5% NaOCl, 2% chlorhexidine, 0.14% calcium hydroxide and polymyxin B) on endotoxins in root canals. Only calcium hydroxide and polymyxin B were able to neutralize endotoxins when used as irrigants, while NaOCl and chlorhexidine were not effective. Regarding the analysis of the action of propolis extract as root canals irrigating on Escherichia coli *and endotoxin, it was found that propolis extract promoted complete elimination of* E. coli *and significant reduction of endotoxins, but only with the use of medications containing calcium hydroxide the endotoxin levels were low. The following study,* in vivo, *analyzed the effectiveness of endodontic treatment for endotoxin reduction in root canals with primary infection. It was observed that the combination of chlorhexidine with limewater (0.14% calcium hydroxide) as irrigant was the most effective in reducing endotoxins and that the intracanal medication, calcium hydroxide paste and chlorhexidine, neutralized the cytotoxic effects of the content of root canals. In a more recent* in vitro *study, it was assessed different intracanal medications action of neutralizing the effects of lipoteichoic acid (LTA) to induce the production of pro-inflammatory cytokines and nitric oxide in macrophages. It was observed that calcium hydroxide-containing intracanal medications were the most effective in neutralizing the cytotoxic effects of LTA, as observed in previous studies with LPS. Ordering to enhance the endodontic therapy, in vitro antimicrobial activity of 2.5% NaOCl, 2% chlorhexidine, natural extracts* (Aloe vera *and* Zingiber officinale) *and* Ricinus communis *oil was evaluated on* Candida albicans *and* Enterococcus faecalis *in root canals and it was verified antimicrobial potential for* Z. officinale *extract for intracanal use. In another state, it was*

analyzed antimicrobial and anti-endotoxin activity of Z. officinale *extract and it was found that this extract is a strong antimicrobial agent for* C. albicans, E. faecalis *and* E. coli, *but it is not able to neutralize endotoxins. In the presence of the great diversity of plants and their restricted use in Dentistry, it was analyzed plant extracts* (Equisetum arvense, Glycyrrhiza glabra, Punica granatum, Stryphnodendron barbatimam *and* Arctium lappa) *cytotoxicity and antimicrobial activity against microorganisms of Dental interest* (Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Candida albicans, Candida tropicalis *and* Candida glabrata) *in planktonic cultures and biofilm. All extracts were effective against the microorganisms evaluated, at different concentrations, being* G. glabra *and* A. lappa *extract the least cytotoxic for macrophages while* E. arvense *extract showed cytotoxicity higher than 50%.* P. americana *extract was evaluated on* C. albicans *biofilm and showed antifungal potential, although it was cytotoxic to macrophages in high concentrations. As a general conclusion, the studies demonstrated that: a) in order to neutralize endotoxins (LPS) and lipoteichoic acid (LTA), calcium hydroxide is the most effective antimicrobial agent, allowing neutralization of their cytotoxic effects; b) ginger* (Z. officinale), *barbatimam* (S. barbatiman), *burdock* (A. lappa), *pomegranate* (Punica granatum), *licorice* (G. glabra), *horsetail* (E. arvense) *and avocado* (P. american) *are important antimicrobial agents against bacteria and yeast of Dental interest, being burdock and licorice extracts the least cytotoxic to macrophages. On the basis of the evidences available, it can be concluded that the plant extracts showed antimicrobial potential that should be further explored and more widespread in Dental clinic use.*

Keywords: Antimicrobials. Plant extracts. Calcium hydroxide. Endotoxins. Lipoteichoic acid. Dentistry.

1 INTRODUÇÃO

As principais alterações patológicas que acometem a polpa, os tecidos periapicais e a mucosa bucal é de etiologia microbiana, sendo que diferentes micro-organismos e seus produtos apresentam significante papel na indução e manutenção destas lesões. Na área endodôntica, estudos demonstraram importante relação entre infecção polimicrobiana dos canais radiculares, especialmente causada por bactérias anaeróbias Gram-negativas, e sinais e sintomas clínicos, como dor espontânea, dor à palpação, edema e exsudato purulento (Gomes et al., 2007; Siqueira Jr et al., 2008; Siqueira & Roças, 2013; Ahmed et al., 2013). Estas bactérias possuem na membrana externa da parede celular endotoxinas que são complexos lipopolissacarídicos (LPS), responsáveis por amplificar a resposta inflamatória, imunológica e citotóxica, sendo de grande importância métodos de remoção ou neutralização de LPS nas infecções bucais. Endotoxinas são detectadas em 100% das coletas iniciais de canais radiculares com polpa necrosada, com níveis significativamente maiores em dentes com sintomatologia (Martinho et al., 2008; Martinho et al., 2011; Oliveira et al., 2012; Cardoso et al., 2015; Herrera et al., 2015). Com isso, o tratamento destas infecções deve não somente destruir micro-organismos como também inativar suas endotoxinas e demais produtos tóxicos.

Desde 1975, quando Schein & Shilder relataram pela primeira vez a participação de LPS nas infecções endodônticas, busca-se métodos eficazes para sua neutralizacão. Hipoclorito de sódio e clorexidina, que são substâncias utilizadas durante o preparo dos canais radiculares, possuem ótima atividade antimicrobianana, entretanto, apresentam pouca ou nenhuma ação sobre LPS (Tanomaru et al., 2003;

Oliveira et al., 2007, Vianna et al., 2007; Martinho et al., 2008; Gomes et al., 2009; Maekawa et al., 2011; Oliveira et al., 2012; Marinho et al., 2015). Por outro lado, estudos *in vitro* e *in vivo* demonstraram que o hidróxido de cálcio e polimixina B como medicação intracanal ou irrigantes dos canais radiculares detoxificaram endotoxinas, alterando a propriedade de LPS de induzir produção de anticorpos por linfócitos B e citocinas pró-inflamatórias por macrófagos (Oliveira et al., 2005; 2007; 2012). Outras pesquisas demonstraram significativa redução dos níveis de LPS nos canais radiculares quando hidróxido de cálcio foi usado como medicação (Maekawa et al., 2011; Xavier et al., 2013; Sousa et al., 2014; Adl et al., 2015), porém, as substâncias químicas auxiliares mais utilizadas durante o preparo dos canais não são efetivas sobre LPS, sendo necessário ampliar os estudos de substâncias alternativas, como extratos naturais, que apresentem efetiva ação antimicrobiana e que possam contribuir para ampliação da terapia endodôntica. Própolis e extratos de plantas tem sido estudados como agentes antimicrobianos e anti-inflamatórios e têm demonstrado resultados promissores, incluindo desinfecção dos canais radiculares (Valera et al., 2010; Zare-Jahromi et al., 2012; Tyagi et al., 2013; Bhandari et al., 2014; Valera et al., 2015).

Apesar da população mundial utilizar desde a antiguidade extratos de plantas para o tratamento de diversas enfermidades, diante da grande diversidade de plantas, são poucas as pesquisas que comprovam sua eficácia. Na Odontologia, o uso clínico de extratos naturais é muito restrito, de modo que estudos científicos específicos precisam ser realizados para promover sua inclusão em enxaguatórios bucais, dentifrícios, irrigantes e medicações intracanais, pomadas, entre outros, visando direcionar suas indicações terapêuticas. Em 2015, Farooqui et al. relataram que as combinações sinérgicas de agentes antimicrobianos com diferentes mecanismos de ação têm possibilitado estratégias de sucesso no combate a infecções envolvendo bactérias multirresistentes. Estes autores verificaram que a combinação sinérgica do extrato de

Juglans regia (nogueira) e oxacilina reverteu a resistência a oxicilina de *Staphylococcus aureus*. Anand et al. (2015) analisaram a atividade antimicrobiana de extratos de *Anacardium occidentale* (caju) e *Mangifera indica* (manga) sobre micro-organismos de interesse odontológico (*Enterococcus faecalis, S. aureus, Streptococcus mutans, Escherichia coli* e *Candida albicans*) e verificaram que os extratos de manga e caju promoveram significativa redução dos biofilmes microbianos e foram menos citotóxicos para cultura de fibroblastos gengivais em comparação com enxaguatórios bucais (clorexidina 0,2% e iodopovidona 0,2%). Outros estudos demonstraram efetiva ação antimicrobiana de extratos de plantas, como *Pineapple*, *Emex spinosa , Olea europaea, Mastic gum*, *Inula viscosa, Camellia sinensis, Punica granatum, Psidium guajava, Arctium lappa, Acacia nilotica* e *Schinus terebinthifolius Raddi, Persea americana*, *Anacardium occidentale*, sobre micro-organismos cariogênicos, periodontopatógenos e sobre leveduras do gênero *Candida* (Anand et al., 2015; Shekar et al., 2015; Jesus et al., 2015; Praveen et al., 2014, Donia et al., 2014; Oliveira et al., 2014; Vieira et al. 2014; Karygianni et al., 2014; Araghizadeh et al., 2013; Oliveira et al., 2013).

Em 2015, Shekar et al. revisaram dez extratos de plantas no cuidado de saúde bucal abordando o cenário atual e necessidades futuras e concluíram que a efetiva ação antimicrobiana da combinação de extratos de plantas sobre patógenos cariogênicos e periodontopatogênicos pode levar ao desenvolvimento de métodos inovadores capazes de inibir simultaneamente as duas doenças bucais mais comuns, além de retardar o desenvolvimento de resistência microbiana. Apesar do uso clínico ainda ser restrito na Odontologia, os estudos com extratos de plantas estão crescendo muito. Só no ano de 2015, ao cruzar as palavras “*plant extract*” e “*dentistry*”, observou-se mais de 60 artigos publicados no *PubMed*, demonstrando que este tema é bem atual e relevante.

Os extratos de plantas apresentam diferentes finalidades terapêuticas e diante da grande diversidade os estudos envolvendo substâncias naturais representam importante campo de pesquisa que pode trazer grandes benefícios à terapia odontológica, sendo de grande interesse estudar seus efeitos sobre micro-organismos e seus produtos, incluindo os envolvidos nas infecções endodônticas e periodontais, cárie e candidose. Estes estudos são importantes para comprovar os diferentes efeitos benéficos dos extratos, os quais podem ser inseridos em formulações de uso odontológico, tais como medicações e irrigantes intracanais, enxaguatórios bucais, dentifrícios, gel, pomadas, entre outros. Assim, com a necessidade atual de ampliar as estratégias antimicrobianas, o uso de extratos de plantas na Odontologia, para prevenção e tratamento de infecções bucais, é uma terapia alternativa que pode trazer inúmeros benefícios ainda pouco explorados.

2 PROPOSIÇÃO

A proposta desta tese foi demonstrar os resultados de estudos realizados *in vitro* e *in vivo* dos efeitos de agentes antimicrobianos e extratos naturais, para uso externo na Odontologia, sobre diferentes micro-organismos de interesse odontológico e sobre seus produtos (LPS e ácido lipoteicóico - LTA). Sua estrutura foi dividida em capítulos que correspondem aos artigos publicados ou enviados para publicação, seguindo a sequência abaixo:

a) Avaliação *in vitro* dos efeitos do hidróxido de cálcio e polimixina B sobre endotoxinas em canais radiculares. (*artigo relacionado ao Processo FAPESP 00/09427-7, publicado no periódico Journal of Dentistry, 2005 Feb;33(2):107-14.* doi:10.1016/j.jdent.2004.08.008*, QUALIS A1, Impacto 2,749, citações no ISI=18, Scopus= 18, Google Scholar= 31*);

b) Efeitos *in vitro* de irrigantes endodônticos sobre endotoxinas em canais radiculares. (*artigo relacionado ao Processo FAPESP 02/06467-3, publicado no periódico Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics, 2007 jul 104(1):135-42.* doi:10.1016/j.tripleo.2006.11.037*, QUALIS A2, Impacto 1,261, citações ISI=14, Scopus= 25, Google Scholar= 41*);

c) Ação de própolis e medicações contra *Escherichia coli* e endotoxina em canais radiculares. (*artigo relacionado ao*

Processo FAPESP 05/57668-7, *publicado no periódico Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics, 2010 oct 110(4):e70-4.* doi: 10.1016/j.tripleo.2010.01.029., *QUALIS A2, Impacto 1,261, citações no ISI=11, Scopus= 18, Google Scholar= 27*);

d) Eficácia do tratamento endodôntico para redução de endotoxinas em canais radiculares com infecção primária e avaliação dos efeitos citotóxicos (*artigo relacionado aos Processos FAPESP* 05/57668-7 e 08/53863-8, *publicado no periódico Journal of Endodontics, 2012 Aug;38(8):1053-7. doi:* 10.1016/j.joen.2012.04.015. *QUALIS A1, Impacto 2,788, citações no ISI=7, Scopus= 13, Google Scholar= 20*);

e) Atividade antimicrobiana *in vitro* de substâncias químicas auxiliares e extratos naturais sobre *Candida albicans* e *Enterococcus faecalis* em canais radiculares. (*artigo publicado no periódico Journal of Applied Oral Science, 2013 mar-apr 21(2):118-23.* doi: 10.1590/ 1678-7757201302135. *QUALIS A2, Impacto 0,923, citações no ISI=3, Scopus= 3, Google Scholar= 11*);

f) Citotoxicidade de extratos de plantas brasileiras contra micro-organismos orais de interesse para Odontologia. (*artigo publicado no periódico BMC Complementary & Alternative Medicine, 2013 Aug;15(13):208. doi:10.1186/1472-6882-13208 QUALIS B1, Impacto 2,02, citações no Scopus= 02, Google Scholar= 8*);

g) Controle de micro-organismos de interesse odontológico com extrato de *Arctium lappa* L. (bardana) não citotóxico para cultura de macrófagos (RAW 264.7). (*artigo relacionado ao Processo FAPESP* 07/55867-8, *publicado no periódico Archives of Oral Biology, 2014 Aug;59(8):808-14. doi:* 10.1016/ j.archoralbio.2014.05.013 *QUALIS A2, Impacto 1,735, citações no ISI=1, Scopus= 1, Google Scholar= 3*);

h) Acão *in vitro* da atividade antimicrobiana e anti-endotoxina do extrato de *Zingiber officinale* como substância química auxiliar e como medicação associada ao hidróxido de cálcio e clorexidina. (*artigo publicado no periódico Acta Odontologica Scandinavica, 2015; 73(7):556-61. doi:* 10.3109/00016357.2014.949846. *QUALIS B2, Impacto 1,03)*;

i) Avaliação *in vitro* da atividade antimicrobiana do extrato glicólico de *Persea americana* sobre biofilme de *Candida albicans* e avaliação de sua citotoxicidade. (*artigo relacionado ao processo FAPESP 15/08776-3, aceito para publicação no Scientific World Journal, 2015 oct 15:1-5. doi:* 10.1155/2015/531972, *QUALIS B2, Impacto 1,73)*;

j) *Avaliação in vitro* da ação de medicações intracanais em neutralizar os efeitos do ácido lipoteicóico (LTA) de induzir a produção de citocinas pró-inflamatórias e óxido nítrico em macrófagos. (*artigo relacionado aos Processos FAPESP* 11/23202-2, *12/16451-9, 12/16450-2, enviado para publicação no periódico Journal of Endodontics, QUALIS A1, Impacto 2,971*).

3 ARTIGOS

3.1 *In vitro* effects of calcium hydroxide and polymyxin B on endotoxins in root canals*

ABSTRACT

Objectives. To evaluate the effects of intracanal medicaments on endotoxins in root canals. Methods. Seventy-five freshly extracted maxillary incisors were used in this study. The crowns of teeth were sectioned near the CEJ in order to standardize the root length to 14 mm. The root canals were instrumented to an apical size #50 file and irrigated with 1% sodium hypochlorite solution and sterilized with 60 Co gamma irradiation. Standardized suspension containing Escherichia coli endotoxin was inoculated into the 60 root canals. The specimens were randomly assigned to 5 groups (n=15), according to the intracanal medicament used: (G1) calcium hydroxide; (G2) polymyxin B; (G3) combination neomycin–polymyxin B-hydro- cortisone; (G4) positive control (no intracanal medicament); (G5) negative control (no endotoxin and no intracanal medicament). After 7 days, the detoxification of endotoxin was evaluated by Limulus lysate assay and antibody production in B-lymphocytes culture. Results. Groups 1, 2 and 5 presented the best results by Limulus lysate and were significantly different to groups 3 and 4 ($p<0.05$). Stimulation of antibodies production in cell culture by groups 1 and 6 was smaller and statistically different than groups 2, 3, 4 and 5 ($p<0.05$). Groups 2 and 5 induced a small increase in the antibodies

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production in relation to the groups 1 and 6. Groups 3 and 4 induced a significant increase of antibodies production ($p<0.05$). Conclusions. The calcium hydroxide and polymyxin B intracanal medicaments detoxified endotoxin in root canals and altered the properties of LPS to stimulate the antibody production by B-lymphocytes. The combination neomycin–polymyxin B-hydrocortisone did not detoxified endotoxin.

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In vitro effects of calcium hydroxide and polymyxin B on endotoxins in root canals

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KEYWORDS
Endotoxin;
Root canal;
Calcium hydroxide;
Polymyxin B

Summary *Objectives*. To evaluate the effects of intracanal medicaments on endotoxins in root canals.

Methods. Seventy-five freshly extracted maxillary incisors were used in this study. The crowns of teeth were sectioned near the CEJ in order to standardize the root length to 14 mm. The root canals were instrumented to an apical size #50 file and irrigated with 1% sodium hypochlorite solution and sterilized with 60 Co gamma irradiation. Standardized suspension containing *Escherichia coli* endotoxin was inoculated into the 60 root canals. The specimens were randomly assigned to 5 groups ($n=15$), according to the intracanal medicament used: (G1) calcium hydroxide; (G2) polymyxin B; (G3) combination neomycin-polymyxin B-hydrocortisone; (G4) positive control (no intracanal medicament); (G5) negative control (no endotoxin and no intracanal medicament). After 7 days, the detoxification of endotoxin was evaluated by Limulus lysate assay and antibody production in B-lymphocytes culture.

Results. Groups 1, 2 and 5 presented the best results by Limulus lysate and were significantly different to groups 3 and 4 ($p<0.05$). Stimulation of antibodies production in cell culture by groups 1 and 6 was smaller and statistically different than groups 2, 3, 4 and 5 ($p<0.05$). Groups 2 and 5 induced a small increase in the antibodies production in relation to the groups 1 and 6. Groups 3 and 4 induced a significant increase of antibodies production ($p<0.05$).

Conclusions. The calcium hydroxide and polymyxin B intracanal medicaments detoxified endotoxin in root canals and altered the properties of LPS to stimulate the antibody production by B-lymphocytes. The combination neomycin-polymyxin B-hydrocortisone did not detoxified endotoxin.

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Introduction

The fundamental role of bacteria in the aetiology of endodontic lesions has been intensively studied since 1965.[1] Analyses of the microflora in infected root canals with necrotic pulps have revealed that Gram-negative anaerobic bacteria are the predominant microorganisms.[2,3] Tani-Ishii et al.[4] characterized the root canal microflora present during the active phase of lesion development in mice and demonstrated that the microflora becomes increasingly Gram-negative and anaerobic with the emergence of *Peptostreptococcus*, *Bacteroides*, *Prevotella* and *Neisseria* during the period of rapid lesion expansion.

Gram-negative bacteria contain endotoxin in their outer cell membrane.[5] This endotoxin or lipopolysaccharides (LPS) is released during cell duplication or after cell death. Endotoxins are responsible for several important biological effects as: polymorphonuclear leucocytes (PMNs), release of collagenase, macrophages activation and release of bioactive inflammatory mediators such as the ciclooxygenase pathway products, platelet activating factor (PAF), tumour necrosis factor (TNF), interleukins (IL-1, IL-6 and IL-8), superoxide (O_2^-), nitric oxide, interferon α, β and γ,[6,7,8] complement system activation, B-lymphocytes polyclonal activation, cytotoxicity, hemodinamics modifications, fever induction and osteoclasts attraction.[9,10] These factors are important in the development and maintenance of inflammatory reaction and bone resorption in the periapical region.[11]

Many investigators reported the important role of endotoxin in the pathogenesis of periapical lesions.[8,12–14] Dahlén et al.,[15] Nelson-Filho et al.[16] and Silva et al.[17] studied the effect of endotoxin on the periapical tissues of monkeys or dogs. These authors inserted LPS into root canals of animals and evaluated the radiographic and histological appearance of the periapical region. Inflammatory reactions and bone resorption occurred in the periapical tissues of all the experiment teeth. Khabbaz et al.[18,19] demonstrated that endotoxins are present in carious lesions of symptomatic and asymptomatic teeth and in the pulpal tissue of the carious teeth, indicating that LPS may play an important role in the pathogenesis of human pulpal diseases.

The major goal of endodontic treatment is the elimination of bacteria from the root canal system by mechanical and chemical means and a wide variety of antimicrobial agents have been used for this purpose.[6] However, it is possible that even though bacteria are removed from the root canal, endotoxins capable of maintaining or to induce the apical periodontitis can remain,[8] interfering in the success of endodontic treatment. Therefore, the treatment of infected root canals should not only be concerned with bacterial death, but also the inactivation of endotoxin.[16,17]

Calcium hydroxide has been recommended for use as intracanal medicament based on its high alkalinity, tissue dissolving property and antimicrobial effects on Gram-positive and Gram-negative bacteria, commonly found in infected root canals.[20] Safavi and Nichols[6,7] and Barthel et al.[8] demonstrated that calcium hydroxide alters some biological properties of bacterial LPS as the ability to stimulate prostaglandin E_2 and TNF-α production by monocytes.

Polymyxin B (PmB), a cyclic cationic polypeptide antibiotic, prevents the lethal effect of endotoxin in chick embryos, mice, dogs, goats, foal, horse.[21–24] In these animal models, polymyxin B inhibits LPS-induced intravascular coagulation, the generalized Shwartzman reaction, LPS-induced macrophage production of interferon-γ, TNF-α and interleukin-1 and endotoxin-mediated shock.[25] Polymyxin B presents important results in endotoxin systemic detoxifying, however, their effects on LPS in root canal system have received little attention.

Despite of the presence of endotoxins in teeth with vital pulp or with necrotic pulp and apical periodontitis has already been showed since 1975 by Schein and Schilder,[3] little research has been performed to evaluate the effectiveness of intracanal medicaments on bacterial LPS using teeth as system model. The purpose of this study was to evaluate in vitro the effects of intracanal medicaments: calcium hydroxide, polymyxin B and the combination neomycin–polymyxin B-hydrocortisone on endotoxins in root canals.

Materials and methods

Specimen preparation

Seventy-five recently extracted maxillary central or lateral incisors were used in the study. These teeth were collected from patients of School of Dentistry/UNESP at São José dos Campos and selection of teeth was made on the basis of relative dimensions and similarity in morphology. Debris, calculus and soft tissue remnants on the root surfaces were cleaned using a Gracey curette and all teeth were stored in saline solution (NaCl 0.85%) until used. The apical 3 mm of each root were transversely sectioned using a water-cooled diamond disc and the crown was sectioned at a point in order to

standardize the root length to approximately 14 mm. Working length was determined by subtracting 1 mm from this measurement.

Canals were enlarged to a size #50 K-file (Maillefer, Michigan, USA), which served as the master apical file. Step-back cleaning and shaping was performed with recapitulation followed by irrigation with 1% sodium hypochlorite solution and the cervical third was enlarged with size #3 and #4 Gates-Glidden burs. During instrumentation, the root canals were irrigated with 5 ml of 1% sodium hypochlorite for each file used.

The canals were dried with sterile paper points (Dentsply Ind. Com. LTDA, RJ, Brazil) and apical region was sealed with light-cured resin composites (3M Dental Products, St Paul, USA). The outer surfaces of the specimens were covered with two layers of epoxy adhesive (Araldite—Brascola, SP, Brazil), except the cervical opening.

Specimen sterilization

All the specimens were sterilized by autoclaving (20 min at 121 °C) and randomly assigned to five cell culture plates (96-wells, Costar, NY, USA), with 15 teeth each (Fig. 1A). The specimens and all the materials used in the experiment were irradiated with 60 Co gamma-rays for degradation of pre-existing LPS.[30]

Contamination with endotoxin

Escherichia coli 055:B5 endotoxin (Sigma, St Louis, USA) was used for the experiments. Under sterile laminar flow, 10 μl of a standard solution containing endotoxin were inoculated into the root canals of 60 specimens using a micropipette. Teeth were sealed with pyrogen-free cotton ball and the plates containing the specimens were closed, sealed and incubated for 24 h at 37 °C in a humidified atmosphere.

Experimental groups

After 24 h, the cervical sealing was removed and the canals lumen was filled with intracanal medicaments.

The 75 specimens were divided into five groups ($n=15$), according to the intracanal medicament used, as follows:

Group 1: calcium hydroxide (Calen, S.S. White, Rio de Janeiro, Brazil), inserted with ML syringe (SS White, Rio de Janeiro, Brazil);
Group 2: polymyxin B solution (10,000 UI/ml) (Ophtalmos Fórmulas Oficiais Ltda, São Paulo, Brazil), inserted with non-pyrogenic 1.0 ml plastic syringes and 13 mm needles (Injex, São Paulo, Brazil);
Group 3: the combination neomycin–polymyxin B-hydrocortisone (OTOSPORIN, Glaxo Wellcome, SP, Brazil), inserted with non-pyrogenic 1.0 ml plastic syringes and 13 mm needles (Injex, São Paulo, Brazil);
Group 4 (control group): did not receive any medicament (positive control);
Group 5 (control group): did not receive endotoxin and any medicament (negative control).

All the specimens were sealed with a pyrogen-free cotton ball. The plates containing the specimens were closed, sealed and incubated at 37 °C in

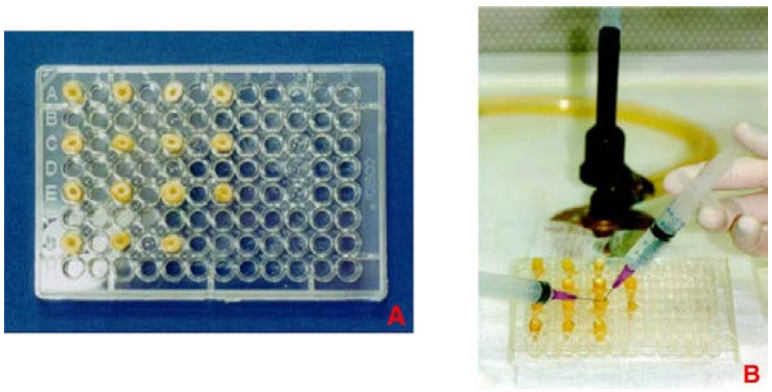

Figure 1 Model system: (A) Distribution of teeth ($n=15$) in cell culture plates (96 wells); (B) Irrigation with pyrogen-free water to remove all intracanal medicament.

a humidified atmosphere for 7 days. After this period, the root canals were irrigated with 3 ml of pyrogen-free water (Fig. 1B) to remove all intracanal medicaments and dried with pyrogen-free paper points. Each specimen was filled with pyrogen-free water and 130 μl of root canal content was collected with a non-pyrogenic 1.0 ml plastic syringe (Injex, São Paulo, Brazil) to verify the detoxification of endotoxin.

Detoxification of endotoxin—Limulus amebocyte lysate assay

The detoxification of endotoxin was evaluated by Limulus amebocyte lysate assay (Sigma, USA). Limulus is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*. When exposed to minute quantities of endotoxins, the lysate increases in opacity as well as viscosity, forming a hard gel. An aliquot of canal content (100 μl) was put into a pyrogen-free tube containing 100 μl of Limulus lysate, according to the manufacture's instructions. The tube was gently mixed and incubated at 37 °C for 1 h. These procedures were repeated for all the specimens. After 1 h incubation, the tubes were gently removed and slowly inverted 180° while observing for evidence of gelation. The formation of a hard gel, which permits complete inversion of the tube without disruption of gel, was considered as positive test. All other results: soft gels, turbidity, increase in viscosity, clear liquid were considered negative test.

Detoxification of endotoxin—antibody production in lymphocyte B culture

Three BALB/c mice with approximately 6 months of age were sacrificed and the spleen of each animal was removed and macerated in a pyrogen-free Falcon tube containing RPMI 1640 medium (Sigma Chemical Company, St Louis, USA), to obtain B-lymphocytes. The tube was centrifuged at $534.8 \times g$ for 10 min and the pellet was resuspended in 30 ml of RPMI 1640 medium supplemented with 10% bovine fetal serum. In order to prevent contamination of the culture, sterile conditions were maintained by sterile laminar flow.

For cell viability, the trypane blue exclusion assay (0.4%) (Sigma, St Louis, USA) and a buffer to promote the erythrocytes rupture were used. The viable cells count was 4.36×10^6 cells/ml. Approximately 1×10^6 viable cells (250 μl) were transferred for each well of non-pyrogenic cell culture plates (24-well, Costar, NY, USA). These plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO_2). B-lymphocytes of each well of cell cultures plates were stimulate with the remaining 30 μl of each root canal content. Fifteen wells contained only pure cell culture consisted the cell culture control group (G6). The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 4 days. The enzyme-linked immunosorbent assay (ELISA) was used to determine of total IgM levels in the culture supernatant.

ELISA was performed in three microplates (96-well, Hemobag, São Paulo, Brazil). All the plates were coated with mouse anti-immunoglobulin M (Sigma, St Louis, USA) in the concentration of 0.5 mg/ml. The plates were incubated for 2 h at 37 °C and maintained in the refrigerator until used.

In the day of the experiment, the plates were blocked with 0.5% gelatin (G) in phosphate buffered saline (PBS) and 0.2% BSA (45 min at 37 °C). Culture supernatants (100 μl) were added to each well and incubated for 2 h at 37 °C, followed by washing with 0.1% Tween-20 in PBS (PBS-T). All the tests were performed in duplicate. Anti-mouse IgM peroxidase conjugate, diluted 1000 times with PBS-GT (0.5% gelatin and 0.1% Tween 20 in PBS), was added to the plates and incubated for 1 h at 37 °C. After washing with PBS-T, peroxidase enzyme activity was detected by addition of the substrate, 50 μl of *o*-phenylenediamine (1 mg/ml) in a solution of 0.1 M citrate and 0.03% H_2O_2 for 10 min. Finally, the reaction was stopped with 2.5 N H_2SO_4. The optical density (OD) of the wells was read at an absorbance of 490 nm on a Bio-Rad Microplate reader Model 3550 (Bio-Rad Laboratories, Hercules, CA). For each specimen, the mean values of OD were obtained and the results were statistically analysed.

Statistical analysis

The results obtained with Limulus lysate assay were statistically analysed using non-parametric Mann-Whitney test, attributing scores 1 to the specimens that presented positive results and score 0 to the specimens that presented negative results. The ELISA results were statistically analysed using ANOVA and Tukey's test. In all cases, p values < 0.05 were considered as statistically significant.

Results

The results of Limulus lysate assay are presented in Table 1. No gel formation was observed in groups 1 and 5. Three specimens of group 2 and all

Table 1 Results obtained by Limulus lysate assay.

	G1	G2	G3	G4	G5
01	−	+	+	+	−
02	−	−	+	+	−
03	−	−	+	+	−
04	−	+	+	+	−
05	−	−	+	+	−
06	−	−	+	+	−
07	−	+	+	+	−
08	−	−	+	+	−
09	−	−	+	+	−
10	−	−	+	+	−
11	−	−	+	+	−
12	−	−	+	+	−
13	−	−	+	+	−
14	−	−	+	+	−
15	−	−	+	+	−

G1, calcium hydroxide; G2, polymyxin B; G3, combination neomycin–polymyxin B-hydrocortisone; G4, positive control; G5, negative control.

specimens of groups 3 and 4 presented gel formation. Fig. 2A and B shows the formation or not of hard gel by Limulus amebocytes lysate. There were no statistically significant differences among groups 1, 2 and 5 ($p > 0.05$) and between groups 3 and 4 ($p > 0.05$). However, the groups 1, 2 and 5 were significantly different from groups 3 and 4 ($p < 0.05$).

The values of OD obtained by ELISA for all the experimental groups are presented in Table 2. Stimulation of antibodies (IgM) production in B-lymphocytes culture by groups 1 and 6 was smaller and statistically different than groups 2, 3, 4 and 5 ($p < 0.05$). Groups 2 and 5 induced a small increase in the antibodies (IgM) production in relation to the groups 1 and 6. Groups 3 and 4 induced a significant increase of IgM production, statistically different to the other groups ($p < 0.05$).

Discussion

Kakehashi et al.[1] firstly showed the essential role of bacteria and their products in the development of periapical lesions. Elimination of microorganisms from root canals system by antimicrobial agents has been a major goal of endodontic treatment.[26] However, treatment of root canals in teeth with pulp necrosis should not only be concerned with bacterial death, but also the inactivation of endotoxin.[16,17] Endotoxin has an important role in the inflammatory and immunologic responses of pulpal and periapical tissues, including bone resorption.[8,11,12]

The presence of endotoxin in teeth with vital or necrotic pulps has already been proven by many researchers,[3,18,27] however, few studies have evaluated the effects of intracanal medicaments on LPS using teeth as a model system. Endotoxins as well as bacteria are capable to diffuse through dentinal tubules which are present not only in the canal lumen but also in the entire root canal system.[28,29] In the present research, we used single-root human teeth extracted for several reasons, independently of diagnosis, because the endotoxin possibly present in these teeth was degraded by 60 °C gamma irradiation.[30] Selection of teeth was made on the basis of relative dimensions and similarity in morphology and the apical 3 mm of each root were removed to minimize variations such as cracks, apical resorptions and ramifications.

The results of this study showed that calcium hydroxide medication intracanal for 7 days has a detoxifying effect on endotoxin in root canal system. These results are similar to other studies using different methodology that demonstrated calcium hydroxide could alter the biological properties of LPS.[6–8,16]

Safavi and Nichools[6] related that calcium hydroxide treatment of LPS release elevated quantities of

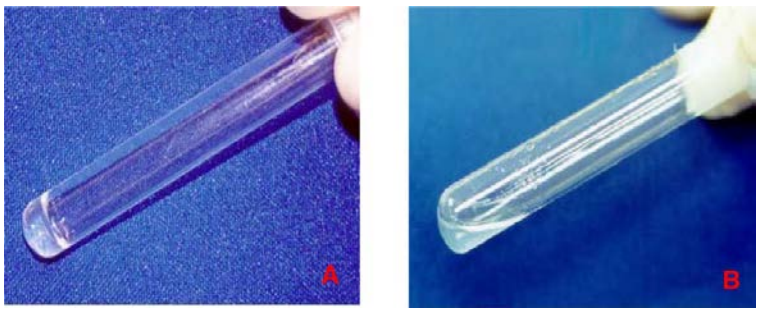

Figure 2 Results obtained by Limulus lysate assay: (A) gel formation; (B) No gel formation.

Table 2 Optical density (OD) values obtained in groups.

	G1	G2	G3	G4	G5	G6
01	0.690	1.033	1.206	1.292	0.763	0.725
02	0.825	0.924	1.230	1.284	0.722	0.491
03	0.909	0.994	1.270	1.260	0.822	0.504
04	0.680	0.996	1.254	1.287	0.994	0.489
05	0.841	0.866	1.200	1.268	0.779	0.614
06	0.735	0.878	1.121	1.308	0.819	0.656
07	0.730	0.933	1.115	1.350	0.849	0.562
08	0.873	0.875	1.205	1.196	0.806	0.675
09	0.856	0.695	1.079	1.314	0.963	0.678
10	0.739	0.865	1.152	1.287	0.952	0.491
11	0.556	0.875	1.288	1.242	0.990	0.764
12	0.569	0.671	1.191	1.265	1.004	0.766
13	0.735	0.753	1.181	1.273	0.966	0.698
14	0.740	0.831	1.185	1.197	0.945	0.782
15	0.399	0.846	1.127	1.213	1.007	0.764
Means	0.725	0.869	1.187	1.269	0.892	0.643
SD	0.136	0.103	0.059	0.045	0.100	0.111

G1, calcium hydroxide; G2, polymyxin B; G3, combination neomycin–polymyxin B-hydrocortisone; G4, positive control; G5, negative control; G6, cell culture control group.

hydroxy fatty acids by hydrolysis of ester bonds in the lipid moiety of bacterial LPS. This result suggests that detoxification of LPS by calcium hydroxide may be one of the mechanisms by which this agent exerts its beneficial effects in clinical endodontics. According to Safavi and Nichools[7] the prostaglandin E_2 production was inhibited in monocytes culture stimulated with calcium hydroxide-treated LPS. These experiments suggested that the biological properties of LPS require the presence of ester-linked hydroxy fatty acid and these linkages are destroyed by treatment with calcium hydroxide. In 1997, Barthel et al.[8] also evaluated whether the toxic potential of an *E. coli* LPS could be reduced or eliminated by calcium hydroxide. It was concluded that calcium hydroxide is able to eliminate the ability of an *E. coli* LPS to stimulate TNF-α production in peripheral blood monocytes. In this study, we verified that calcium hydroxide is able to alter the ability of endotoxin to stimulate antibodies production in B-lymphocytes culture. The absence of hard gel formation in the Limulus amebocyte lysate assay showed also that calcium hydroxide is able to detoxify LPS, probably by hydrolysis of the link ester-hydroxy fatty acids.

Polymyxin B was also effective in LPS detoxifing in root canal system. Only three teeth treated with this medicament induced the gel formation in the Limulus lysate assay and B-lymphocytes activation was significantly smaller than groups 3 and 4 ($p < 0.05$). These results are in accordance with other authors[22,24] that used polymyxin B treatment in Gram-negative microorganisms sepsis and endotoxic shock.

The polymyxin B neutralizing effect in LPS activity was observed for the first time by Neter[31] that incubated LPS with polymyxin B and verified that the erythrocytes agglutination, normally induced by LPS, was inhibited. Since then, other authors have demonstrated that polymyxin B can inhibit the endotoxic lethality in chicken, rats, rabbits, dogs and horses.[22–24] Rifkind and Palmer[21] and Rifkind[22] showed that polymyxin B prevents endotoxic lethality in chicken embryos and rats, and neutralize the Schwartzman's reaction in rabbits. They postulated a mechanism of action in which polymyxin B, a cationic antibiotic, covalently couples with the electronegative charge of the A lipid in endotoxin molecule. Morrison and Jacobs[32] confirmed this theory in vitro. Polymyxin B has the ability to bind with high affinity to the A lipid portion, altering the three-dimensional conformation of the LPS molecule. This conformational alteration possibly enables the binding of the complex endotoxin–polymyxin B to monocytes CD14 receptor, inhibiting the liberation of inflammatory mediators such as the TNF.[23] In the present study, polymyxin B enabled the B-lymphocytes activation by LPS and also inhibited the gel formation in Limulus amebocyte lysate assay, probably due to the conformational alteration of the endotoxin–polymyxin B complex.

Endotoxins may also evoke pain due to Hageman factor activation[33] or by neurotoxic

properties. Schein and Schilder,[3] Horiba et al.[27] and Khabbaz et al.[19] showed that symptomatic teeth present greater amount of endotoxin than asymptomatic teeth. Many dentists use the combination neomycin–polymyxin B-hydrocortisone to treat teeth with vital pulp due to its satisfactory anti-inflammatory action and capacity of preserving the periapical tissues.[34] In the present study, this medicament showed gel formation in all the tested specimens and high values of OD, inducing significant increase in antibodies (IgM) production. This value was very similar to those obtained in G4 (no medication). These results disagree with those obtained by other authors[35,36] that used this medicament when treating of supurative medium otitis caused by Gram-positive and negative microorganisms. However, none of these studies evaluated the neutralizing activity of the combination neomycin–polymyxin B-hydrocortisone on LPS. Lopes and Siqueira[37] studied the antimicrobial activity of this medicament on salivary microorganisms. After aerobiosis and anaerobiosis incubation, it was observed that this medication produced inhibitory effects only in oxygen presence. Therefore, it was effective against aerobic and facultative microorganisms but not against strictly anaerobic bacteria. In the present study, we investigated the detoxifying capacity of the combination neomycin–polymyxin B-hydrocortisone on the LPS and did not observe any activity. This absence of activity could be due to the solution pH, as we observe that polymyxin B presented a pH value of approximately 6.4 while the combination neomycin–polymyxin B-hydrocortisone presented a pH value of 5.4. Another possibility is the interference of one of the components on the polymyxin B. These and other alterations could have affected the endotoxins detoxifying action. In this way, the combination neomycin–polymyxin B-hydrocortisone should have limited use in the endodontics.

Conclusions

Under the conditions of this study, it can be concluded that the calcium hydroxide and polymyxin B intracanal medicaments detoxified endotoxin in root canals and altered the properties of LPS to stimulate the antibodies production by B-lymphocytes. The combination neomycin–polymyxin B-hydrocortisone did not detoxify endotoxin.

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3.2 *In vitro* effects of endodontic irrigants on endotoxins in root canals*

ABSTRACT

Objective. The objective of this study was to evaluate the effects of endodontic irrigants on endotoxins in root canals. *Study design.* Ninety-eight single-root human teeth were used. *Escherichia coli* endotoxin was inoculated into 84 root canals. All root canals were enlarged and assigned to 7 groups (n = 14), according to solution used. Group 1 (G1): 2.5% NaOCl; G2: 5.25% NaOCl; G3: 2% chlorhexidine; G4: 0.14% calcium hydroxide; G5: polymyxin B; G6: positive control, saline solution; G7: negative control (no endotoxin). Two samplings of root canal were accomplished: immediate and after 7 days. Detoxification of endotoxin was evaluated by Limulus assay and antibody production in B-lymphocyte culture. Results were analyzed by Kruskal-Wallis/Dunn and ANOVA/Tukey. *Results.* At the immediate and second samplings, groups G4, G5, and G7 presented the best results, significantly different from groups G1, G2, G3, and G6 ($P < 0.05$). *Conclusions.* Calcium hydroxide and polymyxin B detoxified endotoxin in root canals and altered properties of LPS to stimulate the antibody production by B-lymphocytes. Sodium hypochlorite and chlorhexidine did not detoxify endotoxin.

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In vitro effects of endodontic irrigants on endotoxins in root canals

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Objective. The objective of this study was to evaluate the effects of endodontic irrigants on endotoxins in root canals.
Study design. Ninety-eight single-root human teeth were used. *Escherichia coli* endotoxin was inoculated into 84 root canals. All root canals were enlarged and assigned to 7 groups (n = 14), according to solution used. Group 1 (G1): 2.5% NaOCl; G2: 5.25% NaOCl; G3: 2% chlorhexidine; G4: 0.14% calcium hydroxide; G5: polymyxin B; G6: positive control, saline solution; G7: negative control (no endotoxin). Two samplings of root canal were accomplished: immediate and after 7 days. Detoxification of endotoxin was evaluated by Limulus assay and antibody production in B-lymphocyte culture. Results were analyzed by Kruskal-Wallis/Dunn and ANOVA/Tukey.
Results. At the immediate and second samplings, groups G4, G5, and G7 presented the best results, significantly different from groups G1, G2, G3, and G6 ($P < .05$).
Conclusions. Calcium hydroxide and polymyxin B detoxified endotoxin in root canals and altered properties of LPS to stimulate the antibody production by B-lymphocytes. Sodium hypochlorite and chlorhexidine did not detoxify endotoxin. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;104:135-42)**

Bacteria and their by-products have a fundamental role in the initiation and perpetuation of pulpal and periradicular disease. Analyses of the microflora in infected root canals with necrotic pulps have revealed that Gram-negative anaerobic bacteria are the predominant microorganisms,[1,2] mainly during the active phase of rapid lesion expansion.[3]

Gram-negative bacteria contain endotoxin in their outer cell membrane.[4] This endotoxin or lipopolysaccharide (LPS) is released during cell duplication or after cell death. Endotoxins are responsible for several important biological effects, such as polymorphonuclear leucocytes (PMN), release of collagenase, macrophage activation, and release of bioactive inflammatory mediators such as the cyclooxygenase pathway products; platelet-activating factor (PAF); tumor necrosis factor (TNF); interleukins (IL-1, IL-6, and IL-8); superoxide (O_2^-); nitric oxide; interferon α, β, and γ[5-7]; complement system activation; B-lymphocyte polyclonal activation; cytotoxicity; hemodinamics modifications; fever induction; and osteoclast attraction.[8] These factors are important in the development and maintenance of inflammatory reaction and bone resorption in the periapical region.[9]

The inoculation of LPS into root canals of many animals (monkeys, dogs, cats) induced inflammatory reactions and bone resorption in the periradicular tissues of all the experiment teeth,[10-15] demonstrating the important role of endotoxin in the pathogenesis of periapical lesions. Khabbaz et al.[16,17] showed the presence of endotoxins in carious lesions of symptomatic and asymptomatic teeth and in the pulpal tissue of the carious teeth, suggesting that LPS may play an important role in the pathogenesis of human pulpal diseases.

The major goal of endodontic treatment is the elimination of bacteria from the root canal system by mechanical and chemical means and a wide variety of antimicrobial agents have been used for this purpose.[5] However, it is possible that even though bacteria are removed from the root canal, endotoxins capable of maintaining or inducing apical periodontitis can remain,[7] interfering in the success of endodontic treatment. Therefore, the treatment of infected root canals should not only be concerned with bacterial death, but also the inactivation of endotoxin.[11,12]

During instrumentation of root canal, frequent irrigation is recommended with the main purpose of removing debris from the canal, killing microorganisms,

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and dissolving tissue. Therefore, several irrigation solutions have been used in endodontic treatment. Sodium hypochlorite has been widely recommended as an irrigant solution for the chemomechanical debridement of root canals because of its solvent activity for necrotic and vital tissues and also because of its ability to be an effective agent against a broad spectrum of bacteria.[18,19] Chlorhexidine gluconate has been recommended as an irrigant solution based on its antibacterial effects, substantivity, and relative absence of cytotoxicity.[18] The antimicrobial action of sodium hypochlorite and chlorhexidine, at different concentrations, has been related in several studies[20,21]; however, the activity of these irrigant solutions on LPS during instrumentation of the root canal is not well known.

On the other hand, calcium hydroxide presents a detoxifying effect on endotoxin when used as intracanal medicament for at least 7 days.[5,22] Safavi and Nichols[5] related that calcium hydroxide treatment of LPS released elevated quantities of hydroxy fatty acids by hydrolysis of ester bonds in the lipid moiety of bacterial LPS. Calcium hydroxide alters some biological properties of bacterial LPS as the ability to stimulate prostaglandin E_2 and TNF-α production by monocytes and antibodies by B-lymphocytes.[6,7,22] Studies in vivo have demonstrated that intracanal medicament with calcium hydroxide for 30 or 60 days detoxified LPS, neutralizing their effects in the periapical tissues of dogs.[11,12,23]

Polymyxin B (PmB), a cyclic cationic polypeptide antibiotic, prevents the lethal effect of endotoxin in chick embryos, mice, dogs, goats, foals, and horses.[24-26] In these animal models, polymyxin B inhibits LPS-induced intravascular coagulation; the generalized Shwartzman reaction; LPS-induced macrophage production of interferon-γ, TNF-α, and interleukin-1; and endotoxin-mediated shock.[27] Polymyxin B presents important results in endotoxin systemic detoxifying; however, their effects on LPS in the root canal system have received little attention. In 2005, Oliveira et al.[22] showed that polymyxin B as intracanal medicament for 7 days detoxified endotoxin in root canals and altered the properties of LPS to stimulate antibody production by B-lymphocytes. Considering these findings, it would be important to analyze the effectiveness of polymyxin B as an irrigant solution during instrumentation of root canals.

In 1975, Schein and Schilder[1] showed the presence of endotoxins in infected root canals and apical periodontitis; however, little research evaluated the effectiveness of irrigant solutions on bacterial LPS. Considering that endotoxins induce inflammatory reactions and bone resorption in the periapical tissues, the purpose of this study was to evaluate in vitro the effects of the endodontic irrigant solutions sodium hypochlorite, chlorhexidine, calcium hydroxide, and polymyxin B on endotoxins in root canals.

MATERIALS AND METHODS

Specimen preparation

This study was submitted to and approved by the Committee of Research of the University of Dentistry of São José dos Campos - UNESP (number 034/2002).

Ninety-eight freshly extracted single-rooted human teeth were used in the study. These teeth were collected from patients of the School of Dentistry/UNESP at São José dos Campos and selection of teeth was made on the basis of relative dimensions and similarity in morphology. Debris, calculus, and soft tissue remnants on the root surfaces were cleaned using a Gracey curette and all teeth were stored in saline solution (NaCl 0.85%) until used. The apical 3 mm of each root were transversely sectioned using a water-cooled diamond disc and the crown was sectioned at a point in order to standardize the root length to approximately 14 mm. Working length was determined by subtracting 1 mm from this measurement.

For standardization of root canal diameter, initial enlargement was accomplished by a size #30 K-file (Maillefer, Ballaigues, Switzerland), followed by irrigation with saline solution for each file used. The canals were dried with sterile paper points (Dentsply Ind Com LTDA, RJ, Petrópolis, Brazil) and apical region was sealed with light-cured resin composites (3M Dental Products, St Paul, MN). The outer surfaces of the specimens were covered with 2 layers of epoxy adhesive (Araldite - Brascola, Sao Paulo, Brazil), except the cervical opening.

Specimen sterilization

All specimens were sterilized by autoclaving (20 minutes at 121°C) and randomly assigned to 7 cell culture plates (24 wells, Costar, Corning, NY), with 14 teeth each. The teeth were fixed with chemically activated acrylic resin (Fig. 1*A*). The specimens and all the materials used in the experiment were irradiated with 60 Co gamma rays for degradation of preexisting LPS.[28]

Contamination with endotoxin

Escherichia coli 055:B5 endotoxin (Sigma, St Louis, MO) was used for the experiments. Under sterile laminar flow, 10 μL (40 endotoxin units [EU]) of a standard solution containing endotoxin (4000 EU/mL) were inoculated into the root canals of 84 specimens using a micropipette. Teeth were sealed with pyrogen-free cotton ball and the plates containing the specimens were

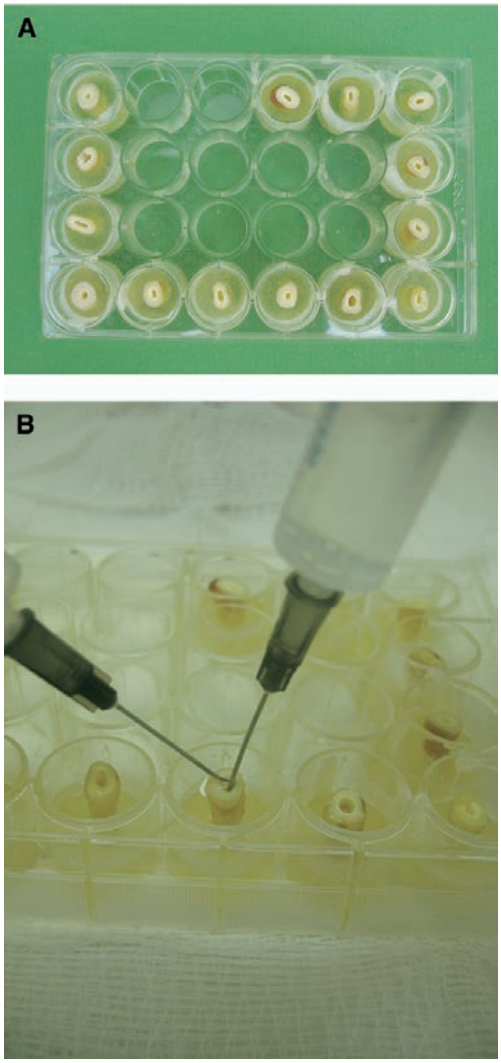

Fig 1. Model system. **A,** Distribution of teeth (n = 14) in cell culture plates (24 wells). **B,** Irrigation of root canal with an endodontic solution.

closed, sealed, and incubated for 24 hours at 37°C in a humidified atmosphere.

Experimental groups

After 24 hours, the cervical sealing was removed and all the root canals were enlarged to a size #80 K-file (Maillefer). Step-back cleaning and shaping was performed with recapitulation followed by irrigation with 3 mL of irrigation solution for each file used (Fig. 1, *B*). The specimens were divided into the following 7 groups (n = 14), according to the endodontic irrigant solution used. Group 1: 2.5% sodium hypochlorite (Aqua Marina Drugstore, São José dos Campos, SP, Brazil); Group 2: 5.25% sodium hypochlorite (Aqua Marina Drugstore); Group 3: 2% chlorhexidine digluconate (Aqua Marina Drugstore); Group 4: 0.14% calcium hydroxide solution (0.14 g of calcium hydroxide in pyrogen-free water; Biodinâmica, PR, Brazil); Group 5: polymyxin B solution (10000 units/mL) (Ophtalmos Fórmulas Oficiais Ltda, São Paulo, Brazil); Group 6 (positive control): pyrogen-free saline solution (Beker, São Paulo, Brazil); Group 7 (negative control): did not receive endotoxin and pyrogen-free saline solution (Beker) was used as irrigant.

Afterwards, the root canals were irrigated with 3 mL of pyrogen-free water to remove all irrigant solution. Each specimen was filled with pyrogen-free water and 2 samplings of root canal content were taken to verify detoxification of endotoxin: (1) First sampling (immediate): 230 μL of root canal content were collected with a nonpyrogenic 0.5-mL plastic syringe (Injex, São Paulo, Brazil) immediately after the instrumentation; (2) Second sampling: 230 μL of root canal content were collected after 7 days of the immediate collection. During this period, the plates containing the specimens were closed, sealed, and incubated at 37°C in a humidified atmosphere.

For all the samplings (immediate and after 7 days), 2 methods were used to verify the detoxification of endotoxin: Limulus amebocyte lysate assay and antibody production in B-lymphocytes culture.

Detoxification of endotoxin: Limulus amebocyte lysate assay

The detoxification of endotoxin was evaluated by Limulus amebocyte lysate assay (Sigma). Limulus is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*. When exposed to minute quantities of endotoxins, the lysate increases in opacity as well as viscosity, forming a hard gel. An aliquot of canal content (100 μL) was put into a pyrogen-free tube containing 100 μL of Limulus lysate, according to the manufacturer's instructions. The tube was gently mixed and incubated at 37°C for 1 hour. These procedures were repeated for all the specimens. After 1 hour of incubation, the tubes were gently removed and slowly inverted 180 degrees while observing for evidence of gelation. The formation of a hard gel, which permits complete inversion of the tube without disruption of gel, was considered a positive test. All other results (soft gels,

Table I. Parameters of semiquantitative analysis of endotoxin presented in the sampling of root canals

Scores	*Dilutions*	*Endotoxin (EU)*
0	0	<0.125
1	1:2	0.125-0.249
2	1:4	0.250-0.499
3	1:8	0.5-0.999
4	1:16	1.0-1.999
5	1:32	2.0-3.999
6	1:64	4.0-7.999
7	1:128	8.0-15.999
8	1:256	16.0-31.999
9	1:512	32.0-40.0

turbidity, increase in viscosity, clear liquid) were considered a negative test.

Then, a semiquantitative analysis of endotoxin present in the root canal content was performed. For this purpose, an aliquot of 100 μL of the root canal content was diluted until gel formation was not observed as described in Table I. Considering that the sensitivity of the test is of 0.125 EU/mL as described by the manufacturer, approximate values of endotoxin (EU) in the sampling was determined after dilutions.

Detoxification of endotoxin: antibody production in lymphocyte B culture

Four Swiss mice (*Mus musculus*), approximately 6 months of age, were humanely killed and the spleen of each animal was removed and macerated in a pyrogen-free Falcon tube containing RPMI 1640 medium (Sigma), to obtain B-lymphocytes. The tube was centrifuged at 500*g* for 10 minutes and the pellet was resuspended in 30 mL of RPMI 1640 medium supplemented with 10% bovine fetal serum. To prevent contamination of the culture, sterile conditions were maintained by sterile laminar flow.

For cell viability, the trypane blue exclusion assay (0.4%) (Sigma) and a buffer to promote erythrocyte rupture were used. The viable cells count was 4.22×10^6 cells/mL. Approximately 1×10^6 viable cells (250 μL) were transferred to each well of pyrogen-free cell culture plates (24-well, Costar). These plates were incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. B-lymphocytes of each well of cell culture plates were stimulated with the remaining 30 μL of each root canal content. The 15 wells containing only pure cell culture were considered as the cell culture control group (G8). The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 4 days. The enzyme-linked immunosorbent assay (ELISA) was used to determine total immunoglobulin M (IgM) levels in the culture supernatant.

ELISA was performed in 8 microplates (96-well, Hemobag, São Paulo, Brazil). All the plates were coated with mouse anti-IgM (Sigma) in the concentration of 0.5 mg/mL. The plates were incubated for 2 hours at 37°C and maintained in the refrigerator until used.

On the day of the experiment, the plates were blocked with 0.5% gelatin (G) in phosphate-buffered saline (PBS), and 0.2% bovine serum albumin (BSA) (45 minutes at 37°C). Culture supernatants (100 μL) were added to each well and incubated for 2 hours at 37°C, followed by washing with 0.1% Tween-20 in PBS (PBS-T). All the tests were performed in duplicate. Anti-mouse IgM peroxidase conjugate, diluted 1000 times with PBS-GT (0.5% gelatin and 0.1% Tween 20 in PBS), was added to the plates and incubated for 1 hour at 37°C. After washing with PBS-T, peroxidase enzyme activity was detected by addition of the substrate, 50 μL of *o*-phenylenediamine (1 mg/mL) in a solution of 0.1 M citrate and 0.03% H_2O_2 for 10 minutes. Finally, the reaction was stopped with 2.5 N H_2SO_4. The optical density (OD) of the wells was read at an absorbance of 490 nm on a Bio-Rad Microplate reader Model 3550 (Bio-Rad Laboratories, Hercules, CA). For each specimen the mean values of OD were obtained and the results were statistically analyzed.

Statistical analysis

The results obtained with Limulus lysate assay were statistically analyzed by nonparametric Kruskal-Wallis and Dunn's tests. ELISA results were statistically analyzed using analysis of variance (ANOVA) and Tukey's test. In all cases P values less than .05 were considered as statistically significant.

RESULTS

Results of Limulus lysate assay by qualitative analysis at immediate sampling demonstrated hard gel formation in all specimens of G1 (NaOCl 2.5%), G2 (NaOCl 5.25%), and G3 (2% chlorhexidine). These results were statistically similar to G6 (positive control) ($P < .05$). No gel formation was observed in groups G4 ($Ca(OH)_2$) and G5 (polymyxin B) and the results were statistically similar to G7 (negative control) ($P < .05$). Considering semiquantitative analysis, scores were attributed according to the number of dilutions requested until no hard gel formation (Table I). The results (scores) were analyzed by nonparametric Kruskall-Wallis and Dunn's test. Groups G1, G2, and G3 presented median values of 1 to 1.999 EU of endotoxin, corresponding to dilution 1:16 (score 4). These results were statistically similar to G6 (positive control) ($P > .05$), which presented median values of 2 to 3.999 EU of endotoxin, corresponding to dilution 1:32 (score 5).

Table II. Median values (scores) obtained by Limulus assay for each experimental group (first and second sampling)

	Median	
Groups	*First sampling*	*Second sampling*
G1	4^{A}	4^{A}
G2	4^{A}	4^{A}
G3	4^{A}	4^{A}
G4	0^{B}	2^{B}
G5	0^{B}	2^{B}
G6	5^{A}	5^{A}
G7	0^{B}	0^{B}

Group 1 (G1), 2.5% NaOCl; G2, 5.25% NaOCl; G3, 2% chlorhexidine; G4, 0.14% $Ca(OH)_2$; G5, polymyxin B; G6, positive control; G7, negative control.
*Different letters^{A,B} indicate statistically different medians.

Table III. Mean values of optical density (OD) ± SD presented by each group in the first (immediate) and second (7-day) sampling

Groups	*OD ± SD (first sampling)*	*OD ± SD (second sampling)*
G1	0.638 ± 0.060 ^{A}	0.603 ± 0.072 ^{A}
G2	0.628 ± 0.071 ^{A}	0.598 ± 0.071 ^{A}
G3	0.647 ± 0.057 ^{A}	0.628 ± 0.054 ^{A}
G4	0.292 ± 0.060 ^{B}	0.397 ± 0.063 ^{C}
G5	0.311 ± 0.056 ^{B}	0.410 ± 0.072 ^{C}
G6	0.673 ± 0.067 ^{A}	0.647 ± 0.069 ^{A}
G7	0.318 ± 0.051 ^{B}	0.291 ± 0.035 ^{B}
G8	0.278 ± 0.045 ^{B}	0.263 ± 0.047 ^{B}

Group 1 (G1), 2.5% NaOCl; G2, 5.25% NaOCl; G3, 2% chlorhexidine; G4, 0.14% $Ca(OH)_2$; G5, polymyxin B; G6, positive control; G7, negative control.
*Different letters^{A,B,C} indicate statistically different medians.

The groups G4, G5, and G7 presented median values less than 0.125 EU (score 0) (Table II).

At the sampling after 7 days, groups G4 and G5 presented hard gel formation in almost all specimens; however, after dilutions of root canal content, it could be verified that endotoxin amounts in groups G4 and G5 (0.250 to 0.499 EU, score 2) were significantly lower than the other experimental groups ($P < .05$). There were no statistically significant differences among groups G4, G5, and G7 (negative control) ($P > .05$). Groups G1, G2, and G3 presented median values of endotoxin (1 to 1.999 EU, score 4) similar to G6 (2 to 3.999 EU, score 5) ($P > .05$) and were significantly different from groups G4, G5, and G7 ($P < .05$) (Table II).

In relation to results obtained for the antibody production in lymphocyte B culture, the values of OD obtained by ELISA were statistically analyzed using ANOVA and Tukey's test. At the immediate sampling, mean values of OD concerning antibody (IgM) production in B-lymphocyte culture in the groups G1 (0.638 ± 0.060), G2 (0.628 ± 0.071), G3 (0.647 ± 0.057), and G6 (0.673 ± 0.067) were significantly higher than groups G4 (0.292 ± 0.060) and G5 (0.311 ± 0.056) ($P < .05$). Groups G4 and G5 presented low IgM production, similar to groups G7 (0.318 ± 0.051) and G8 (cell culture control group) (0.278 ± 0.045) ($P > .05$) (Table III).

At the second sampling, groups G1 (NaOCl 2.5%), G2 (NaOCl 5.25%), G3 (2% chlorhexidine), and G6 (positive control) induced significant increase of IgM production and presented mean values of OD of 0.603 ± 0.072, 0.598 ± 0.071, 0.628 ± 0.054, and 0.647 ± 0.069, respectively. These results were statistically different in relation to the other groups ($P < .05$). IgM production by groups G4 (0.397 ± 0.063) and G5 (0.410 ± 0.072) was significantly higher than control groups G7 (0.291 ± 0.035) and G8 (0.263 ± 0.047) ($P < .05$); however, significantly lower than groups G1, G2, G3, and G6 ($P < .05$) (Table III).

DISCUSSION

Elimination of microorganisms from root canal systems by antimicrobial agents has been a major goal of endodontic treatment. However, treatment of root canals in teeth with pulp necrosis should not only be concerned with bacterial death, but also the inactivation of endotoxin.[11,12] Endotoxin has an important role in the inflammatory and immunologic responses of pulpal and periradicular tissues, including bone resorption.[7,9,13]

The presence of endotoxin in teeth with vital or necrotic pulps has already been proven by many researchers[1,17,29]; however, few studies have evaluated the effects of irrigation solutions on LPS using teeth as a model system. Endotoxins as well as bacteria are capable of diffusing through dentinal tubules, being present not only in the canal lumen but also in the entire root canal system.[30] In the present research, we used single-root human teeth extracted for several reasons, independently of diagnosis, because the endotoxin possibly present in these teeth was degraded by 60Co gamma irradiation.[28] Selection of teeth was made on the basis of relative dimensions and similarity in morphology and the apical 3 mm of each root was removed to minimize variations such as cracks, apical resorptions, and ramifications.[22]

In this study 2 methods were used to verify the detoxification of endotoxin: Limulus amebocyte lysate assay and antibody production in B-lymphocyte culture. The use of Limulus amebocyte lysate for the detection of endotoxin evolved from the observation by Bang[31] that a Gram-negative infection of *Limulus*

polyphemus, the horseshoe crab, resulted in fatal intravascular coagulation. Latter, Levin and Bang[32] demonstrated that this clotting was the result of a reaction between endotoxin and a clottable protein in the circulation. Following the development of a suitable anticoagulant for *Limulus* blood, Levin and Bang[33] prepared a lysate from washed amebocytes, which was an extremely sensitive indicator of the presence of endotoxin. Several studies used the Limulus amebocyte lysate assay for demonstration of endotoxins in middle ear effusions, root canals, caries, and vital pulp of human carious teeth.[1,16,17,29,34] LPS is a T-independent polyclonal B-lymphocyte activator and polyclonal B-cell activation usually enhances much more IgM synthesis than IgG.[35] This study evaluated the antibody (IgM) production in B-lymphocyte culture stimulated with root canal content as an indirect method for detection of LPS.

The results of this study showed that calcium hydroxide and polymyxin B as endodontic irrigants had a detoxifying effect on endotoxin in root canal systems. These results are in accordance with other studies that, using different methods, demonstrated that calcium hydroxide could alter the biological properties of LPS[5,6,7,11,22] and that polymyxin B is effective in the treatment of endotoxemia.[25,26]

The irrigation of root canals with 0.14% calcium hydroxide solution or polymyxin B reduced significantly the amount endotoxin in root canals in relation to other solutions (sodium hypochlorite and chlorhexidine), remaining only a small amount of LPS, detected in the second sampling (after 7 days). The presence of this small amount of endotoxin probably occurred because the calcium hydroxide and polymyxin B, although it is effective on LPS, did not penetrate enough in the dentinal tubules in order to act on LPS in deeper portions. Vahdaty et al.[36] compared the antimicrobial effects of chlorhexidine and NaOCl solution on *Enterococcus faecalis* in the dentinal tubules of bovine incisors roots and verified that they significantly reduced the bacterial counts in the first 100 μm of tubules; however, in deeper levels the irrigants were less effective, probably because they had not penetrated deep enough in the time available. Our findings suggest that calcium hydroxide and polymyxin B present important action on LPS; however, the irrigation was not enough to reach every LPS contained in dentinal tubules.

The amount of LPS remaining in groups G4 and G5, in the second sampling, induced a small increase in IgM production, significantly smaller than other experimental groups ($P < .05$), showing that calcium hydroxide and polymyxin B alter the biological properties of LPS in B-cell polyclonal activation. Safavi and Nichools[5] related that calcium hydroxide treatment of LPS release elevated quantities of hydroxy fatty acids by hydrolysis of ester bonds in the lipid moiety of bacterial LPS. This way, the treatment with calcium hydroxide detoxifies LPS, inhibiting their pathological effects on cell activation, inflammatory mediators, interleukins, tumor necrosis factor (TNF-α) production, and induction of periapical lesions in animals.[6,7,11,12,22] These experiments suggested that the biological properties of LPS require the presence of ester-linked hydroxy fatty acids and these linkages are destroyed by treatment with calcium hydroxide. The detoxification of LPS by calcium hydroxide may be one of the mechanisms by which this agent exerts its beneficial effects in clinical endodontics. In this study, we verified that calcium hydroxide was able to alter the properties of LPS to stimulate polyclonal antibody production by B-lymphocytes. The absence of hard gel formation in the Limulus amebocyte lysate assay, in the first collection, showed that calcium hydroxide is able to detoxify LPS, probably by hydrolysis of the link ester: hydroxy fatty acids.

The effectiveness of polymyxin B on LPS presented in this study is in agreement with other authors,[25,26,37] who used polymyxin B in the treatment of endotoxemia in animals and with Oliveira et al.,[22] who demonstrated effectiveness of polymyxin, as intracanal medication for 7 days, in detoxifying of LPS in root canals. Hong et al.[38] verified that systemic administration of polymyxin B in rats reduced the extent of periapical lesion-associated bone resorption by 76% to 80%. Rifkind and Palmer[24] and Rifkind[25] postulated a mechanism of action in which polymyxin B, a cationic antibiotic, covalently couples with the electronegative charge of the A lipid in the endotoxin molecule. Morrison and Jacobs[39] confirmed this theory in vitro. Polymyxin B has the ability to bind with high affinity to the A lipid portion, altering the 3-dimensional conformation of the LPS molecule. This conformational alteration possibly enables the binding of the complex endotoxin-polymyxin B to the monocyte CD14 receptor, inhibiting the liberation of inflammatory mediators. In the present study, polymyxin B enabled B-lymphocyte activation by LPS and also inhibited gel formation in Limulus amebocyte lysate assay, in the first sampling, probably due to the conformational alteration of the endotoxin-polymyxin B complex. The polymyxin B solution used in this study contained 1 mg/mL of polymyxin B that corresponds to 10000 units/mL, the same concentration used in otologic medications.

Sodium hypochlorite (2.5% and 5.25%) and chlorhexidine 2% did not detoxify LPS in root canals in our study. The results presented by these groups in the Limulus lysate assay and in the B-cell stimulation were similar statistically to the positive control group ($P >$

.05), demonstrating a high amount of LPS in relation to G4 (calcium hydroxide) and G5 (polymyxin B) groups ($P < .05$). Sodium hypochlorite and chlorhexidine present antimicrobial effectiveness on Gram-negative bacteria[20,21]; however, in the present study, no effect was observed on LPS.

These results are in agreement with the study of Tanomaru et al.,[23] who showed that biomechanical preparation with sodium hypochlorite and chlorhexidine solutions as endodontic irrigants did not inactivate the effects of the endotoxin to induce an inflammatory reaction in the periapical region of dogs. Aibel and Stevens[40] verified that the treatment of LPS with 1.2% chlorhexidine for 40 minutes did not inactive its ability to induce the production of IL-6 by macrophages. Buck et al.[41] showed by quantitation of free fatty acid release that 2.625% sodium hypoclorite and 0.12% chlorhexidine solutions alone were not effective on LPS. On the other hand, these authors demonstrated that an alkaline mixture of chlorhexidine, ethanol, and sodium hypochlorite as an irrigation solution for 30 minutes and calcium hydroxide as an intracanal medicament for 1, 2, or 5 days detoxified LPS. Niwa et al.[42] demonstrated that the treatment of endotoxin with mild alkali alters the physical, chemical, and biological properties of endotoxin.

The few studies in the literature that evaluate the effectiveness of irrigant solutions on LPS agree that the irrigant solutions most frequently used in the endodontic practice (sodium hypochlorite and chlorhexidine) are not effective on endotoxin. As these irrigants present important properties as antimicrobial actions on bacteria commonly found in root canal infections, capacity to dissolve tissue (sodium hypochlorite) and substantivity (chlorhexidine), it would be interesting to analyze associations of these irrigants with polymixin B or calcium hydroxide that demonstrate important effectiveness on LPS.

In conclusion, calcium hydroxide and polymyxin B, as endodontic irrigant solutions, detoxified endotoxin in root canals and altered the properties of LPS to stimulate the antibody production by B-lymphocytes. Sodium hypochlorite (2.5% and 5.25%) and chlorhexidine 2% solutions did not detoxify endotoxin.

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3.3 Action of propolis and medications against *Escherichia coli* and endotoxin in root canals*

ABSTRACT

This study evaluated the action of propolis and intracanal medications against *Escherichia coli* and endotoxin. Forty-eight dental roots were contaminated with *E. coli*. The root canals were instrumented with propolis and divided into groups according to the type of intracanal medication: $Ca(OH)_2$, polymyxin B, or $Ca(OH)_2$ 2% chlorhexidine gel. In the control group, saline solution was used without application of intracanal medication. Counts of colony-forming units were carried out and the endotoxin was quantified by the chromogenic *Limulus* amobocyte lysate assay. The results were evaluated by analysis of variance and the Dunn test (5%). Root canal irrigation with propolis was effective to completely eliminate *E. coli* and reduce the amount of endotoxins. All intracanal medications contributed to the significant decrease in endotoxins. Only intracanal medications may reduce the amount of endotoxins in the root canals. The greatest efficacy was observed for medications containing $Ca(OH)_2$.

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Action of propolis and medications against *Escherichia coli* and endotoxin in root canals

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This study evaluated the action of propolis and intracanal medications against *Escherichia coli* and endotoxin. Forty-eight dental roots were contaminated with *E. coli*. The root canals were instrumented with propolis and divided into groups according to the type of intracanal medication: $Ca(OH)_2$, polymyxin B, or $Ca(OH)_2$ + 2% chlorhexidine gel. In the control group, saline solution was used without application of intracanal medication. Counts of colony-forming units were carried out and the endotoxin was quantified by the chromogenic *Limulus* amobocyte lysate assay. The results were evaluated by analysis of variance and the Dunn test (5%). Root canal irrigation with propolis was effective to completely eliminate *E. coli* and reduce the amount of endotoxins. All intracanal medications contributed to the significant decrease in endotoxins. Only intracanal medications may reduce the amount of endotoxins in the root canals. The greatest efficacy was observed for medications containing $Ca(OH)_2$. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:e70-e74)**

Studies have assessed the significant presence of anaerobic microorganisms, especially gram-negative groups, in root canals with necrotic pulp.[1] The endotoxins present on the cell walls of gram-negative bacteria are released during their multiplication or cell death, promoting several biologic effects that lead to inflammation, immunologic reactions, and periapical bone resorption.[2] These substances are able to penetrate and diffuse toward the dentinal tubules, reaching the cementum in a period of 24 hours.[3]

The endotoxins present in the blood stream and other biologic tissues are bonded to the CD-14 receptor on the surface of monocytes and macrophages. When activated, these cells are able to secrete interleukins (IL-1, IL-5, IL-8), tumor necrosis factor α (TNF-α), superoxide (O_2), nitric oxide, interferons α, β, and γ, and lipidic molecules, such as platelet-activating factor and prostaglandins.[4] The released endotoxins may contribute to an increase of neurotransmitters and vasoactive substances around the nerve bundles at the periapical region, causing pain.[5] Therefore, during endodontic therapy, the selection of auxiliary chemical substances and intracanal medication should be based on their action against microorganisms and endotoxins.

Propolis extract shows antiinflammatory, anesthetic, and antimicrobial activities.[6] Some authors[6-8] demonstrated these results in investigations concerning the action of propolis extract against *Streptococcus mutans*. In addition, Duarte et al.[9] showed its influence in the reduction of acid production by *S. mutans* in the dental biofilm, besides its inhibitory action over the F-ATPase activity of *S. mutans*. According to Koo et al.,[10] the growth of several bacteria, such as *S. mutans* and *S. sanguis*, was significantly reduced by 10% propolis extract, which also affected the growth of anaerobic bacteria. Also, Geraldini et al.[11] demonstrated that the use of 10% propolis alcoholic extract, 2% chlorhexidine (CLX) gel, or calcium hydroxide ($Ca(OH)_2$) as root canal dressing promoted similar bacterial reduction when applied in dogs' teeth with induced periapical lesions.

Calcium hydroxide has been widely used as intracanal medication owing to its capacity to induce remineralization,[12] antimicrobial activities[13,14] and effective action against lipopolysaccharides (LPS).[15-17] Some authors provided evidence that it promotes hydrolysis of the lipidic portion of the endotoxin, thus neutralizing its biologic effects.[18]

The association of $Ca(OH)_2$ and 2% CLX has been satisfactorily used as intracanal medication.[19,20] It was also demonstrated that this association shows antimicrobial, repairing, and effective activity on endotoxins present in root canals.[21]

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The medical use of polymyxin B, a cationic polypeptide antibiotic, blocks the biologic effects caused by endotoxins.[22] This medication has been used in the treatment of patients with gram-negative sepsis.[23]

The present study evaluated the antimicrobial action of propolis glycolic extract used as irrigant during biomechanical preparation and intracanal medications against *Escherichia coli* and its endotoxin in the root canals.

MATERIALS AND METHODS

Preparation and contamination of specimens

This study was approved by the Institutional Review Board of São Paulo State University, São José dos Campos, Brazil (protocol no. 067/2005). Forty-eight human single-rooted teeth were used in the study, whose crowns were removed and root canals instrumented at a standardized length of 16 ± 0.5 mm.

Initial root canal instrumentation was made on their full length up to K-file #30, followed by irrigation with 3 mL saline solution after each instrument. Then, the apical region of each tooth was sealed with light-cured composite resin (Z-100; 3M Dental Products, St. Paul, MN) and the roots externally sealed with 2 layers of epoxy adhesive (Araldite; Brascola, São Paulo, Brazil), except for the cervical opening. The specimens were randomly placed in cell culture plates containing 24 wells (Costar, New York, NY). All culture plates and materials used were sterilized by gamma radiation (Embrarad; Cotia, São Paulo, Brazil).

The root canals were contaminated with a suspension (10^6 cells/mL) of *E. coli*. Then, 10 μL of the suspension and 10 μL of brain heart infusion (BHI) broth (Himedia, Mumbai, India) were inoculated into each root canal. A sterile cotton pellet was soaked in the culture medium and placed in the cervical third of root canals. All specimens were stored in an incubator at 37 ± 1°C with humidified atmosphere for 14 days. The BHI broth was placed into the root canals every 3 days.

Experimental groups

After verifying the contamination (initial collection, Si), all specimens were instrumented up to K-file #50 with stepback preparation up to K-file #80. Irrigation was done at each change of instrument using 3 mL 12% propolis glycolic extract (Apis Flora, Ribeirão Preto, Brazil). After biomechanical preparation (BMP), the root canals were irrigated with 3 mL pyrogen-free saline solution and the first sample was collected (S1). The root canals were filled with pyrogen-free saline solution and stored in an incubator at 37°C for 7 days. The second sample was then collected (S2). The specimens were divided into 3 experimental groups (n = 12 each) according to the intracanal medication used:

- Calcium hydroxide paste (Pasta Calen; SS White, Rio de Janeiro, Brazil).
- Polymyxin B (PB) solution (Ophtalmos Fórmulas Oficiais, São Paulo, Brazil).
- Association of $Ca(OH)_2$ powder (Biodinâmica Química e Farmacêutica, Ibiporã, Brazil) and 2% CLX gel (Byofórmula-Farmácia de Manipulação, São José dos Campos, Brazil) at a ratio of 1:1.

The control group (n = 12) was irrigated with the pyrogen-free saline solution without intracanal medication.

After filling with the root canal dressing medication, all samples were stored at 37°C for 14 days. After that period, the medications were removed with 10 mL pyrogen-free saline solution. Collection of the third sample (S3) was carried out after this period. The root canals were filled with pyrogen-free saline solution and stored in an incubator at 37°C for 7 days. Following that, the fourth collection (S4) was performed.

All sample collections (Si, S1, S2, S3, and S4) were carried out in the same standard way: The root canals were filled with pyrogen-free saline solution, and 100 μL of the root canal content were collected for microbiologic analysis and quantification of endotoxins.

The antimicrobial activity was determined by serial dilutions of the collected samples and double plating on BHI agar plates. Then they were incubated at 37°C for 48 hours, and the number of colony-forming units (CFU/mL) of *E. coli* was counted.

The quantification of endotoxins (EU/mL) was performed by the kinetic chromogenic *Limulus* amebocyte lysate assay (Cambrex Bio Ciência Brasil, São Paulo, Brazil). The results were evaluated by analysis of variance and the Dunn test (5%).

RESULTS

Contamination of the root canals was confirmed at Si. According to the microbiologic test, there was no colony growth of *E. coli* in all samples collected. Considering that the CFU/mL values were equal to zero, it can be stated that the propolis glycolic extract showed great efficacy against *E. coli*; thus, statistical analysis of the microbiologic results was not necessary (Table I).

In relation to the saline solution (control group), there was a reduction in the number of microorganisms immediately after BMP (S1), which then resumed their growth during the study (S4; Table I).

According to the results of endotoxin quantification, there was significant difference between samples S1 and S2 with propolis and with saline solution (Table II).

The mean, standard deviation, and median values obtained in the EU/mL counts, as well as the homogeneous groups of samples S3 and S4, are shown in Table

Table I. Mean ± SD values of colony-forming units/mL (CFU/mL) (log10)

Irrigating solution	*Si*	*S1*	*S2*	*ICM*	*S3*	*S4*
Propolis	8.65 ± 0.69	0.0	0.0	$Ca(OH)_2$	0.0	0.0
				PB	0.0	0.0
				$Ca(OH)_2$ + CLX	0.0	0.0
Saline solution (control)	8.16 ± 0.73	4.57 ± 0.41	6.23 ± 0.74	Saline solution	4.22 ± 0.94	4.55 ± 1.06

Si, Initial collection; *S1*, first sample; *S2*, second sample; *ICM*, intracanal medication; *S3*, third sample; *S4*, fourth sample; *$Ca(OH)_2$*, calcium hydroxide paste; *PB*, polymyxin B; *CLX*, chlorhexidine.

Table II. Descriptive statistical mean, standard deviation, and median values and homogeneous groups of the quantity of *Escherichia coli* endotoxin (EU/mL) for samples S1 and S2

	S1			*S2*		
Irrigating solution	*Mean ± SD*	*Median*	*Homogeneous groups**	*Mean ± SD*	*Median*	*Homogeneous groups**
Propolis	3,222 ± 6,462	1335	B	2,969 ± 3,355	1715	A
Saline solution	815 ± 713	534	A	6,108 ± 5,537	4524	B

Abbreviations as in Table I.
*Different letters indicate significant difference ($P < .05$).

Table III. Descriptive statistical mean, standard deviation, and median values and homogeneous groups of the quantity of *Escherichia coli* endotoxin (EU/mL) for samples S3 and S4

		S3			*S4*		
Irrigating solution	*ICM*	*Mean ± SD*	*Median*	*Homogeneous groups**	*Mean ± SD*	*Median*	*Homogeneous groups**
Propolis	$Ca(OH)_2$	13.67 ± 2.7	12.6	B	12.83 ± 4.1	12.05	C
	PB	123.30 ± 79.7	103.9	A	615.1 ± 331.5	592.0	AB
	$Ca(OH)_2$ + CLX	20.10 ± 15.73	11.95	B	26.58 ± 21.48	16.20	BC
Saline solution	Saline solution	2,720 ± 2,467	2,160.0	A	2,890 ± 1,655	2,790.0	A

Abbreviations as in Table I.
*Different letters indicate significant difference ($P < .05$).

III. In the S3 samples, $Ca(OH)_2$ + 2% CLX gel group and $Ca(OH)_2$ group presented the lowest endotoxin values. These groups were similar and statistically different from PB and saline solution. In the S4 sample, $Ca(OH)_2$ was similar to $Ca(OH)_2$ + 2% CLX gel and different from PB and saline solution. PB was similar to saline solution and $Ca(OH)_2$ + 2% CLX gel. For both samples S3 and S4, $Ca(OH)_2$ and $Ca(OH)_2$ + 2% CLX gel showed the lowest endotoxin values.

DISCUSSION

Cleaning and disinfection of the root canals are fundamental in the treatment of pulp necrosis and periapical lesions. The endodontic treatment must not only eliminate microorganisms but also inactivate the toxic byproducts as endotoxins.[16] Several substances used during BMP do not show efficacy against endotoxins, underscoring the need to evaluate other substances, which should present antimicrobial activity as well as absence of cytotoxicity, capacity to dissolve tissues, and effects against endotoxins.

Studies have shown that even after BMP, microorganisms and endotoxins can remain in the root canals, requiring the use of intracanal medications that may act on the dentin and resorption lacunae, which are hardly accessed during BMP.

The present microbiologic analysis did not reveal difference between groups irrigated with propolis; there was no growth of *E. coli* colonies or other microorganisms in all of the samples collected after BMP. This corroborates the results of Ferreira et al.,[24] who evaluated the in vitro antimicrobial activity of propolis against anaerobic bacteria, such as *Prevotella nigrescens*, *Fusobacterium nucleatum*, and *Enterococcus faecalis*, as well as the findings of Önçag et al.,[25] who investigated the efficacy of propolis as intracanal medication in teeth contaminated with *E. faecalis* for 7 days. Some authors, such as Al-Shaher et al.,[26] Silva et al.,[27] Ozan et al.[28] and Gardjeva et al.,[29] showed that, in addition to the well known properties of propolis, such as anesthetic, antiinflammatory, and antimicrobial activities, it shows low cytotoxicity. Silva et al.[27] verified

that propolis was the least irritating substance compared with both saline solution and otosporin. The present study revealed the efficacy of propolis against *E. coli*; however, irrigation with propolis glycolic extract was not effective enough to neutralize the endotoxins. Because the endotoxins are released during bacterial death and the propolis extract was able to completely eliminate the *E. coli*, the statistically significant difference between propolis and saline solution in sample S1 suggests a large release of endotoxins in the root canals. Conversely, the saline solution only decreased the amount of microorganisms in the root canal, and therefore a smaller amount of endotoxins was released.

There was a reduction in the amount of endotoxins after use of intracanal medications $Ca(OH)_2$ paste, PB, and $Ca(OH)_2$ + 2% CLX gel for 14 days. The $Ca(OH)_2$ paste and the association of $Ca(OH)_2$ + 2% CLX gel showed similar values and more effective results compared with PB.

These results are similar to the reports of Oliveira et al.,[30] Nelson Filho et al.,[15] and Silva et al.[16] Calcium hydroxide is able to inactivate the endotoxin (LPS) by the hydrolysis reaction of lipid A, resulting in greater release of free fatty acids.[18] The association of $Ca(OH)_2$ + CLX combines the properties of neutralization of LPS and antimicrobial action, owing to the synergistic effect between both substances.[19,20]

The results with PB corroborate the findings of Oliveira et al.,[30] who used this antibiotic as intracanal medication and as irrigant in teeth contaminated with *E. coli* endotoxin. Also, Rifkind and Palmer[31] suggested that some cationic polypeptide antibiotics, such as PB, can neutralize the endotoxins by the direct combination to the electronegative charge of lipid A in the endotoxin molecule.

In the S4 sample, the $Ca(OH)_2$ paste showed the greatest efficacy, with the smallest values obtained compared with other medications. The association of $Ca(OH)_2$ + 2% CLX gel also showed effectiveness against endotoxins, being similar to $Ca(OH)_2$ and PB.

The present results allow the conclusion that propolis is effective against *E. coli* after biomechanical preparation. However, it was not able to eliminate endotoxins present in the root canal. Conversely, intracanal medications, particularly $Ca(OH)_2$, have shown significant results in the reduction of endotoxins.

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3.4 Efficacy of endodontic treatment for endotoxin reduction in primarily infected root canals and evaluation of cytotoxic effects*

ABSTRACT

Introduction: Endotoxins are one of the etiologic agents involved in the pathogenesis of apical periodontitis. The objectives of this clinical study were to investigate the effects of endodontic treatment by using different irrigants on endotoxins in root canals with pulp necrosis and apical periodontitis and to evaluate the cytotoxic effects. Methods: Thirty-six root canals were selected. Samples were collected before (S1) and after instrumentation (S2). The root canals were divided into 3 groups (n = 12) according to the irrigant combination used: CLX + LW, 2% chlorhexidine gel + calcium hydroxide (0.14%, limewater); CLX + PmB, chlorhexidine + polymyxin B; CLX (control), chlorhexidine + saline. The third sampling (S3) was performed after ethylenediaminetetraacetic acid and S4 after intracanal medication (CLX + calcium hydroxide for 14 days). Endotoxins were quantified by the chromogenic Limulus amebocyte lysate assay, and cytotoxic effects were evaluated by the production of cytokines (interleukin-1ß, tumor necrosis factor a) in macrophages (RAW 264.7) stimulated with the root canal content. Results: Endotoxins were detected in all root canals before instrumentation (S1). Group CLX + LW presented the greatest endotoxin reduction after instrumentation (99.18%), which was similar to group CLX + PmB (96.42%, $P > .05$) and different from group CLX (90.78%, $P < .05$). The intracanal medication promoted important endotoxin neutralization, with a reduction of 99.2% to 100%. The root canal content induced a higher production of tumor necrosis factor-a and interleukin-1b in S1 samples compared with samples obtained after treatment. Conclusions: The combination of CLX and limewater as irrigant was the most effective in reducing endotoxins in root canals, and intracanal medication was important to neutralize the cytotoxic effects.

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Efficacy of Endodontic Treatment for Endotoxin Reduction in Primarily Infected Root Canals and Evaluation of Cytotoxic Effects

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Abstract

Introduction: Endotoxins are one of the etiologic agents involved in the pathogenesis of apical periodontitis. The objectives of this clinical study were to investigate the effects of endodontic treatment by using different irrigants on endotoxins in root canals with pulp necrosis and apical periodontitis and to evaluate the cytotoxic effects. **Methods:** Thirty-six root canals were selected. Samples were collected before (S1) and after instrumentation (S2). The root canals were divided into 3 groups (n = 12) according to the irrigant combination used: CLX + LW, 2% chlorhexidine gel + calcium hydroxide (0.14%, limewater); CLX + PmB, chlorhexidine + polymyxin B; CLX (control), chlorhexidine + saline. The third sampling (S3) was performed after ethylenediaminetetraacetic acid and S4 after intracanal medication (CLX + calcium hydroxide for 14 days). Endotoxins were quantified by the chromogenic *Limulus* amebocyte lysate assay, and cytotoxic effects were evaluated by the production of cytokines (interleukin-1β, tumor necrosis factor α) in macrophages (RAW 264.7) stimulated with the root canal content. **Results:** Endotoxins were detected in all root canals before instrumentation (S1). Group CLX + LW presented the greatest endotoxin reduction after instrumentation (99.18%), which was similar to group CLX + PmB (96.42%, $P > .05$) and different from group CLX (90.78%, $P < .05$). The intracanal medication promoted important endotoxin neutralization, with a reduction of 99.2% to 100%. The root canal content induced a higher production of tumor necrosis factor α and interleukin-1β in S1 samples compared with samples obtained after treatment. **Conclusions:** The combination of CLX and limewater as irrigant was the most effective in reducing endotoxins in root canals, and intracanal medication was important to neutralize the cytotoxic effects. *(J Endod 2012;38:1053–1057)*

Key Words

Calcium hydroxide, cytokines, endotoxin, macrophages, polymyxin B, root canal

Most pathologic alterations involving the pulp and periapical tissues present a microbial etiology, with bacteria and their products playing a significant role in the induction and maintenance of these lesions. Studies demonstrated a relationship between polymicrobial infection of root canals, especially infections caused by anaerobic gram-negative bacteria, and clinical signs and symptoms such as spontaneous pain, pain on palpation, edema, and purulent exudate (1–6).

Endotoxins consist of lipopolysaccharide (LPS) complexes that are part of the outer membrane of the cell wall of gram-negative bacteria. Endotoxins are the main virulence factor of these bacteria (7) and exert various biological effects that result in the amplification of inflammatory and immune responses (8). Studies suggest that endotoxins are involved in the pathogenesis of pulpal and periapical inflammation (6, 9–15). A positive correlation between endotoxin concentration in the root canal and the presence of endodontic signs and symptoms has been shown (16). Endotoxins have been detected in 100% of first samplings of root canals with necrotic pulp, with levels significantly higher in symptomatic teeth (17–21). Therefore, treatment of infected root canals should not only destroy bacteria but should also inactivate their endotoxins and other toxic products.

Sodium hypochlorite and chlorhexidine, which are used as auxiliary chemical solutions during biomechanical preparation, have shown an excellent antimicrobial activity, but little or no effect on LPS has been reported (12, 15, 17–19, 22). In an *in vitro* study (15), 2.5% and 5.25% sodium hypochlorite and 2% chlorhexidine were unable to inactivate LPS in root canals. However, the application of 0.14% calcium hydroxide (limewater) and polymyxin B as irrigation agents significantly reduced the level of endotoxins in root canals. It would therefore be interesting to evaluate *in vivo* the combined application of irrigants with antimicrobial properties such as chlorhexidine gel and substances able to inactivate endotoxins such as calcium hydroxide and polymyxin B in an attempt to enhance the benefits of endodontic therapy. In addition, it would be important to investigate the *in vivo* effect of the administration of intracanal medications (calcium hydroxide + 2% chlorhexidine gel) for 14 days on LPS. We hypothesized that these medications abolish the cytotoxic effects induced by endotoxins and other bacterial factors such as the production of different cytokines (interleukin [IL]-1β and tumor necrosis factor [TNF]-α) involved in the process of periapical bone resorption.

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The objectives of the present clinical study were to investigate the effects of biomechanical preparation by using different combinations of irrigation agents and intracanal medication on endotoxins in root canals with pulp necrosis and apical periodontitis and to evaluate the cytotoxic effects of the root canal content in macrophages.

Materials and Methods

Patient Selection

Thirty-six patients seen at the Endodontics Clinic of the São José dos Campos Dental School (UNESP), São José dos Campos, São Paulo, Brazil, participated in this study. The age of the patients ranged from 19–55 years. Thirty-six single-rooted teeth (superior incisors, superior and inferior canines, and inferior premolars) with pulp necrosis and radiographically visible apical periodontitis were selected. Patients who had received antibiotic therapy during the last 3 months and teeth with periodontal pockets larger than 4 mm were excluded. The project was approved by the Ethics Committee of the São José dos Campos Dental School, UNESP (protocol 096/2007-PH/CEP), and all patients signed a free informed consent form.

The presence of caries, spontaneous pain, pain on percussion or palpation, a history of pain, periodontal health (probing), and presence of a fistula, edema, and abscess were evaluated. In addition, the size of the apical periodontitis and the internal condition of the root canal (presence of a transparent, purulent, or hemorrhagic exudate, presence of pulp tissue remnants, or dry canal) were analyzed.

Experimental Groups

Files, instruments, and all materials used in this study were treated with Co^{60} gamma radiation (20 kGy for 6 hours) for sterilization and elimination of preexisting endotoxins (23) (EMBRARAD; Empresa Brasileira de Radiação, Cotia, SP, Brazil).

The teeth were isolated with a rubber dam, and the operative field was disinfected with 30% hydrogen peroxide for 30 seconds, followed by 2.5% sodium hypochlorite for an additional 30 seconds. The sodium hypochlorite solution was neutralized with 5% sodium thiosulfate (16, 17, 19–21, 24).

Access to the pulp cavity was made with sterile apyrogenic high-speed burs (KG Sorensen, Cotia, SP, Brazil) under irrigation with sterile apyrogenic saline solution. For collection of the first root canal sample, which was collected immediately after opening of the crown and before application of any antimicrobial solution (S1), the canals were filled with sterile apyrogenic saline for 1 minute. Care was taken to avoid overflow of the solution. The content was then aspirated with an insulin syringe and needle. This procedure was repeated until a final volume of 100 μL was obtained.

Next, the cervical and middle thirds of the root canals were prepared by the crown-down technique by using Endo-Eze oscillating files (Ultradent Products, South Jordan, UT), according to the manufacturer's instructions. The files of the Endo-Eze system were adapted to the Endo-Eze contra-angle in a handpiece (Kavo do Brasil, Ltda, Saguaçú Joinville, SC, Brazil). K-files size 15 or 20 (Dentsply-Maillefer, Ballaigues, Switzerland) were always used between each file of the oscillating system.

The lumen of the canal was identified by using a K-file size 10 (Dentsply-Maillefer). Next, cervical interferences were eliminated with the 13/.060 instrument of the Endo-Eze system by using the same principles of the crown-down pressureless technique. Instrumentation was continued by using an oscillating 13/.045 file, K-file (#15 or 20), oscillating 13/.035 file, K-file (#15 or 20), and oscillating 10/.025 file until reaching a depth that was 3 mm shorter than the full length of the root canal calculated from preoperative radiographs.

During preparation of the cervical and middle thirds, the root canal was filled with 2% chlorhexidine gel as adjunct, followed by irrigation and aspiration of 5 mL sterile apyrogenic saline after use of the oscillating instrument. This procedure was repeated at each file change. The working length (1 mm from the radiographic apex) was calculated on the basis of a radiograph after introducing a file into the root canal.

Apical preparation (0.5–1 mm beyond the radiographic apex) was performed with 4 K-files. The teeth were then divided into 3 groups (n = 12) according to the combination of adjunct substances used during apical root canal preparation (Table 1): CLX + LW, 2% chlorhexidine gel + apyrogenic saline, followed by limewater [0.14% $Ca(OH)_2$]; CLX + PmB, 2% chlorhexidine gel + apyrogenic saline, followed by polymyxin B; CLX, 2% chlorhexidine gel + apyrogenic saline.

In group CLX + LW, 2% chlorhexidine gel was applied during the first 2 files used for apical preparation, followed by irrigation with 5 mL apyrogenic saline. Next, 5 mL limewater [0.14% $Ca(OH)_2$] was used as irrigation agent for the last 2 files to neutralize possible endotoxins after the death of gram-negative bacteria. After the last file, the canals were filled with limewater, which was mixed for 3 minutes (#25 K-file) before final irrigation with apyrogenic saline.

In group CLX + PmB, 2% chlorhexidine gel was applied during the first 2 files used for apical preparation, followed by irrigation with 5 mL apyrogenic saline. Next, polymyxin B + saline were used as irrigation agent for the last 2 files to neutralize possible endotoxins after the death of gram-negative bacteria. After application of the last file, the canals were filled with polymyxin B, which was mixed for 3 minutes (#25 K-file) before final irrigation with apyrogenic saline.

In group CLX (control), 2% chlorhexidine gel was applied during the 4 files used for apical preparation, followed by irrigation with 5 mL apyrogenic saline at each file change.

At the end of biomechanical preparation, all root canals were irrigated with 5 mL sterile apyrogenic saline, and a second sample was collected (S2). All root canals were flooded with 17% ethylenediaminetetraacetic acid (EDTA), which was mixed with a file for 3 minutes and then removed with 5 mL sterile apyrogenic saline. A third root canal sample was then obtained (S3).

The canals were dried with absorbent (sterile and apyrogenic) paper points and filled with intracanal medication consisting of a paste of 2% chlorhexidine gel and calcium hydroxide pro-analysis (equal volumes) (Table 1). The medication was introduced into the root canals with a lentulo spiral run at low speed, and a provisional restoration of glass ionomer cement was placed. Fourteen days after use of the intracanal medication, the surgical field was isolated and disinfected,

TABLE 1. Groups Treated with Different Combinations of Adjunct Substances during Apical Root Canal Preparation

Groups (n = 12)	Adjunct substances	Intracanal medication
CLX + LW	2% chlorhexidine gel + apyrogenic saline, followed by limewater [0.14% $Ca(OH)_2$]	Paste of 2% chlorhexidine gel and $Ca(OH)_2$ pro-analysis
CLX + PmB	2% chlorhexidine gel + apyrogenic saline, followed by polymyxin B	Paste of 2% chlorhexidine gel and $Ca(OH)_2$ pro-analysis
CLX	2% chlorhexidine gel + apyrogenic saline	Paste of 2% chlorhexidine gel and $Ca(OH)_2$ pro-analysis

TABLE 2. Levels of Endotoxins (mean ± standard deviation, EU/mL) in the 4 Root Canal Samplings Obtained from Groups CLX + LW, CLX + PmB, and CLX

Group	S1	S2	S3	S4
CLX + LW	1,157 ± 1,471	9.53 ± 7.44	14.37 ± 10.45	3.17 ± 4.55
CLX + PmB	6,955 ± 14,021	14.04 ± 11.18	14.26 ± 10.49	2.52 ± 4.59
CLX	569 ± 677	40.8 ± 52.7	29.72 ± 25.76	1.55 ± 2.03

and the provisional restoration was removed. Next, the root canals were irrigated with 10 mL apyrogenic saline. The solution was aspirated, and the root canal content was again collected (S4).

For completion of endodontic treatment, all root canals were filled with gutta-percha points and AH+ cement (Dentsply) by using the active lateral condensation technique. The teeth were restored, and the cases will be followed during a period of 2 years for evaluation of treatment success.

Collection of Root Canal Content

Four root canal samplings were performed: S1, immediately after coronal opening; S2, after biomechanical preparation; S3, after EDTA application; and S4, after removal of the intracanal medication. All samples were submitted to the quantification of endotoxins and the evaluation of cytotoxic effects in macrophage cultures (RAW 264.7).

Quantification of Endotoxins (LPS) by the Kinetic Chromogenic *Limulus* Amebocyte Lysate Assay

The kinetic chromogenic *Limulus* amebocyte lysate (LAL) (Lonza, Walkersville, MD) assay was used for the quantification of endotoxins. *Escherichia coli* endotoxin was used as standard. A positive control (root canal sample contaminated with a known amount of endotoxin) was included for each sample to determine the presence or absence of interfering agents. For the test, 100 μL of apyrogenic water (reaction blank), 5 standard endotoxin solutions (0.005–50 EU/mL), the root canal samples, and the positive controls were added to a 96-well apyrogenic plate. The tests were carried out in quadruplicate. The plate was incubated at 37°C ± 1°C for 10 minutes in the Kinetic-QCL reader, which was coupled to a microcomputer with the WinKQCL software. Next, 100 μL of the chromogenic reagent was added to each well. After the beginning of the kinetic test, the software continuously monitors absorbance at 405 nm in each microplate well and automatically calculates the log/log linear correlation between the reaction time of each standard and the corresponding endotoxin concentration.

The results were analyzed by Friedman analysis of variance and Dunn test for the evaluation of the percent reduction of endotoxins within the same group. Comparisons between experimental groups were done by the Kruskal-Wallis and Dunn tests. The level of significance was set at 5% for all tests.

Evaluation of the Cytotoxic Effects of Root Canal Content by the Production of Cytokines (IL-1β and TNF-α) in Macrophage Cultures

A mouse macrophage line (RAW 264.7) obtained from the cell bank of Associação Técnico Científica Paul Ehrlich (APABCAM, Rio de Janeiro, Brazil) was maintained in Dulbecco modified Eagle medium (DMEM) (LGCBio, São Paulo, Brazil) enriched with 10% fetal bovine serum (FBS) (Invitrogen Brasil Ltda, São Paulo, SP, Brazil). The culture medium was changed every 2 days, and the cells were maintained at 37°C in a 5% CO_2 atmosphere.

For the tests, a cell suspension was prepared by mechanical removal of the cells with a cell scraper (Corning Costar, Cambridge, MA). Next, the culture medium containing the cells was transferred to tubes and centrifuged at 9000 rpm for 5 minutes at 25°C. The supernatant was discarded, and the pellet was resuspended in DMEM + 10% FBS. Cell viability was evaluated by trypan blue exclusion, and viable cells were counted in a Neubauer chamber. Each well of a 24-well polystyrene plate (Corning Costar) was inoculated with 1 × 10^5 viable cells, and DMEM + 10% FBS was added until a final volume of 500 μL. These cells were activated with the samples collected from the root canals and incubated at 37°C in a 5% CO_2 atmosphere. After 24 hours, the supernatants were collected, and cytokines (IL-1β and TNF-α) were quantified by an enzyme-linked immunosorbent assay. The DuoSet anti–IL-1β or anti–TNF-α kit (R&D Systems, Minneapolis, MN) was used according to manufacturer's instructions. After determination of optical densities, the levels of IL-1β (pg/mL) and TNF-α (pg/mL) in the macrophage culture supernatants were calculated by using the GraphPad Prism 5.0 (La Jolla, CA) program. The results were analyzed statistically by analysis of variance and the Tukey test, adopting a level of significance of 5%.

Results

Quantification of Endotoxins (LAL Assay)

Endotoxins were detected in 100% of S1 samples (baseline) (Table 2). A significant reduction of endotoxin levels was observed at S2 in all groups (CLX + LW, 99.18%; CLX + PmB, 96.42%; CLX, 90.78%) (Table 3). This reduction was significantly higher in group CLX + LW (instrumentation with chlorhexidine gel + limewater) than in group CLX (chlorhexidine gel + saline solution) ($P < .05$) and was similar compared with group CLX + PmB (chlorhexidine gel + polymyxin B) ($P > .05$). No significant difference in the reduction of endotoxin levels was observed for CLX + PmB when compared with groups CLX + LW and CLX ($P > .05$) (Table 3). Group CLX presented the lowest reduction in endotoxin levels.

A slight increase of endotoxin levels was observed after the application of EDTA (S3) in groups CLX + LW and CLX + PmB and a small reduction in group CLX (Table 3). However, the difference was not statistically significant ($P > .05$). A significant reduction of endotoxin levels was observed after the intracanal medication (chlorhexidine + calcium hydroxide paste) (S4) in all groups (CLX + LW, 100%; CLX + PmB, 100%; CLX, 99.2%), with no significant difference between groups ($P > .05$) (Table 3).

Evaluation of Cytotoxic Effects

Production of IL-1β. Mean IL-1β production was higher at S1 compared with the other samplings, with no significant difference between the experimental groups ($P > .05$) (Table 4). The root canal content collected after biomechanical preparation (S2) promoted low production of IL-1β in all groups, with no significant difference between

TABLE 3. Percent Reduction or Increase of Endotoxin Levels between Root Canal Samplings Obtained from Groups CLX + LW, CLX + PmB, and CLX

Sampling	CLX + LW	CLX + PmB	CLX
S1 to S2	−99.18%^{A}	−96.42%^{AB}	−90.78%^{B}
S2 to S3	+52.3%^{C}	+16.1%^{C}	−13.1%^{C}
S3 to S4	−100%^{AD}	−100%^{AD}	−99.20%^{BD}

Different superscript letters ^{A,B,C,D} indicate statistically significant differences ($P < .05$).

TABLE 4. Levels of IL-1β (mean $\pm$ standard deviation, pg/mL) in Macrophage Culture Supernatants After Activation with the Root Canal Content

Sampling	CLX + LW	CLX + PmB	CLX
S1	50.34 $\pm$ 71.69^{A}	86.02 $\pm$ 130.51^{A}	43.28 $\pm$ 92.94^{A}
S2	4.26 $\pm$ 5.74^{B}	3.41 $\pm$ 6.93^{B}	5.07 $\pm$ 7.43^{B}
S3	3.14 $\pm$ 3.71^{B}	3.81 $\pm$ 7.37^{B}	3.17 $\pm$ 4.84^{B}
S4	2.36 $\pm$ 3.52^{B}	1.91 $\pm$ 6.62^{B}	1.33 $\pm$ 3.87^{B}

Different superscript letters ^{A,B} indicate statistically significant differences ($P < .05$).

groups ($P > .05$). No statistical differences in IL-1β production were observed after EDTA application (S3). Root canal content collected after the intracanal medication (S4) promoted little or no IL-1β production, with no significant difference between groups ($P > .05$) (Table 4).

Production of TNF-α. The root canal content collected at S1 promoted high production of TNF-α in practically all cases, but no significant difference was observed between groups ($P > .05$) (Table 5). A lower mean production of TNF-α was observed after biomechanical preparation (S2) compared with S1. However, TNF-α levels continued to be high in all groups, and there was no significant difference between the experimental groups ($P > .05$). TNF-α production after EDTA application (S3) was similar to S2, with high production in all groups and no significant difference between groups ($P > .05$). A reduction of TNF-α production was observed after the intracanal medication (S4) in many cases when compared with the previous samplings, but production continued to be relatively high in various samples. There was no significant difference between the 3 groups ($P > .05$) (Table 5).

Discussion

Quantification of endotoxins revealed their presence in all root canals with necrotic pulps, in agreement with the literature (16–20, 25). The endotoxin concentration observed in the first sampling (S1) ranged from 29.2–42,400 EU/mL. These results agree in part with those reported by other authors (17, 18), who obtained endotoxin concentrations ranging from 10–200 EU/mL (mean, 151.61 EU/mL) and from 17–696 EU/mL (mean, 228 EU/mL), respectively. Similar levels were observed in most of the patients studied here. However, some samples presented much higher levels, in agreement with Jacinto et al (16), who found endotoxin concentrations ranging from 2,390–22,100 EU/mL in necrotic root canals. These differences between results might be attributed to the sensitivity of the test that detects minimum variations in endotoxin levels, the method of sampling of root canal content, time of sampling, and anatomical diversity of root canals, among others.

With respect to clinical data, only 8 of the 36 patients reported painful symptoms. Six of these patients had high endotoxin levels in the first sampling (S1), in agreement with studies showing a correlation between clinical signs and symptoms and higher endotoxin levels (16, 18, 21, 25). However, endotoxin levels were not elevated in the other 2 patients reporting pain. Regarding the size of the lesions, 2 patients with extensive lesions (>10 mm) accompanied by an exudate presented the highest levels of endotoxins in the first sampling (42,400 and 29,200 EU/mL). However, no elevated endotoxin levels were observed in 3 other patients with lesions measuring $\geq$10 mm. In the remaining patients, the small variation in the extent of apical periodontitis and endotoxin concentration did not permit any correlation of the data.

A significant reduction in endotoxin levels compared with the first sampling (S1) was observed after biomechanical preparation (S2), irrespective of the irrigation agent used. This reduction was more than 90% in all cases. These results agree with other authors (17–19) who suggested that chemomechanical preparation reduces the levels of endotoxins in root canals. However, these authors reported mean reductions of 44.4%–47.12% or 57.98%–59.99% when 2% chlorhexidine or 2.5% NaOCl was used, respectively. In the present study, the mean reduction was 90.78% when 2% chlorhexidine was used as adjunct. These divergent results might be explained by the different techniques used for root canal preparation, including the size of the instruments used for apical preparation and the volume of irrigants. In the present study, 4 files of the Endo-Eze system were used for preparation of the cervical and middle thirds and 4 K-files for preparation of the apical third. Thus, part of the reduction in endotoxin levels seems to be due to the process of instrumentation itself, irrigation and aspiration of root canal content, irrespective of the irrigant used.

In the present study, a significantly higher reduction in endotoxin levels was observed when limewater (0.14% calcium hydroxide) was used in combination with 2% chlorhexidine gel during biomechanical preparation of the root canals. The use of limewater for the last 2 files during apical preparation promoted a significantly higher neutralization of endotoxins (mean of 99.18%) than the use of 2% chlorhexidine gel alone throughout preparation (90.78%). In group CLX + LW, no endotoxins were detected in 3 root canals after instrumentation. These results indicate that the use of limewater during the final steps of root canal instrumentation is feasible and has benefits for endodontic therapy, neutralizing most endotoxins after the death of gram-negative bacteria. Further studies combining limewater with different irrigants during biomechanical preparation are needed to evaluate whether this combination increases the success of endodontic treatment. All cases of this study will be followed up for a minimum period of 2 years to evaluate clinical success or failure. In group CLX + PmB, the use of polymyxin B for the last 2 files during apical preparation also resulted in an important reduction of endotoxins in root canals (mean of 96.42%), demonstrating that final application of polymyxin B is able to neutralize a large quantity of endotoxins. However, the difference was not significant when compared with the CLX group.

No endotoxins (levels <0.005 EU/mL) were detected in 20 root canals after the intracanal medication (chlorhexidine gel + calcium hydroxide) for 14 days, corresponding to a reduction of 99.20% and 100%. These results disagree with those of Vianna et al (17), who found that *in vivo* administration of intracanal medication (calcium hydroxide paste, chlorhexidine gel, or chlorhexidine gel + calcium hydroxide paste) for 7 days only promoted a mean endotoxin reduction in root canals of 1.4%. According to these authors, the use of intracanal medications for 7 days does not bring any improvement. The longer

TABLE 5. Levels of TNF-α (mean $\pm$ standard deviation, pg/mL) in Macrophage Culture Supernatants after Activation with the Root Canal Content

Sampling	CLX + LW	CLX + PmB	CLX
S1	1132.26 $\pm$ 782.27^{A}	1115.37 $\pm$ 538.36^{A}	1046.02 $\pm$ 580.79^{A}
S2	628.57 $\pm$ 426.30^{B}	518.64 $\pm$ 420.05^{B}	791.75 $\pm$ 403.23^{B}
S3	674.26 $\pm$ 310.48^{B}	532.58 $\pm$ 279.00^{B}	694.67 $\pm$ 237.07^{B}
S4	345.40 $\pm$ 261.53^{C}	291.61 $\pm$ 240.31^{C}	323.94 $\pm$ 273.50^{C}

Different superscript letters ^{A,B,C} indicate statistically significant differences ($P < .05$).

time of application (14 days) in the present study might have promoted a greater action of the intracanal medication deep within the dentinal tubules. The present *in vivo* results confirm previous *in vitro* studies in which application of the intracanal medication (chlorhexidine gel + calcium hydroxide) for 14 days neutralized most of the endotoxins that remained in the root canals after biomechanical preparation (22, 26). The biological properties of LPS require the presence of ester-linked hydroxy fatty acids, and calcium hydroxide cleaves these bonds (27). Studies demonstrated the ability of calcium hydroxide to neutralize endotoxins in root canals when used as intracanal medication (10–12, 22, 26, 28). In this respect, the present results showed that calcium hydroxide did not lose its LPS-neutralizing capacity in the presence of chlorhexidine gel, and that this combination had positive effects on the elimination of endotoxins from root canals, supporting its importance as an intracanal medication.

Analysis of the cytotoxic effects of root canal content showed that samples collected before treatment (S1) promoted a higher mean production of TNF-α and IL-1β than content collected after treatment. Martinho et al (24) showed a correlation between the number of gram-negative bacteria and the levels of IL-1β and TNF-α ($P < .05$). Elevated levels of endotoxin were followed by the secretion of TNF-α, and higher levels of IL-1β and endotoxin were related to a larger size of the radiolucent area (24). These authors concluded that the antigenicity of the endodontic content is not only related to the concentration of endotoxin found in the root canal but also to the number of different species of gram-negative bacteria involved in the infection. In the present study, production of IL-1β was lower than that of TNF-α in all cases, with almost undetectable levels in many samples after treatment (S2, S3, and S4). TNF-α production was relatively high in all cases after biomechanical preparation and EDTA application (S2 and S3), demonstrating the cytotoxic effect of these samples. Mean TNF-α production was lower in samples after the intracanal medication. However, elevated levels were observed in some cases, demonstrating that the production of TNF-α was more sensitive to the presence of several bacterial factors such as endotoxin (LPS), lipoteichoic acid, and peptidoglycan than IL-1β in the cells studied. The results showed no significant difference in the production of IL-1β or TNF-α between the substances used for biomechanical preparation (CLX + LW, CLX + PmB, and CLX), and the intracanal medication was found to promote a lower production of these cytokines.

In conclusion, the present study demonstrated that the combination of 2% chlorhexidine gel and calcium hydroxide 0.14% (limewater) as irrigant was the most effective in reducing endotoxins in root canals, and the use of intracanal medication (2% chlorhexidine gel + calcium hydroxide) for 14 days was important to neutralize the cytotoxic effects.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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3.5 *In vitro* antimicrobial activity of auxiliary chemical substances and natural extracts on *Candida albicans* and *Enterococcus faecalis* in root canals*

ABSTRACT

Objective: The aim of this study was to evaluate the antimicrobial activity of auxiliary chemical substances and natural extracts on *Candida albicans* and *Enterococcus faecalis* inoculated in root canals. Material and Methods: Seventy-two human tooth roots were contaminated with *C. albicans* and *E. faecalis* for 21 days. The groups were divided according to the auxiliary chemical substance into: G1) 2.5% sodium hypochlorite (NaOCl), G2) 2% chlorhexidine gel (CHX), G3) castor oil, G4) glycolic *Aloe vera* extract, G5) glycolic ginger extract, and G6) sterile saline (control). The samples of the root canal were collected at different intervals: confirmation collection, at 21 days after contamination; 1st collection, after instrumentation; and 2nd collection, seven days after instrumentation. Microbiological samples were grown in culture medium and incubated at 37°C for 48 hours. Results: The results were submitted to the Kruskal-Wallis and Dunn (5%) statistical tests. NaOCl and CHX completely eliminated the microorganisms of the root canals. Castor oil and ginger significantly reduced the number of CFU of the tested bacteria. Reduction of CFU/mL at the 1st and 2nd collections for groups G1, G2, G3 and G4 was greater in comparison to groups G5 and G6. Conclusion: It was concluded that 2.5% sodium hypochlorite and 2% chlorhexidine gel were more effective in eliminating *C. albicans* and *E. faecalis*, followed by the castor oil and glycolic ginger extract. The *Aloe vera* extract showed no antimicrobial activity.

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In vitro antimicrobial activity of auxiliary chemical substances and natural extracts on *Candida albicans* and *Enterococcus faecalis* in root canals

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ABSTRACT

Objective: The aim of this study was to evaluate the antimicrobial activity of auxiliary chemical substances and natural extracts on *Candida albicans* and *Enterococcus faecalis* inoculated in root canals. Material and Methods: Seventy-two human tooth roots were contaminated with *C. albicans* and *E. faecalis* for 21 days. The groups were divided according to the auxiliary chemical substance into: G1) 2.5% sodium hypochlorite (NaOCl), G2) 2% chlorhexidine gel (CHX), G3) castor oil, G4) glycolic *Aloe vera* extract, G5) glycolic ginger extract, and G6) sterile saline (control). The samples of the root canal were collected at different intervals: confirmation collection, at 21 days after contamination; 1st collection, after instrumentation; and 2nd collection, seven days after instrumentation. Microbiological samples were grown in culture medium and incubated at 37°C for 48 hours. Results: The results were submitted to the Kruskal-Wallis and Dunn (5%) statistical tests. NaOCl and CHX completely eliminated the microorganisms of the root canals. Castor oil and ginger significantly reduced the number of CFU of the tested bacteria. Reduction of CFU/mL at the 1st and 2nd collections for groups G1, G2, G3 and G4 was greater in comparison to groups G5 and G6. Conclusion: It was concluded that 2.5% sodium hypochlorite and 2% chlorhexidine gel were more effective in eliminating *C. albicans* and *E. faecalis*, followed by the castor oil and glycolic ginger extract. The *Aloe vera* extract showed no antimicrobial activity.

Key words: *Ricinus communis*. *Aloe vera*. *Zingiber officinale*. Sodium hypochlorite. Chlorhexidine.

INTRODUCTION

Micro-organisms activity and their metabolic products have been reported as being part of the etiology of pulp and periapical lesions, which can certainly lead to pulp necrosis and inflammatory reactions. Root canal infections can be caused by a combination of microorganisms[24]. *Enterococcus faecalis* has been frequently isolated from infected pulp and persistent infections in post-endodontic treatment[24]. This type of microorganism has the ability to penetrate into the dentinal tubules and survive in root canals without other bacterial support[16]. Its eradication depends on a high pH value environment (pH=11)[17].

A percentage of yeasts, mainly from the *Candida* genus, ranging from 6 to 55%, can be also found in necrotic pulps[20]. In addition, the presence of *Candida spp*. in refractory periapical granulomas is reported in studies with a polymerase chain reaction (PCR) analysis[21,31]. Yeasts have been particularly

associated with persistent root canal infections that did not respond favorably to conservative root canal therapy[28].

Some properties such as antimicrobial effect, biocompatibility, and ability of tissue dissolving activity are required for irrigating solutions in order to achieve a satisfactory level of cleaning. Sodium hypochlorite has long been recognized as presenting outstanding disinfection properties, and it has been widely used for root canal disinfection[19,28,30]. Amongst its positive properties, sodium hypochlorite is known to be highly irritant to periapical tissues when used at high concentrations[22].

Chlorhexidine, in liquid or gel formats, has great potential to be used as an endodontic auxiliary chemical substance during the biomechanical preparation. It has shown great efficacy against microorganisms found in root canals[7,8,11,29]. On the other hand, it does not present tissue dissolving activity. For that matter, alternative solutions have been proposed, aiming to associate antimicrobial efficacy, tissue dissolving action and biocompatibility[14,26,27].

More recently, there have been an increasing number of studies focused on the use of phytotherapic substances for medical purposes. It is known that plant extracts and several types of teas have been used in popular medicine since remote times. However, their real properties and applications have not been scientifically investigated yet. Several companies, groups and developed countries have shown an increasing interest towards the biodiversity of tropical and subtropical countries such as Brazil.

The aim of this study was to evaluate *in vitro* the antimicrobial activity of auxiliary chemical substances and natural extracts against *Candida albicans* and *Enterococcus faecalis* inoculated in root canals.

MATERIAL AND METHODS

The present study was approved by the Institutional Review Board from Univ. Estadual Paulista – UNESP, São José dos Campos, Brazil (approval n. 093/2005). A total of seventy-two freshly extracted human single-rooted teeth were used in this study. All samples were cleaned and stored in saline prior to use. The crown portion was removed and the length of instrumentation was standardized at 16±0.5 mm.

The root canals were initially over-instrumented to 0.5 mm beyond the apex by means of a #25 K-file (Dentsply Ind. Com. Ltda, Petrópolis, RJ, Brazil), and post-instrumented to 1 mm from the apex with a #30 K-file. The root canals were filled with 17% EDTA solution for 3 minutes and rinsed with 5 mL of saline solution. The apex was sealed using Z-100 composite resin (3M – Saint Paul, USA) and the roots were externally sealed with epoxy adhesive (Araldite, Brascola, São Paulo, SP, Brazil), except for the cervical opening. All samples were included in transparent light-cured acrylic resin (Dencor Artigos Odontológicos Clássico – São Paulo, SP, Brazil). The specimens were distributed on cell plates (24 wells) (Costar, Corning, New York, USA) and further sterilized by Cobalt-60 gamma radiation[6].

The microorganisms strains used were *Candida albicans* (ATCC 18804) and *Enterococcus faecalis* (ATCC 29212). Both microorganisms were seeded on Petri dishes containing Sabouraud Dextrose Agar (SDA) (Himedia Laboratories, Mumbai, India) for *C. albicans*, and Brain Heart Infusion (BHI) (Himedia Laboratories, Mumbai, India) for *E. faecalis*. The SDA dishes were incubated in a bacteriological oven at 37±1ºC for 24 hours, while the BHI dishes were incubated for a period of 48 hours.

Standardized saline solution suspensions of *C. albicans* and *E. faecalis* were prepared (10^8 cells/mL) by means of a spectrophotometric technique (λ=530 nm, DO=1.258, and λ=760 nm, DO=1.258, respectively). The root canals were contaminated with 10 µL of each microorganism suspension and 10 µL of BHI broth (Himedia Laboratories, Mumbai, India), resulting in 30 µL of inoculated medium in the root canals. A sterile cotton pellet embedded in BHI broth was placed at the entrance of the canals. The samples were stored in an incubator at 37±1ºC in a humid atmosphere for 21 days. During this period, a small amount of BHI broth was placed in the root canals every three days[19].

After the contamination period, samples of all specimens were collected to confirm the contamination of the root canals (confirmation collection). The samples were then divided into 6 experimental groups (n=12), according to the auxiliary chemical substances used.

Group 1 – 2.5% sodium hypochlorite solution (NaOCl) (Byofórmula – Farmácia de Manipulação, São José dos Campos, SP, Brazil);

Group 2 – 2% chlorhexidine gel (Byofórmula – Farmácia de Manipulação, São José dos Campos – SP) and irrigation with saline solution between files Exchange;

Group 3 – Castor oil extract (*Ricinus communis*) (Chemistry Institute of São Carlos – USP, São Carlos, SP, Brazil);

Group 4 – Glycolic ginger extract (*Zingiber officinale*)(Becker – Farmácia de Manipulação, São José dos Campos, SP, Brazil);

Group 5 – Glycolic *Aloe vera* extract (Synthon Especialidades Químicas Ltda.);

Group 6 – Sterile saline solution.

The root canals were biomechanically prepared up to a size #50 K-file and rinsed with 3 mL of irrigating solution after each file.

Microbiological samples were collected immediately post-instrumentation (1st collection) and seven days post-instrumentation (2nd collection).

Root specimens from group 1 were irrigated with 3 mL of 0.6% sodium thiosulphate, previous to the first collection, in order to neutralize the remaining NaOCl, while the root canals from group 2 were irrigated with 3 mL of 0.5% Tween 80+0.07% lecithin to neutralize the remaining chlorhexidine[19].

Each of the three sample collections (confirmation collection, 1st collection, and 2nd collection) was carried out at the same way. A number 30 sterile paper cone (confirmation collection) or a number 50 sterile paper cone (1st and 2nd collection) was placed and left in the root canal for one minute. The paper cone was placed in an Eppendorf test tube containing 0.5 mL sterile saline solution and stirred for 30 seconds. A 0.1 mL aliquot of each content was seeded and duplicated into dishes containing Agar Sabouraud for *C. albicans* and Agar Mitis Salivarius for *E. faecalis*.

Subsequent to the first collection, all the root canals were filled with sterile saline solution, and a sterile cotton pellet was placed at the entrance of the canals. The samples were stored in an incubator at 37±1°C with a humid atmosphere for 7 days prior to the second collection.

Characteristic grown colonies of *E. faecalis* and *C. albicans* were counted and confirmed by the Gram-color staining method. Descriptive statistics,

Table 1- Colony forming units *per* mL (CFU/mL) (log10) for confirmation, first, and second collections

Groups	Confirmation collection		1st collection		2nd collection	
	C. albicans	*E. faecalis*	*C. albicans*	*E. faecalis*	*C. albicans*	*E. faecalis*
G1 2.5% NaOCl	6.09	6.12	0	0	0	0
G2 2% CLX gel	6.17	6.01	0	0	0	0
G3 Castor oil	6.16	6.24	0.50	1.57	2.80	3.49
G4 Ginger	6.29	8.22	0	2.42	3.50	3.49
G5 *Aloe vera*	5.95	7.33	4.90	5.39	5.68	5.73
G6 Saline	6.08	6.17	4.85	5.03	5.53	5.46

Table 2- Percentage reduction values of *C. albicans* and *E. faecalis* CFU/mL, during the first and the second collections in relation to the confirmation collection. The homogeneous groups are also presented

Groups	Confirmation X 1st collection				Confirmation X 2nd collection			
	C. albicans		*E. faecalis*		*C. albicans*		*E. faecalis*	
	Median	HG*	Median	HG*	Median	HG*	Median	HG*
G1 NaOCl 2,5%	100	A	100	A	100	A	100	A
G2 2% CLX gel	100	A	100	A	100	A	100	A
G3 Castor oil	100	A	100	A	99.9	A	99.9	A
G4 Ginger	100	A	99.9	AB	98.8	A	99.9	A
G5 *Aloe vera*	98.5	B	98.66	BC	76.7	B	77	B
G6 Saline	95.1	B	93.21	C	74.8	B	82.6	B

*Homogeneous groups: different letters show statistical significant difference ($p<0.05$)

and the Kruskal-Wallis test and the Dunn's test (5%) were used to evaluate the results. The statistical analysis was based on the percentage of reduction.

RESULTS

The mean values of colony forming units *per* mL (CFU/mL) for each group were determined and are shown in Table 1.

The reduction or complete elimination of *C. albicans* and *E. faecalis* for the first and second collection compared to the confirmation collection, according to the Dunn's test (5%), is shown in Table 2.

DISCUSSION

The root canals within the present study were inoculated with *C. albicans* and *E. faecalis* for 21 days. The literature shows this contamination period is the reference for obtaining mature biofilms in the dentin[2,32]. Wang, et al.[32] (2012) evaluated the antibacterial effect of different disinfecting solutions on young and established *E. faecalis* biofilms in dentin canals using a novel dentin infection model. They verified that within the dentin canals, endodontic medications less easily kill bacteria in established biofilms than bacteria in young biofilms.

The results obtained in the present study showed a range of effects against the microorganisms tested, for both the 1st collection and 2nd collection.

The irrigation of the root canals with 2.5% NaOCl or 2% chlorhexidine gel during the instrumentation process, resulted in negative microbiological collections immediately post-instrumentation (1st collection) and also for the period of seven days post-instrumentation (2nd collection). It shows that those substances are capable of eliminating both *E. faecalis* and *C. albicans*. Valera, et al.[29,30] (2009, 2010) also assessed 1% NaOCl and 2% clorhexidine gel as significantly reducing the quantity of *C. albicans* and *E. faecalis* inoculated into the root canals.

It is evident that both 2.5% NaOCl solution and 2% chlorhexidine gel have antimicrobial activity and great capacity of penetration into the dentinal tubules, but the efficacy of NaOCl seven days post biomechanical preparation has not been established by other studies[19,28]. In the present study, the residual effect of those substances have not been evaluated, due to the neutralization process performed after the biomechanical preparation: roots irrigated with 2.5% NaOCl were neutralized by 3 mL of a 0.6% sodium thiosulphate solution; while roots irrigated with 2% chlorhexidine gel were neutralized by 3 mL of a 0.5% Tween 80+0.07% lecithin[19]. The neutralization process before the microbiological collection was required once some residues from the irrigating solutions might have remained and inhibited the growing process of microorganisms when using culture medium. However, even with the neutralization process it is possible to evaluate whether microorganisms remained in the tubules after the mechanical preparation and, in this study, the biomechanical preparation completely eliminated *C. albicans* and *E. faecalis*.

The efficacy of NaOCl as an irrigating solution has been reported by other studies[3,8,19,30] at different concentrations. Sodium hypochlorite has been capable of promoting biosynthetic cell alterations and phospholipids damage. These properties are related to the formation of chloramines, which interfere with cell metabolism leading to an oxidizing action. As a consequence, irreversible enzymatic inhibition of the sulphidrila present in bacterial enzymes and degradation of fatty acids and lipids are expected[8]. Gomes, et al.[11] (2001) verified a better performance of chlorhexidine on *E. faecalis* in comparison to 2.5% NaOCl.

The efficacy of chlorhexidine has been already reported in previous studies[8,11,19,29]. The antimicrobial activity of chlorhexidine is based on its positively charged molecule that interacts with negatively charged phosphate groups present on the bacterial cell wall, allowing the chlorhexidine molecule to penetrate the bacteria and leading to intracellular toxic effects[7-9]. This substance acts on Gram-positive and Gram-negative microorganisms. Due to its cationic properties, this biguanide is able to connect to hydroxyapatite, dental biofilm, oral mucosa and salivary proteins. When its concentration decreases in the oral environment, this substance is released from those structures. Such a characteristic is called substantivity, and promotes a durable effect to clorhexidine[8,9]. In concentrations ranging from 0.2 to 2%, chlorhexidine presents a wider antimicrobial spectrum, lower toxicity, better diffusion through the dentinal tubules, biocompatibility, and it has been more efficient in removing the smear layer compared to sodium hypochlorite, which makes this substance a good choice for endodontic therapy[7,8,25].

Castor oil detergent (*Ricinus communis*) has shown antimicrobial activity and biocompatibility, non-toxic results, detergent properties, which are important requirements for an irrigant solution[4,5,10,15,27]. The literature has reported that irrigation with castor oil extract is capable of removing debris, showing similar results to 1% NaOCl[18]. Valera, et al.[27] (2012) showed a significant decrease in the number of *Escherichia coli* in the root canals after irrigation with castor oil extract during the biomechanical preparation. These findings suggest that this substance could be utilized in endodontic therapy.

When analyzing the first collection data of the

present study, it could be verified that castor oil was able to completely eliminate *C. albicans* and it was also able to significantly reduce the amount of *E. faecalis*. On the other hand, in the second collection data, the development of two microorganisms (0.1%), especially *E. faecalis*, demonstrated that this solution is promising for endodontic purposes. However, more studies are required to clarify its antimicrobial activity mechanism.

The medical use of *Aloe vera* has been supported by its antimicrobial, anti-inflammatory and regenerative properties[12]. Gontijo, et al.[12] (2013) evaluated *in vivo* dentine-pulp behaviour of rats after direct pulp capping with *Aloe vera*. They observed the presence of acute inflammatory infiltrate (light to moderate) in the first day, while for the calcium hydroxide group (positive control), the presence of acute inflammatory infiltrate was severely related to superficial necrosis. Athiban, et al.[1] (2012) detected the *in vitro* antimicrobial activity of *Aloe vera* over *E. faecalis, E. coli* and *Staphylococcus aureus.* It was concluded *Aloe vera* could be effectively used for decontaminating GP points within a short application time. In the present study, *Aloe vera* did not show antimicrobial efficacy, once it was not able to eliminate all the microorganisms determined in the first sample collection. Moreover, it allowed a growth of microorganisms between the first and second data collection. According to the comparative Dunn's test (5%), the *Aloe vera* group showed similar results compared to the saline group.

The use of ginger (*Zingiber officinale*) has been appreciated since remote times, and it has been widely used in alcoholic drinks, seasonings and in popular medicine. It was verified that this extract was effective in eliminating microorganisms. The antimicrobial activity of the ginger extract on three Gram-negative anaerobes, *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia* was observed[23]. The effective action of glycolic and alcoholic ginger extract on *S. mutans*, *Staphylococcus aureus*, *E. coli* and *C. albicans*[13] were also reported. This fact might contribute to the treatment of some diseases caused by these types of microorganisms present in the oral cavity. The real mechanism of action of the ginger extract has not yet been elucidated in the literature.

In this study, the irrigation of the root canals with glycolic ginger extract resulted in the negative development of *C. albicans* for the first sample collection (immediately post instrumentation). Although a positive increase of *C. albicans* was observed in the second sample collection, no statistical differences were detected. This result suggests that microorganisms situated deeper in the dentinal tubules were not affected by the irrigating agent, and therefore they were able to recolonize the root canal lumen after seven days. Although *E. faecalis* was not completely eliminated in the first sample collection, the reduction was close to 100%. In the second sample collection, there was an increase in the number of microorganisms in comparison to the first collection, demonstrating no residual effect. In spite of the growth of microorganisms, the results obtained for the ginger group was statistically similar to 2.5% NaOCl and 2% chlorhexidine gel. The present observed antimicrobial effect of the ginger extract may be related to the very low concentration used. Further investigations on higher concentrations of these substances would be necessary to elucidate the action of the ginger over the microorganisms.

The saline solution (control) used in this experiment was the reference for evaluation of the antimicrobial action of the other substances. Due to its absence of antimicrobial effect, it was possible to assume that the physical action of the instrumentation leads to a considerable decrease in the amount of microorganisms in the root canals.

The results obtained in the present study show that phytotherapic substances might be used in the future as alternative irrigating solutions for endodontic treatment, since they are natural products and do not disturb the environment. As previously mentioned, it is important to support further investigations to identify the most suitable concentration of these substances and their effects over other types of microorganisms and their products.

CONCLUSION

According to the methodology used and the results obtained in this experiment, it could be concluded that: 2.5% NaOCl and 2% chlorhexidine gel were the most effective irrigating solutions against *C. albicans* and *E. faecalis*, for both the first and second sample collections. They were able to completely eliminate the microorganisms from the root canals. Castor oil extract and glycolic ginger extracts were able to significantly decrease the amount of microorganisms, not being able to completely eliminate them 7 days post biomechanical preparation. The *Aloe vera* natural extract, did not show antimicrobial efficacy with the methodology used.

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3.6 Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry*

ABSTRACT

Background: With the emergence of strains resistant to conventional antibiotics, it is important to carry studies using alternative methods to control these microorganisms causing important infections, such as the use of products of plant origin that has demonstrated effective antimicrobial activity besides biocompatibility. Therefore, this study aimed to evaluate the antimicrobial activity of plant extracts of *Equisetum arvense* L., *Glycyrrhiza glabra* L., *Punica granatum* L. and *Stryphnodendron barbatimam* Mart. against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Candida albicans, Candida tropicalis,* and *Candida glabrata,* and to analyze the cytotoxicity of these extracts in cultured murine macrophages (RAW 264.7). Methods: Antimicrobial activity of plant extracts was evaluated by microdilution method based on Clinical and Laboratory Standards Institute (CLSI), M7-A6 and M27-A2 standards. The cytotoxicity of concentrations that eliminated the microorganisms was evaluated by MTT colorimetric method and by quantification of proinflammatory cytokines (IL-1β and TNF-α) using ELISA. Results: In determining the minimum microbicidal concentration, *E. arvense* L., *P. granatum* L., and *S. barbatimam* Mart. extracts at a concentration of 50 mg/mL and *G. glabra* L. extract at a concentration of 100 mg/mL, were effective against all microorganisms tested. Regarding cell viability, values were 48% for *E. arvense* L., 76% for *P. granatum* L, 86% for *S. barbatimam* Mart. and 79% for *G. glabra* L. at the same concentrations. About cytokine production after stimulation with the most effective concentrations of the extracts, there was a significant increase of IL-1β in macrophage cultures treated with *S. barbatimam* Mart.

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(3.98 pg/mL) and *P. granatum* L. (7.72 pg/mL) compared to control (2.20 pg/mL) and a significant decrease of TNF-α was observed in cultures treated with *G. glabra* L. (4.92 pg/mL), *S. barbatimam* Mart. (0.85 pg/mL), *E. arvense* L. (0.83 pg/mL), and *P. granatum* L. (0.00 pg/mL) when compared to control (41.96 pg/mL). Conclusions: All plant extracts were effective against the microorganisms tested. The *G. glabra* L. extract exhibited least cytotoxicity and the *E. arvense* L. extract was the most cytotoxic.

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Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry

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Abstract

Background: With the emergence of strains resistant to conventional antibiotics, it is important to carry studies using alternative methods to control these microorganisms causing important infections, such as the use of products of plant origin that has demonstrated effective antimicrobial activity besides biocompatibility. Therefore, this study aimed to evaluate the antimicrobial activity of plant extracts of *Equisetum arvense* L., *Glycyrrhiza glabra* L., *Punica granatum* L. and *Stryphnodendron barbatimam* Mart. against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Candida albicans*, *Candida tropicalis*, and *Candida glabrata*, and to analyze the cytotoxicity of these extracts in cultured murine macrophages (RAW 264.7).

Methods: Antimicrobial activity of plant extracts was evaluated by microdilution method based on Clinical and Laboratory Standards Institute (CLSI), M7-A6 and M27-A2 standards. The cytotoxicity of concentrations that eliminated the microorganisms was evaluated by MTT colorimetric method and by quantification of proinflammatory cytokines (IL-1β and TNF-α) using ELISA.

Results: In determining the minimum microbicidal concentration, *E. arvense* L., *P. granatum* L., and *S. barbatimam* Mart. extracts at a concentration of 50 mg/mL and *G. glabra* L. extract at a concentration of 100 mg/mL, were effective against all microorganisms tested. Regarding cell viability, values were 48% for *E. arvense* L., 76% for *P. granatum* L, 86% for *S. barbatimam* Mart. and 79% for *G. glabra* L. at the same concentrations. About cytokine production after stimulation with the most effective concentrations of the extracts, there was a significant increase of IL-1β in macrophage cultures treated with *S. barbatimam* Mart. (3.98 pg/mL) and *P. granatum* L. (7.72 pg/mL) compared to control (2.20 pg/mL) and a significant decrease of TNF-α was observed in cultures treated with *G. glabra* L. (4.92 pg/mL), *S. barbatimam* Mart. (0.85 pg/mL), *E. arvense* L. (0.83 pg/mL), and *P. granatum* L. (0.00 pg/mL) when compared to control (41.96 pg/mL).

Conclusions: All plant extracts were effective against the microorganisms tested. The *G. glabra* L. extract exhibited least cytotoxicity and the *E. arvense* L. extract was the most cytotoxic.

Keywords: *Equisetum arvense* L, *Glycyrrhiza glabra* L, *Punica granatum* L, *Stryphnodendron barbatimam* Mart, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*

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Background

Herbal drugs have been used since ancient times as remedies for the treatment of a variety of diseases. Despite advances in modern medicine, plants still make an important contribution to health care [1]. Herbal extracts or essential oils prepared from medicinal plants contain different compounds with numerous biological activities confirmed by *in vitro* and *in vivo* studies, such as antibacterial, antifungal, antiviral, antiprotozoal, anthelminthic, antiseptic, anti-inflammatory, antitumor, antioxidative, antiallergic, anticonvulsant, antidepressant, contraceptive, antimutagenic, analgesic, and diuretic properties [2-4]. Some compounds are used for the treatment of diabetes, nervous system disorders such as Alzheimer's disease, dental diseases, male infertility, and erectile dysfunction [5], among other therapeutic properties.

Studies have demonstrated the antimicrobial activity of *Equisetum arvense* L. (Equisetaceae), *Glycyrrhiza glabra* L. (Fabaceae), *Punica granatum* L. (Punicaceae), and *Stryphnodendron barbatimam* Mart. (Leguminosae) [6-11]. However, knowledge of the toxicity of these extracts is scarce. Scientific knowledge of the antimicrobial properties of plant species may contribute to the development of drugs against microorganisms of medical interest, particularly in dentistry. In this respect, the environmental conditions of the oral cavity favor infection and inflammation caused by opportunistic microorganisms such as *Staphylococcus* spp., *Streptococcus* spp., and *Candida* spp.

Staphylococcus aureus can cause endodontic infections, parotid gland infections, mandibular osteomyelitis, mucositis, apical abscesses, and postoperative complications after dental implant placement [12-14]. Moreover, methicillin-resistant *S. aureus* (MRSA) have emerged as causative agents of serious infections in hospitals [15].

Staphylococcus epidermidis is an important agent of hospital-acquired infections and possesses pathogenic properties such as adhesion to implanted materials and biofilm formation [16]. Microorganisms organized in biofilms are more resistant to antimicrobial agents and components of host immunity [17,18].

Streptococcus mutans is an important etiological agent of dental caries in humans that attaches to the tooth surface and forms a biofilm [13,14]. For this purpose, the microorganism produces glucosyltransferases and synthesizes an adherent and water-insoluble glucan from sucrose that permits it to adhere firmly to the tooth surface [19]. The colonization of the oral cavity with *S. mutans* depends on factors such as salivary flow, saliva buffering capacity, and presence of salivary immunoglobulins [20].

Biofilm formation is an important virulence factor for *Candida* species because it increases resistance to antifungal therapy [21,22]. *C. albicans* is the most pathogenic species in humans and is commonly identified in dental prostheses and oral candidiasis. These yeasts colonize the oral mucosa, tongue and palate. Oral, vaginal or systemic candidiasis is a common infection in immunocompromised patients that can be fatal [13,14,21,23,24].

Alternative microbiological control measures that are suitable for use in humans are of the utmost importance in view of the emergence of antimicrobial-resistant strains. Therefore, the present study evaluated the effectiveness of herbal extracts of *E. arvense* L., *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart. against clinical strains of *Staphylococcus* spp., *S. mutans* and *Candida* spp. In addition, the cytotoxic effects of the extract were investigated based on cell viability and cytokine production (interleukin 1β [IL-1β] and tumor necrosis factor α [TNF-α]) in cultured mouse macrophages (RAW 264.7).

Methods

The study was approved by the Ethics Committee of the São José dos Campos Dental School, UNESP (protocol 008/2012-PA/CEP).

Antimicrobial activity

Dry powders of *E. arvense* L., *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart. were purchased from the Oficina de Ervas (Ribeirão Preto, São Paulo, Brazil) and the extracts were prepared in propylene glycol (200 mg/mL).

Nine clinical strains and reference strains (ATCC) of *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), *S. mutans* (ATCC 35688), *C. albicans* (ATCC 18804), *C. tropicalis* (ATCC 13803) and *C. glabrata* (ATCC 90030) obtained from the Laboratory of Microbiology and Immunology, Institute of Science and Technology, were used. The clinical strains were identified after isolation from the oral cavity of patients with pulmonary tuberculosis. A total of 60 strains were tested.

Bacteria and yeast were cultured in brain-heart infusion (BHI – Himedia, Mumbai, Maharashtra, India) and Sabouraud-dextrose broth (Himedia), respectively, for 24 h at 37°C. *S. mutans* was incubated under microaerophilic conditions (5% CO_2). The microbial suspensions were prepared in sterile saline (0.9% NaCl) at a standard concentration of 5×10^5 cells/mL for bacteria and 1×10^3 to 5×10^3 cells/mL for yeast.

The microdilution method was performed according to NCCLS guidelines [25,26], changes name to Clinical and Laboratory Standards Institute (CLSI). For this purpose, 10 dilutions of the plant extracts (100 to 0.19 mg/mL) were prepared in Mueller-Hinton medium (Himedia) for bacteria and in RPMI 1640 medium (Himedia) plus MOPS buffer (Sigma Aldrich, St. Louis, Missouri, USA), pH 7.0 ± 0.1, for yeast. Next, 5 μL of the bacterial suspension or 100 μL of the yeast suspension was inoculated into each well of 96-well plates (TPP, Trasadingen, Schaffhausen,

Switzerland) and the plates were incubated for 24 h at 37°C (under microaerophilic conditions for *S. mutans*).

After determination of the Minimum Inhibitory Concentration (MIC), 100 μL of this concentration and one above and one below this concentration were seeded onto BHI or Sabouraud-dextrose agar plates to determine the Minimum Microbicidal Concentration (MMC) of the extracts. After 48 h of incubation, the MMC was determined on plates that showed no growth of colonies. The results are reported as the percentage of strains inhibited at each concentration of the plant extract.

Cell culture

Mouse macrophages (RAW 264.7) obtained from the Rio de Janeiro Cell Bank - Associação Técnico Científica Paul Ehrlich (APABCAM - Rio de Janeiro, RJ, Brazil) were cultured routinely in Dulbecco's Modified Eagle Medium (DMEM - LGC Biotechnology, Cotia, SP, Brazil) supplemented with 10% fetal bovine serum (FBS – Gibco, USA) at 37°C in a 5% CO_2 atmosphere. Viable cells were counted by the Trypan blue (0.5%, Sigma-Aldrich, St. Louis, MO, USA) exclusion method.

Cytotoxicity testing (MTT assay)

For the experimental and positive control groups, 8×10^3 viable cells/well were seeded in 96-well plates (Nunc, Kamstrupvej, Roskilde, Denmark) and the plates were incubated for 24 h at 37°C. Next, the cell cultures were exposed to 200 μL of the serial dilutions of the plant extracts according to the concentrations obtained in the microbiological test and incubated for 24 h. At the end of exposure, the cell culture medium was discarded and cell survival was determined by the MTT assay [MTT - (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich].

The plates were washed with phosphate-buffered saline (PBS - Cultilab, Campinas, São Paulo, Brazil) and 100 μL MTT solution (0.5 mg/mL in PBS) was added to each well. After incubation for 1 h, MTT solution was removed and 100 μL dimethyl sulfoxide (DMSO - Sigma-Aldrich) was added to each well. The plates were incubated for 10 min and then shaken on a shaker for an additional 10 min. Optical densities were measured in a multi-well spectrophotometer (Bio-Tek, Winooski, Vermont, USA) at 570 nm. The optical density values obtained for cultures exposed to the extracts were normalized to untreated control cultures (corresponding to 100%).

The results of the cytotoxicity tests were analyzed by ANOVA and the Tukey test ($P \leq 0.05$) using the BioEstat 5.0 software.

Quantification of IL-1β and TNF-α

For analysis of cytokine production, 1×10^6 RAW264.7 cells were seeded in 24-well plates and incubated for 24 h at 37°C. Next, the cell cultures were exposed to concentrations of the plant extracts that were the most effective in the microbiological test. After incubation for 24 h, the supernatant was collected from each well and frozen at −18°C.

The levels of IL-1β and TNF-α in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using the DuoSet ELISA Development System for IL-1β (DY401) and TNF-α (DY410) ((R&D Systems, Minneapolis, Minnesota, USA) according to manufacturer instructions. Optical densities were read on a microplate spectrophotometer at 450 nm and converted into IL-1β and TNF-α concentrations (pg/mL) using the GraphPad Prism 5.0 program.

The results were analyzed by ANOVA and the Tukey test ($P \leq 0.05$) using the BioEstat 5.0 software.

Results

The antimicrobial activity of the plant extracts is shown in Table 1. The *E. arvense* L. extract, at a concentration of 50 mg/mL, eliminated 100% of *S. aureus*, *S. epidermidis*, *C. albicans*, *C. tropicalis*, and *C. glabrata* strains. At a concentration of 25 mg/mL, this extract eliminated 100% of *S. mutans.* The MMC of the *G. glabra* L. extract was 100 mg/mL for *S. aureus*, *S. epidermidis* and *S. mutans*, and 50 mg/mL for yeast (*C. albicans*, *C. tropicalis*, and *C. glabrata*). The *P. granatum* L. extract presented an MMC of 25 mg/mL for *S. aureus* and *S. epidermidis*, 12.5 mg/mL for *S. mutans*, and 50 mg/mL for yeast. The widest variation in MMC was observed for the *S. barbatimam* Mart. extract, with an MMC of 3.13 mg/mL for *S. mutans*, 12.5 mg/mL for *S. epidermidis*, 25 mg/mL for *S. aureus*, and 50 mg/mL for yeasts.

Cytotoxicity of the plant extracts was evaluated in cultured macrophages by the MTT assay. The highest cell survival rates were observed for cultures treated with *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart. The group treated with the *E. arvense* L. extract exhibited the lowest survival rate (<50%) (Table 2).

Table 1 Values of the MMC (mg/mL) of plant extracts for all microorganisms evaluated

Microorganism*	Plant extract			
	E. arvense L.	*G. glabra* L.	*P. granatum* L.	*S. barbatimam* Mart.
S. aureus	50	100	25	25
S. epidermidis	50	100	25	12.5
S. mutans	25	100	12.5	3.13
C. albicans	50	50	50	50
C. tropicalis	50	50	50	50
C. glabrata	50	50	50	50

*Ten strains of each microbial species were evaluated.

Table 2 Percentage of cell viability (± SD) after treatment with the MMC of plant extracts

Concentration (mg/mL)	Plant extract			
	E. arvense L.	*G. glabra* L.	*P. granatum* L.	*S. barbatimam* Mart.
100	-	79 ± 6.18^{B}	-	-
50	48 ± 5.29^{B}	52 ± 2.96^{C}	76 ± 3.10^{B}	86 ± 4.05^{B}
25	45 ± 1.08^{B}	-	57 ± 5.24^{C}	70 ± 4.96^{C}
12,5	-	-	57 ± 3.70^{C}	65 ± 4.91^{C}
3,13	-	-	-	72 ± 6.27^{C}
Control	100^{A}	100^{A}	100^{A}	100^{A}

Different superscript letters indicate statistically significant differences (P < 0.05).

Analysis of cytokine production showed a significant increase of IL-1β production in cultures treated with the *P. granatum* L. and *S. barbatimam* Mart. extracts when compared to the control group. No difference in IL-1β production was observed between cultures treated with the *E. arvense* L. and *G. glabra* L. extracts and the control group (Table 3).

All extracts significantly reduced the production of TNF-α when compared to the control group, with complete inhibition of TNF-α production in the *P. granatum* L.-treated group (Table 3).

Discussion

The present study evaluated the antimicrobial activity of plant extracts not only against reference strains (ATCC), but also against clinical isolates since differences in antimicrobial susceptibility exist between the same species isolated from different patients as demonstrated by the present results. The four extracts exerted microbicidal activity against six microbial species. However, the MMC varied according to the extract and its concentration used and according to bacterial or fungal species studied.

The *S. mutans* isolates showed high sensitivity to the *E. arvense* L. extract, with a concentration of 25 mg/mL inhibiting the growth of all strains. At a concentration of 50 mg/mL, *E. arvense* L. was effective against all *Staphylococcus* spp. and *Candida* spp. isolates. The antimicrobial activity of *E. arvense* L. can be attributed to the presence of various compounds such as the phenolic monoterpene thymol, capable inhibit the growth of microorganisms [8,27]. It was reported that concentrations of *E. arvense* L. hydromethanolic extracts presented a dose-dependent activity in *S. aureus*, at 10–1000 mg/mL, showing a total elimination at 1000 mg/mL [28]. According to Radulovic et al. [27] the essential oil of *E. arvense* L. also showed activity against *S. aureus* and *C. albicans* strains, in agreement with our study, yet it was effective against Gram-negative bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Escherichia coli*, and against *Aspergillus niger*. In vitro tests also demonstrated the inhibitory effect of *E. arvense* L. hydroethanolic extract against *Neisseria gonorrhoeae* [29].

Table 3 Levels of IL-1β and TNF-α (mean ± standard deviation, pg/mL) in macrophage culture supernatants after exposure to plant extracts (100 mg/mL for *G. glabra* L. and 50 mg/ml for *E. arvense* L., *P. granatum* L. and *S. barbatimam* Mart.)

Plant extract	Cytokine production (pg/mL)	
	IL-1β	TNF-α
E. arvense L.	1.32 ± 1.37^{A}	0.83 ± 2.86^{B}
G. glabra L.	1.99 ± 1.46^{A}	4.92 ± 1.46^{B}
P. granatum L.	7.72 ± 1.13^{C}	0.00^{B}
S. barbatimam Mart.	3.98 ± 1.40^{B}	0.85 ± 2.93^{B}
Control	2.20 ± 2.11^{A}	41.96 ± 10.53^{A}

Different superscript letters indicate statistically significant differences (P < 0.05).

The *G. glabra* L. extract inhibited the growth of all bacterial strains studied at a concentration of 100 mg/mL. In contrast, fungal growth was inhibited at a concentration of less than 50 mg/mL, demonstrating that *Candida* spp. were more sensitive to the extract than the bacterial strains. Antifungal activity of this plant was attributed to the presence of glabridin, hispaglabridin B [30] and 18β-glycyrrhetinic acid [9]. Glabridin exhibiting inhibitory activity against *C. albicans* strain and inhibited the growth of this yeast mutant strain resistant to amphotericin B, nystatin and clotrimazole [30]. This compound also showed inhibitory effect on the growth of *S. aureus* strains sensitive and resistant to methicillin, showing MIC at 12.5 mg/mL [31]. The 18β-glycyrrhetinic acid caused inhibitory effect for *C. albicans* at concentrations of 50 and 100 mg/mL [9]. Clinical and reference strains of this yeast was also proven their susceptibility to the extract of *G. glabra* L. prepared in different solvents as ethanol, ethyl acetate, and hexane [32]. According to our study, Hwang et al. [33] also found the antibacterial extract *G. glabra* L regard to *S. mutans*. Statti et al. [34] collected *G. glabra* L. from different regions of Calabria, Italy, and verified an inhibitory effect on bacteria and fungi, and this biological activity varied due to differences in the chemical composition of this plant obtained from different sites.

The *P. granatum* L. extract completely inhibited the growth of *S. mutans*, *Staphylococcus* spp. and *Candida* spp. at concentrations of 12.5, 25 and 50 mg/mL, respectively. Thus, *S. mutans* showed high sensitivity to the extract, whereas *Candida* spp. exhibited low sensitivity to *P. granatum* L., requiring higher concentrations for growth inhibition. Rosas-Piñón et al. [35] studying the medicinal plants from a Mexico region used by local people to treat dental diseases such as toothache, dental caries, periodontal disease and gingivitis, found the antimicrobial activity of 47 plant species on *Porphyromonas gingivalis* and *S. mutans*. According to our study, the authors

verified that *S. mutans* was more sensitive to *P. granatum* L extract, compared to *P. gingivalis*, showing lower inhibitory concentrations of growth. Thus, it is believed that this higher sensitivity of streptococcal is related to its cell composition. Products based on *P. granatum* L., as commercial sauces and juices also showed antimicrobial activity against natural microbiota of vegetables such as lettuce, chives and parsley, as well as on *S. aureus* and *E. coli* that was inoculated on these plants [36]. Our results are in agreement with Abdollahzadeh et al. [37], which verified that *P. granatum* L. extract was effective on oral opportunistic pathogens such as *S. aureus*, *S. epidermidis*, *S. mutans* and *C. albicans*. Also, Fawole et al. [38] showed that *P. granatum* L. extract was effective on *S. aureus*, and this extract also presented antimicrobial activity against *K. pneumoniae* and *E. coli*. Some studies have also been proven the effectiveness of *P. granatum* L. against MRSA, *Listeria monocytogenes*, *Bacillus subtilis*, *S. enteritidis* and *Yersinia enterocolitica* [39]. The bactericidal activity of this plant is due to the presence of tannins and punicalagins [40,41]. A paste based on the extract of *P. granatum* L. [42] was produced and applied on wounds created in guinea pigs and their potential healing was confirmed with total regression of the wound in 20 days, and also effectively control the microbiota of the wound. In vitro tests have demonstrated the antimicrobial activity of this extract on *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *Salmonella anatum*, *S. typhimurium*, *S. pneumoniae*, *C. albicans*, *C. glabrata*, and *A. rubrum Trichopyton niger*. Vasconcelos et al. [7] also used a gel based on *P. granatum* L. extract to control *S. mutans*, *S. sanguis*, *S. mitis* and *C. albicans*, alone or associated with each other, and it has shown inhibition of adhesion of these microorganisms on the glass surface. By inhibiting the adherence of *S. mutans* other species also have their adhesion impaired, since this streptococci fail to provide the support to anchor these pathogens; e.g., *C. albicans* on the tooth surface [43].

The *S. barbatimam* Mart. extract exerted microbicidal activity at different concentrations, which were 3.13 mg/mL for *S. mutans*, 12.5 mg/mL for *S. aureus*, and 25 mg/mL for S. *epidermidis*. However, only the concentration of 50 mg/mL was effective against the *Candida* species tested. The results of our study demonstrate that *S. mutans* was the most sensitive specie to *S. barbatimam* Mart. extract, and *C. albicans* was less sensitive, agreeing with the study by Pereira et al. [44], which the extract of *Stryphnodendron* spp. also had lower and higher MMC against *S. mutans* and *C. albicans*, respectively. The extract of *Stryphnodendro*n spp. produced in aqueous and ethyl-acetate fractions showed significant antifungal activity against clinical isolates of *C. albicans*, *C. parapsilosis* and *C. tropicalis*, and revealed that the presence of tannins affected the integrity of the cell wall of *Candida*, a factor that contributes to decreased adhesion to host cells and germ-tube formation, besides affecting the budding process and stimulate phagocytosis [6].

The present study also analyzed the cytotoxicity of the *E. arvense* L., *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart extracts in macrophage cultures (RAW 264.7) using the most effective concentrations obtained in the microbiological test. Except for *P. granatum* L., there are no studies in the literature investigating the cytotoxicity of these plants by the methods used here. After exposure for 24 h to *G. glabra* L., at concentrations of 100 and 50 mg/mL, reduced cell survival by 79% (± 6.18) and 52% (± 2.96), respectively. The viability of cells treated with *P. granatum* L. at a concentration of 50 mg/mL was 76% (± 3.10), whereas the percentage of surviving cells was 57% (± 5.24) and 57% (± 3.70) at concentrations of 25 and 12.5 mg/mL, respectively. The percentage of surviving cells was 86% (± 4.05), 70% (± 4.96), 65% (± 4.91), and 72% (± 6.27) for macrophages treated with *S. barbatimam* Mart. at concentrations of 50, 25, 12.5 and 3.13 mg/mL, respectively. Cell viability was less than 50% only in the groups treated with *E. arvense* L., i.e., 48% (± 5.29) at 50 mg/mL and 45% (± 1.08) at 25 mg/mL. It has been demonstrated reduction in cell viability after 24 h exposure to antimicrobials used in endodontic therapy such as ciprofloxacin hydrochloride, clindamycin hydrochloride, and metronidazole, with these drugs, showing dose-dependent cytotoxicity [45]. In the present study, cell viability was significantly higher in the groups treated with higher concentrations of *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart. when compared to the groups treated with lower concentrations.

The cytotoxicity of the plant extracts was also investigated by quantifying the production of IL-1β and TNF-α in cell cultures (RAW 264.7) after 24-h exposure to concentrations of the extracts that were most effective against the microorganisms tested. No significant difference in the production of IL-1β was observed between cultures treated with *E. arvense* L. (1.32 ± 1.37 pg/mL) and *G. glabra* L. (1.99 ± 1.46 pg/mL) and the control group (2.20 ± 2.11 pg/mL). In contrast, production of this cytokine was significantly higher in cultures treated with *S. barbatimam* Mart. (3.98 ± 1.40 pg/mL) and *P. granatum* L. (7.72 ± 1.13 pg/mL). The production of TNF-α was significantly lower in cultures treated with *E. arvense* L. (0.83 ± 2.86 pg/mL), *G. glabra* L. (4.92 ± 1.46 pg/mL) and *S. barbatimam* Mart. (0.85 ± 2.93 pg/mL) when compared to the group not stimulated with the extracts (41.96 ± 10.53 pg/mL). The *P. granatum* L. extract completely inhibited the synthesis of TNF-α. Thus, it was observed that the basal levels of TNF-α were higher than those of IL-1β. This behavior also was shown in other studies [46,47].

The study of the cytotoxicity and antimicrobial activity of natural products such as plant extracts is important for the clinical application of these compounds in different areas of health care. For example, in dentistry,

mouthwashes, root canal irrigants, intracanal medications and even toothpastes with antimicrobial activity could be developed that are safe for use in humans. However, animal studies and subsequent clinical trials are needed to confirm the safety of these products.

Conclusion

In conclusion, the *E. arvense* L., *G. glabra* L. *P. granatum* L. and *S. barbatimam* Mart. extracts exerted microbicidal activity against all *Staphylococcus* spp., *S. mutans* and *Candida* spp. strains tested. The *G. glabra* extract exhibited least cytotoxicity and the *E. arvense* extract was the most cytotoxic.

Abbreviations

°C: Degrees celsius; ANOVA: Analysis of variance; ATCC: American Type Culture Collection; BHI: Brain-heart infusion; CLSI: Clinical and Laboratory Standards Institute; CO_2: Carbon dioxide; DMEM: Dulbecco's Modified Eagle Medium; DMSO: Dimethyl sulfoxide; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; h: Hour(s); IL-1β: Interleukin 1-beta; mg/mL: Milligram per milliliter; MIC: Minimum Inhibitory Concentration; MMC: Minimum Microbicidal Concentration; MOPS: 3-(N-morpholino) propanesulfonic acid; MRSA: Methicillin-resistant *S. aureus*; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaCl: Sodium chloride; NCCLS: National Committee for Clinical Laboratory Standards; nm: Nanometers; PBS: Phosphate-buffered saline; pg/mL: Picograms per milliliter; RAW 264.7: Cell line of mouse macrophages; RPMI: Medium developed by Moore et al. in Roswell Park Memorial Institute; TNF-α: Tumor necrosis factor alpha.

Competing interests

No conflict of interests for this study with any person or institution.

Authors' contributions

JRO: conception, design of the experiments, interpretation of data, redaction of the manuscript and attainment of the tables. VCC: design of the experiments. PGFV: conception, design of the experiments. SEAC: redaction and review of the manuscript. CATC: conception and redaction of the manuscript. AOCJ: conception, interpretation of data and redaction of the manuscript. LDO: conception, interpretation of data, critical analysis and redaction of the manuscript. All authors read and approved the final manuscript.

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3.7 Control of microorganisms of oral health interest with *Arctium lappa* L. (burdock) extract non-cytotoxic to cell culture of macrophages (RAW 264.7)*

ABSTRACT

Objectives: To evaluate the antimicrobial activity of *Arctium lappa* L. extract on *Staphylococcus aureus, S. epidermidis, Streptococcus mutans, Candida albicans, C. tropicalis* and *C. glabrata*. In addition, the cytotoxicity of this extract was analyzed on macrophages (RAW 264.7). Design: By broth microdilution method, different concentrations of the extract (250–0.4 mg/ mL) were used in order to determine the minimum microbicidal concentration (MMC) in planktonic cultures and the most effective concentration was used on biofilms on discs made of acrylic resin. The cytotoxicity *A. lappa* L. extract MMC was evaluated on RAW 264.7 by MTT assay and the quantification of IL-1b and TNF-a by ELISA. Results: The most effective concentration was 250 mg/mL and also promoted significant reduction ($\log_{10}$) in the biofilms of *S. aureus* (0.438 ± 0.269), *S. epidermidis* (0.377 ± 0.298), *S. mutans* (0.244 ± 0.161) and *C. albicans* (0.746 ± 0.209). Cell viability was similar to 100%. The production of IL-1b was similar to the control group ($p > 0.05$) and there was inhibition of TNF-a ($p < 0.01$). Conclusions: *A. lappa* L. extract was microbicidal for all the evaluated strains in planktonic cultures, microbiostatic for biofilms and not cytotoxic to the macrophages.

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Control of microorganisms of oral health interest with *Arctium lappa* L. (burdock) extract non-cytotoxic to cell culture of macrophages (RAW 264.7)

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ABSTRACT

Objectives: To evaluate the antimicrobial activity of *Arctium lappa* L. extract on *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus mutans*, *Candida albicans*, *C. tropicalis* and *C. glabrata*. In addition, the cytotoxicity of this extract was analyzed on macrophages (RAW 264.7).

Design: By broth microdilution method, different concentrations of the extract (250–0.4 mg/mL) were used in order to determine the minimum microbicidal concentration (MMC) in planktonic cultures and the most effective concentration was used on biofilms on discs made of acrylic resin. The cytotoxicity *A. lappa* L. extract MMC was evaluated on RAW 264.7 by MTT assay and the quantification of IL-1β and TNF-α by ELISA.

Results: The most effective concentration was 250 mg/mL and also promoted significant reduction ($\log_{10}$) in the biofilms of *S. aureus* (0.438 ± 0.269), *S. epidermidis* (0.377 ± 0.298), *S. mutans* (0.244 ± 0.161) and *C. albicans* (0.746 ± 0.209). Cell viability was similar to 100%. The production of IL-1β was similar to the control group ($p > 0.05$) and there was inhibition of TNF-α ($p < 0.01$).

Conclusions: *A. lappa* L. extract was microbicidal for all the evaluated strains in planktonic cultures, microbiostatic for biofilms and not cytotoxic to the macrophages.

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1. Introduction

Arctium lappa L. belongs to the *Asteraceae* family and its root is commonly consumed as food in many parts of Asia and it is used in folk medicine for the treatment of infectious diseases. Studies have demonstrated that in addition to the antimicrobial activity, this plant has also anti-inflammatory, antiallergic, antioxidant and antiviral activity and also acts as a hepatoprotective agent.[1–4] The extract of *A. lappa* L. has displayed potent antimicrobial activity against microorganisms that cause endodontic infections.[5,6] Thus, it is important to expand the studies analyzing its cytotoxic potential and antimicrobial action (planktonic and biofilm) on different microorganisms (bacteria and yeasts) involved in infections of the oral cavity as *Staphylococcus* spp., *S. mutans* and *Candida* spp., to expand its possible therapeutic indication. The expanded study of this extract may facilitate future dental applications, not only endodontic (dressing and irrigating solutions), as well as toothpastes, mouthwashes and ointments.

The dental biofilm is an ecosystem comprised of many species of microorganisms, about 750 different species, involved in a polysaccharide extracellular matrix, and all this microbial mass is adhered to solid surfaces as the tooth tissue, for instance. The composition and organization of the biofilm are strongly influenced by the source and availability of nutrients, as well as by the pH of the ambient.[7,8] The biofilm growth and the development of infections such as caries and periodontal diseases are related to the intra and inter-specific communication of the microorganisms.[8–10] The dental biofilm is, probably, the most studied natural biofilm.[11] Among the staphylococci species isolated in the oral cavity, 24–36% are *S. aureus*. These microorganisms are considered as belonging to the transient microbiota. They are important agents of endodontic infections, mandibular osteomyelitis, apical abscesses and mucositis.[12–14] *S. epidermidis* exhibits a high capacity of adherence to implanted materials and, consequently, they are responsible for infections and the development of its biofilm is an important pathogenic factor.[15] *S. mutans* is an important causative agent of caries, acting mainly in the form of biofilm.[13,14] *C. albicans* is the most common fungal pathogen that causes infections in humans, causing invasive infections in patients with immunologic abnormalities and severe infections related to implanted materials which may be contaminated with yeast, especially in the form of biofilm.[13,14,16,17]

There are many studies that have been conducted in order to investigate biological activities of medicinal plants such as antimicrobial, antiviral, antiparasitic, anti-inflammatory, antitumor, antiallergic, among others activities. Thus, it is remarkable the search for alternative methods of treatment and control of diseases which should be effective in curing without causing side effects to patients, or if such effects occur, they are minimized. Thus, the purpose of this study was to evaluate the antimicrobial effect of *A. lappa* L. glycolic extract on bacteria and yeasts isolated from the oral cavity as *S. aureus*, *S. epidermidis*, *S. mutans*, *C. albicans*, *C. tropicalis* and *C. glabrata* in planktonic form, as well as its effect on biofilms of *S. aureus*, *S. epidermidis*, *S. mutans* and *C. albicans*. We also evaluated the cytotoxicity of the most effective concentration of the extract on these microorganisms in regard to the cell viability and the production of cytokines IL-1β and TNF-α in macrophages (RAW 264.7).

2. Materials and methods

This study was approved by the Ethics Committee of the Institute of Science and Technology – UNESP, protocol 008/2010-PA/CEP.

A total of 60 microbial strains were used, nine clinical samples and standard strains (ATCC) of *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), *S. mutans* (ATCC 35688), *C. albicans* (ATCC 18804), *C. tropicalis* (ATCC 13803) and *C. glabrata* (ATCC 90030), from the Laboratory of Microbiology and Immunology, Institute of Science and Technology, UNESP.

A. lappa L. glycolic extract of was purchased commercially (Byofórmula, São José dos Campos, SP, Brazil) in the concentration of 500 mg/mL prepared in propylene glycol.

2.1. Antimicrobial activity in planktonic cultures

In order to determine the minimum inhibitory concentration (MIC), it was used broth microdilution method, according to NCCLS (2002, 2003).[18,19] Initially bacterial strains were seeded in Brain Heart Infusion agar (BHI – Himedia, Mumbai, Maharashtra, India) and incubated for 24 h at 37 °C with CO_2 at 5% for strains of *S. mutans*. Then, the inoculums were standardized in sterile saline (0.9% NaCl) in the concentration of 5×10^5 cells/mL. The fungal strains were subcultured on Sabouraud-dextrose (Himedia) for 24 h at 37 °C. In order to standardize the inoculums, it was prepared a standard solution containing 1×10^6 cells/mL with spectrophotometer (Micronal B-582, São Paulo, SP, Brazil). Thereafter, this solution was diluted 1:50, followed by a 1:20 dilution to obtain a suspension of approximately 5×10^2 to 2.5×10^3 cells/mL.

Ten serial 1:2 dilutions were made from the extract into a 96-well plate (from 250 to 0.4 mg/mL) from 100 μL of extract in 100 μL of culture medium Mueller–Hinton (Himedia) for bacteria and RPMI 1640 (Himedia) buffered with MOPS (Sigma–Aldrich, St. Louis, MO, USA) at pH 7.0 ± 0.1, for the yeast. Subsequently, 5 μL of the bacterial inoculum or 100 μL of the standardized suspension of yeast were added to each well of 96-well plate. A well for positive control (medium with inoculum) and another well for negative control (medium alone) were included. The plates were incubated for 24 h at 37 °C in microaerophilic conditions for *S. mutans*. The MIC was determined in the well of lowest concentration, in which turbidity was not observed. Aiming to find the CMM, 100 μL of CIM, a concentration above and one below were seeded on BHI or Sabouraud-dextrose plates and incubated at 37 °C for 48 h, in microaerophilic conditions for *S. mutans*.

The antimicrobial activity of propylene glycol was also tested in this study using the broth microdilution test in the same concentrations used for the evaluation of the extract.

2.2. Antimicrobial activity in biofilm

Standard strains of *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), *S. mutans* (ATCC 35688) and *C. albicans* (ATCC 18804)

were used. Inocula were standardized in sterile saline (0.9% NaCl) at the concentration of 1×10^6 cells/mL with spectrophotometer. In each well of a 24-well plate, it was included 2 mL of BHI broth supplemented with 5% sucrose, a disc of acrylic resin (Classical, Sao Paulo, SP, Brazil) sterilized for biofilm growth and 100 μL of the standardized suspension. Ten discs were used for the control group and 10 for each test group. The plates were taken to incubation for 5 days at 37 °C with 5% CO_2 for *S. mutans*. After incubation the discs underwent three washes in sterile saline (0.9% NaCl), under stir, in order to discard the cells that had not adhered to the biofilm. The treated group got in contact for 5 min with the most effective concentration in the test planktonic assay (250 mg/mL) and after passing through the last washing, each disc was placed separately in tubes containing sterile saline solution (NaCl 0.9%). The groups were taken to ultrasonic homogenizer (Sonoplus HD 2200, 50 W, Bandelin Electronic, Heinrichstraße, Berlin, Germany) for 30 s with approximately 25% power to disaggregate the biofilm from the discs. There were four decimal dilutions of inoculum suspensions, which were seeded in duplicate (100 mL of each dilution) in BHI or Sabouraud-dextrose plates. These plates were incubated for 48 h at 37 °C in microaerophilic conditions for *S. mutans*. Then, colony forming units per millilitre (CFU/mL) were counted and the results were converted to $\log_{10}$.[20]

2.3. Cell culture

It was used macrophages cell line RAW 264.7, derived from the Rio de Janeiro Cell Bank – Associação Técnico Científica Paul Ehrlich (APABCAM – Rio de Janeiro, RJ, Brazil) cultured in Dulbecco modified Eagle medium (DMEM – LGC Biotechnology, Cotia, SP, Brazil) supplemented with 10% foetal bovine serum (FBS – Invitrogen, Grand Island, NY, USA) and maintained under a humidified atmosphere at 37 °C, 5% CO_2. Viable cell counts were made by the method of exclusion with Trypan blue (0.5%, Sigma–Aldrich).

2.4. Cytotoxicity testing (MTT assay)

In 96-well plate (Nunc, Kamstrupvej, Roskilde, Denmark), it was added 200 μL of DMEM + 10% FBS containing 8×10^3 viable cells per well. After incubation at 37 °C, 5% CO_2 for 24 h for adhesion of the cells, the supernatant was discarded and it was added *A. lappa* L. extract of diluted in DMEM + 10% FBS in the microbicide concentrations, previously determined during the planktonic microbiological test. For growth control, it was used culture medium with cell suspension and, for negative control, it was used culture medium without cells. After 24 h exposure, cell viability was measured by MTT assay. After incubation, the supernatant was discarded, the wells were washed with sterile phosphate buffer saline (PBS – Cultilab, Brazil) and it was added to the solution of MTT [3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium – Sigma Aldrich], in the concentration of 0.5 mg/mL. It was performed 12 repetitions for each group. After incubation for 1 h at 37 °C, the solution was removed and it was added 100 μL of dimethyl sulfoxide (DMSO – Sigma–Aldrich). The plates returned to incubator for 10 min protected from light and were subsequently brought to the shaker for another 10 min. The absorbance of the wells was read under the wavelength of 570 nm in a microplate spectrophotometer (Bio-Tek, Winooski, VT, USA). The values of optical densities (OD) were converted into percentage of cell viability, compared with the control group.

2.5. Quantification of IL-1β and TNF-α

Macrophages were cultured in 24-well plate at the concentration of 10^6 viable cells per 1 mL of DMEM + 10% FBS for 24 h at 37 °C (5% CO_2) for cell adherence. For this analysis, the cells were exposed to the more effective extract concentration for all microorganisms evaluated. Thus, in the treated group, it was added 1 mL of extract at the concentration of 250 mg/mL, diluted in DMEM + 10% FBS. Cells of the control group had contact only with the culture medium. All plates were inoculated at 37 °C (5% CO_2) and after 24 h, supernatants were collected. It was performed 12 repetitions for each group.

The production of IL-1β and TNF-α was evaluated by enzyme linked immune sorbent assay (ELISA) using commercial kits (R & D Systems, Minneapolis, MN, USA), and the quantification of IL-1β (DY401) and TNF-α (DY410) was done according to the manufacturer.

After this, the optical densities (OD) were read in the microplate spectrophotometer at the wavelength of 450 nm. Values were converted into concentration of cytokines (pg/mL) according to the standard curve for IL-1β or TNF-α, with the assistance of GraphPad Prism 5.0.

2.6. Statistical analysis

The data obtained from the antimicrobial activity in biofilms and cytotoxicity tests were represented as mean values and standard deviation, statistically analyzed by ANOVA and complemented by Tukey test, with significance level of 5% ($p \leq 0.05$), through the statistical programme BioEstat 5.0.

3. Results

In planktonic cultures, *A. lappa* L. extract at the concentration of 250 mg/mL eliminated all microbial strains. At the concentration of 125 mg/mL of the extract, it was also shown total effectiveness against strains of *C. albicans* and *C. glabrata* (Table 1). Regarding propylene glycol, it was found that none of the tested concentrations showed antimicrobial activity.

The utilization of 250 mg/mL of the *A. lappa* L. extract provided significant reduction in the number of CFU/mL ($\log_{10}$) in microbial biofilms. On *S. aureus*, the extract reduced 0.438 ± 0.269 $\log_{10}$, equivalent to 7% (±4), on *S. epidermidis* the reduction was 0.377 ± 0.298 $\log_{10}$ (9%), on *S. mutans*, 0.244 ± 0.161 $\log_{10}$ ($3 \pm 2\%$), and on *C. albicans* the reduction was 0.746 ± 0.209 $\log_{10}$ ($14 \pm 4\%$) (Figs. 1 and 2). Significantly, *C. albicans* biofilm showed greater reduction rate compared to the bacterial strains of *S. aureus* ($p < 0.05$), *S. epidermidis* ($p < 0.01$) and *S. mutans* ($p < 0.01$) that had similar reductions with each other ($p > 0.05$) (Fig. 2).

Cell viability (%) of the *A. lappa* L. extract concentrations showed average values similar to the control group (100%) for the groups treated with 250 mg/mL (97.83 ± 6.95%), 125 mg/mL

Table 1 – Number of microbial strains eliminated in different concentrations of *A. lappa* L. extract (500 mg/mL) and determination of the effective MMC (mg/mL) for 100% of the strains for each microorganism evaluated, nine strains were clinical and one standard (ATCC).

Microorganisms	Concentration (mg/mL)									
	250	125	62.5	31.3	15.6	7.8	3.9	1.9	0.9	0.4
S. aureus	10[a]	2	1	–	–	–	–	–	–	–
S. epidermidis	10[a]	1	–	–	–	–	–	–	–	–
S. mutans	10[a]	–	–	–	–	–	–	–	–	–
C. albicans	10	10	9	5[a]	–	–	–	–	–	–
C. tropicalis	10[a]	8	4	1	–	–	–	–	–	–
C. glabrata	10	10	7[a]	2	–	–	–	–	–	–

[a] Effective MMC for standard strain (ATCC); –, ineffective concentration.

(95 ± 6.12%) and 62.5 mg/mL (94.17 ± 5.44%). However, the group treated with 31.3 mg/mL of the extract was the only one that showed significant reduction in cell viability to 93.58% (±4.34%) (Fig. 3).

Regarding the production of cytokines, the group treated with *A. lappa* L. extract (250 mg/mL) produced IL-1β (1.97 ± 3.84 pg/mL) similarly to the control group (5.70 ± 6.40 pg/mL) ($p > 0.05$), however, the extract significantly inhibited the production of TNF-α (2.38 ± 1.15 pg/mL), since, in the control group there was production of 31.55 ± 13.82 pg/mL this cytokine, as shown in Fig. 4.

4. Discussion

The *A. lappa* L. extract showed antimicrobial activity in both, planktonic and biofilm, cultures. Researchers have shown that its components arctiin and arctigenin have been responsible for some of its biological activities,[21] including possible antimicrobial activity, and antiviral activity on influenza virus is already proven.[4]

In this study, the highest CMM obtained in planktonic cultures (250 mg/mL), when tested on biofilms, afforded significant reduction of CFU/mL in all groups (Figs. 1 and 2), however, there was not complete elimination of the microorganisms. Thus, it was verified that this extract has significant antimicrobial activity but it is necessary to use higher concentrations than the one used in CMM on the planktonic cultures, in order to remove microorganisms under biofilm form. These results agree with several studies that reported the concentration of an antimicrobial agent should be higher for microorganisms adhered when compared with those which are in planktonic cultures,[22] and that biofilms may be ten to a thousand times more resistant to an antimicrobial agent and also to the host immunity components when compared to planktonic cultures.[23]

Besides the standard strains (ATCC) clinical strains were also used in this study in order to verify the behaviour of both in relation to susceptibility to *A. lappa* L. extract, since there may be differences between microorganisms of the same species, but isolated from different patients. This behaviour was demonstrated in this study, as Table 1 shows.

Among the studied microorganisms, the yeasts have demonstrated higher susceptibility to the natural extract. All the strains of *C. albicans* and *C. glabrata* were eliminated with the concentration of 125 mg/mL, 90% of *C. albicans* and 70% of *C. glabrata* were eliminated with the concentration of 62.5 mg/mL. Regarding the bacteria and *C. tropicalis*, there was total elimination only at the first dilution (250 mg/mL). *S. mutans* had the most resistant strains, once all of them were eliminated only for the highest dilution (Table 1). These results agree with Holetz et al.,[24] whom found that *A. lappa* L. extract also had antimicrobial activity on *S. aureus*, *C. albicans*, *C. tropicalis* and other microorganisms. However, in that study,

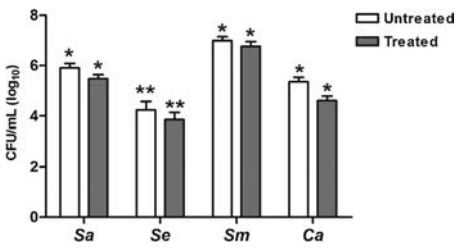

Fig. 1 – Number of CFU/mL (log_{10}) obtained in the untreated group (control – 0.9% NaCl) and treated group with 250 mg/mL of *A. lappa* L. extract for 5 min (*n* = 10) on ATTC strains of *S. aureus* (*Sa*), *S. epidermidis* (*Se*), *S. mutans* (*Sm*) and *C. albicans* (*Ca*). ANOVA and Tukey test ($p \leq 0.05$). *$p < 0.01$; **$p < 0.05$.

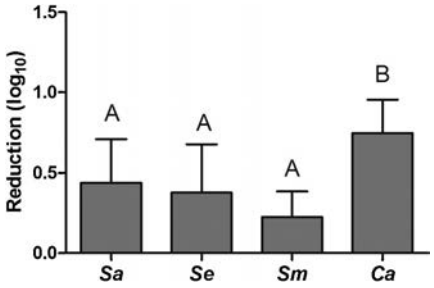

Fig. 2 – Reduction (log_{10}) of the biofilms of *S. aureus* (*Sa*), *S. epidermidis* (*Se*), *S. mutans* (*Sm*) and *C. albicans* (*Ca*) after treatment with 250 mg/mL of *A. lappa* L. extract for 5 min (*n* = 10). ANOVA and Tukey test ($p \leq 0.05$). Different letters indicate statistically significant differences.

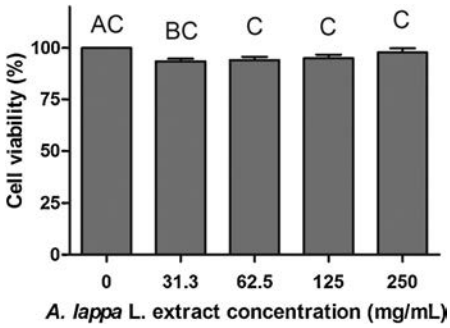

Fig. 3 – Cell viability (%) presented for macrophages untreated (0 mg/mL) and treated for 24 h with *A. lappa* L. extract concentrations (31.3, 62.5, 125 and 250 mg/mL) predetermined during the antimicrobial evaluation in planktonic cultures ($n = 10$). ANOVA and Tukey test ($p \leq 0.05$). Different letters indicate statistically significant differences.

the extract was more effective for bacterial strains unlike our results, where the fungal strains showed increased susceptibility in comparison with the bacteria. Santos et al.[25] found that *Streptococcus* spp. was not sensitive to *A. lappa* L. extract and this extract was effective only against *C. guilliermondii*. On the other hand, in a study using agar diffusion method, *A. lappa* L. extract showed antimicrobial activity against microorganisms of endodontic interest, acting positively on bacteria like *S. aureus*; however, without antifungal effect against *C. albicans*.[5] In our study, *A. lappa* L. extract was effective for both bacteria and *Candida* species.

Regarding biofilms, significant reductions in CFU/mL for each one of the microorganisms studied were observed after treatment with the extract of *A. lappa* L. (250 mg/mL) compared to the untreated group (0.9% NaCl). These reductions are associated with two major factors: the concentration of the extract used and the time of exposure of biofilm microbial cells at this concentration (5 min). This time was

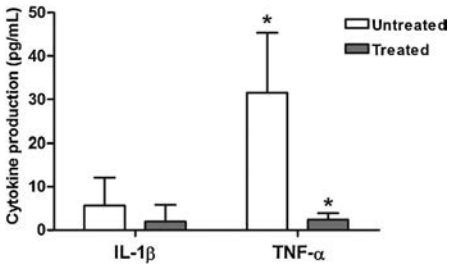

Fig. 4 – Production of IL-1β and TNF-α (pg/mL) for macrophages obtained in the untreated group (control – NaCl 0.9%) and treated group with concentration of 250 mg/mL of the *A. lappa* L. extract ($n = 10$) for 24 h. ANOVA and Tukey test ($p \leq 0.05$). $^{*}p < 0.01$.

used looking for the possibility of integrating this plant or its bioactive compound, which showed antimicrobial power, in oral care products like toothpastes or mouthwashes, where the maximum exposure period is in this range. It was noted that the biofilm of *C. albicans* showed the highest reduction mean of CFU/mL after treatment with *A. lappa* L. extract, which was about 14%. There were significant reductions in the number of CFU/mL in bacterial groups compared to the control group: 7% for *S. aureus*, 9% for *S. epidermidis* and only 3% for *S. mutans*. Biofilms of *C. albicans* formed on surfaces are related to many manifestations of candidosis affecting both mucosal and whole organism.[11] Due to the fact that resistance to traditional antifungal agents has increased, we believe that the use of alternative methods such as the use of herbal medicines can act to control this microorganism. Drug resistance is given by the presence of specific genes in yeast that are not presented regulated during the formation and development of biofilms.[16,26,27] In several *Candida* species, biofilm formation is an important virulence factor by offering them resistance to antifungal therapy, by the difficulty of diffusion of substances, including the resistance to components of the host immune system.[28] In dentures, it is quite frequent biofilm formation of *Candida* spp.[17] Pereira et al.,[20] while studying the effects of photodynamic inactivation of microbial cells through photosensitizer and irradiation of laser on biofilms of *S. aureus*, *S. mutans* and *C. albicans*, have noted the effective control of biofilms with significant reductions in CFU/mL as well as in the present study where it was used a natural product. However, these authors have demonstrated that the highest reduction was found in the group of *S. aureus*; whereas, in the present study, the extract was more effective for group *C. albicans*.

In the present study, biofilms formed on acrylic resin discs were disaggregated by ultrasonic homogenizer. In recent studies, the same instrument was used for this purpose and no damage on the structures of the microbial cells was observed on untreated group, which was subjected to ultrasonic waves. It could be seen due to the images obtained through scanning electron microscopy (SEM). It was also noted that only the groups which were subjected to some kind of treatment evidenced reduction in the number of biofilm cells.[20,29–31] Trentin et al.[32] studied the antibiofilm effect of various plant extracts from Brazilian Caatinga (semi-arid region) on *S. epidermidis* and, using SEM, they were able to demonstrate that such products caused structural damage on this microorganism while the untreated group showed no structural change.

As our extract was initially prepared using propylene glycol as a solvent, a possible influence on antimicrobial activity of the extract of *A. lappa* L. on standard strains (*C. albicans*, *S. aureus*, *S. mutans*, *S. epidermidis*) in planktonic cultures was previously evaluated. Although Kinnunen and Koskela[33] has demonstrated antimicrobial activity of propylene glycol, the concentrations used in our study did not show this biological activity. In 2006, Gentil et al.[6] evaluated the antimicrobial activity of the ethyl acetate fraction (AcOEt) extracted from *A. lappa* L., propylene glycol and calcium hydroxide as an intracanal medication in teeth contaminated with *Pseudomonas aeruginosa*, *Escherichia coli*, *Lactobacillus acidophilus*, *S. mutans* and *C. albicans*, for 7, 14 and 30 days. According to their results,

propylene glycol did not show antimicrobial activity in any of the periods evaluated and it was found severe microbial growth. In 2012, Bonvehí and Gutiérrez[34] evaluated the antimicrobial activity of propolis extract in different solvents (ethanol and propylene glycol) on bacteria and yeasts, and according to their results, the control group containing 100% propylene glycol showed no antimicrobial activity for *S. mutans*, *Streptococcus pyogenes*, *S. aureus*, *Helicobacter pylori*, *Salmonella enterica*, *E. coli*, *C. albicans* and *Saccharomyces cerevisiae*. According to the literature and our previous results, the concentration of propylene glycol in the dilutions of the extract did not affect the antimicrobial activity of *A. lappa* L.

During the evaluation of the cytotoxicity of the *A. lappa* L. extract, the groups treated with concentrations of 250, 125 and 62.5 mg/mL of the extract showed similar cell viability to the control group and only in the group treated with 31.3 mg/mL significant reduction was observed in the cell viability (Fig. 3). These results agree with the study of Sohn et al.,[35] whom demonstrated that the concentrations used for treatment (1, 10 and 100 μg/mL) were not cytotoxic to cell cultures after 24 h while verifying the antiallergic and anti-inflammatory effects of the *A. lappa* L. butanol extract. By MTT assay, it was shown that the treatment of the cells with plant extract was not cytotoxic, since cell viability was greater than 94%.

Regarding the production of pro-inflammatory cytokines by cell cultures, it was found that *A. lappa* L. extract did not stimulate macrophages to produce cytokines; on the contrary, the extract significantly inhibited the production of TNF-α and IL-1β levels remained similar to the untreated group (Fig. 4). Okada et al.[36] also showed that the extract from the seeds of *A. lappa* L. completely inhibited the production of TNF-α of adipocytes cultures, compared to the control group. Hernández et al.[37] investigated the anti-inflammatory effect of the extract of *Heliopsis longipes*, a traditional plant of Mexican medicine and demonstrated that RAW 264.7 cells treated with concentrations of 250 and 500 μg/mL of extract produced TNF-α levels similar to the control group. At another moment, the groups activated with LPS (10 ng/mL) and INF-γ (2 U/ml) and treated with different concentrations of the extract of *H. longipes* (1, 10, 100, 250 and 500 μg/mL) showed gradual and significant reductions of the cytokine in the treatments with 250 and 500 μg/mL, compared to the untreated group and with the ones activated by LPS and INF-γ. Sohn et al.[35] demonstrated that the use of the *A. lappa* L. butanol extract promoted inhibition on the production of IL-4 and IL-5 by murine splenocytes culture in the concentration of 100 μg/mL, suggesting probable immunostimulatory effect on T cells.

Hence, the use of natural products such as *A. lappa* L. extract in order to control opportunistic microorganisms may be an alternative therapy, since there are no antimicrobial strains resistant to conventional usage. Thus, it can be concluded that *A. lappa* L. extract was microbicidal for all the evaluated strains in planktonic cultures (*S. aureus*, *S. epidermidis*, *S. mutans*, *C. albicans*, *C. tropicalis* and *C. glabrata*) and induced significant reduction in biofilms of *S. aureus*, *S. epidermidis*, *S. mutans* and *C. albicans*. Moreover, the extract was not cytotoxic to macrophages, presenting cell viability above 94%, inhibition of TNF-α and similar production of IL-1β to the group not exposed to the extract.

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None.

Competing interests

None declared.

Ethical approval

This study was approved by the Ethics Committee of the Institute of Science and Technology – UNESP, protocol 008/2010-PA/CEP.

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3.8 *In vitro* antimicrobial and anti-endotoxin action of *Zingiber officinale* as auxiliary chemical and medicament combined to calcium hydroxide and chlorhexidine*

ABSTRACT

Objective. This study was conducted in vitro to compare the effectiveness of *Zingiber Officinale* as an auxiliary chemical substance followed by placement of different intra-canal medication in removing endotoxins and cultivable micro-organisms from infected root canals. Materials and methods. Seventy-two root canals were contaminated with *Enterococcus faecalis*, *Candida albicans* and *Escherichia coli* for 28 days. After, the teeth were instrumented using *Zingiber Officinale* and divided into six groups according to the intra-canal medication: chlorhexidine gel; calcium hydroxide + chlorhexidine gel; glycolic ginger extract; calcium hydroxide + glycolic ginger extract; calcium hydroxide + saline solution and saline solution (control). Sample collections were performed after root canal contamination (Baseline; S1), after instrumentation (S2), 7 days after instru- mentation (S3), after 14 days with intra-canal medication (S4) and 7 days after removal of intra-canal medication (S5). The results were analyzed by the Kruskal-Wallis and Dunn tests. Results. It was observed that in S2 and S3 there was significant reduction of the micro-organisms and the quantity of endotoxins after instrumentation. In samples S4 and S5 there was complete elimination of micro-organisms and significant reduction of endotoxins. Conclusion. It was concluded that *Zingiber Officinale* as an auxiliary chemical substance was effective on the micro-organisms tested, yet was unable to eliminate the endotoxins. Similarly, the intra-canal medication were effective on micro-organisms, yet did not completely eliminate the endotoxins.

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ORIGINAL ARTICLE

In vitro antimicrobial and anti-endotoxin action of *Zingiber Officinale* as auxiliary chemical and medicament combined to calcium hydroxide and chlorhexidine

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Abstract

Objective. This study was conducted *in vitro* to compare the effectiveness of *Zingiber Officinale* as an auxiliary chemical substance followed by placement of different intra-canal medication in removing endotoxins and cultivable micro-organisms from infected root canals. ***Materials and methods.*** Seventy-two root canals were contaminated with *Enterococcus faecalis, Candida albicans* and *Escherichia coli* for 28 days. After, the teeth were instrumented using *Zingiber Officinale* and divided into six groups according to the intra-canal medication: chlorhexidine gel; calcium hydroxide + chlorhexidine gel; glycolic ginger extract; calcium hydroxide + glycolic ginger extract; calcium hydroxide + saline solution and saline solution (control). Sample collections were performed after root canal contamination (Baseline; S1), after instrumentation (S2), 7 days after instrumentation (S3), after 14 days with intra-canal medication (S4) and 7 days after removal of intra-canal medication (S5). The results were analyzed by the Kruskal-Wallis and Dunn tests. ***Results.*** It was observed that in S2 and S3 there was significant reduction of the micro-organisms and the quantity of endotoxins after instrumentation. In samples S4 and S5 there was complete elimination of micro-organisms and significant reduction of endotoxins. ***Conclusion.*** It was concluded that *Zingiber Officinale* as an auxiliary chemical substance was effective on the micro-organisms tested, yet was unable to eliminate the endotoxins. Similarly, the intra-canal medication were effective on micro-organisms, yet did not completely eliminate the endotoxins.

Key Words: *endotoxins, micro-organisms, root canal irrigants, root canal medicaments, Zingiber Officinale*

Introduction

Root canals of teeth with necrotic pulps associated with pulp and periapical lesions are a reservoir of micro-organisms and their byproducts, which induce periapical inflammatory reaction [1]. Endodontic infections are predominantly polymicrobial, involving facultative and strict anaerobes, aerobes and fungi [2–5].

Gram-negative bacteria present an endotoxin (lipopolysaccharide) in their cell wall, which is released during duplication or cell death, causing inflammatory reactions and resorption of periradicular tissues, which may be associated with endodontic signs and symptoms [6,7]. *Escherichia coli* presents endotoxins in the cell wall with the basic structure of the lipid component, which represents the active center responsible for the toxicity of LPS [8]. For this reason, even though this bacteria is not commonly found in root canals with necrotic pulp, it has been used to evaluate substances that may be able to neutralize endotoxins [9].

Among the micro-organisms frequently found in the root canal system, *Enterococcus faecalis* is the most prevalent species and is associated with persistent periapical lesions, being able to survive even in adverse conditions, indicating its importance in the etiology of endodontic treatment failure [10,11].

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Candida albicans is also frequently isolated in root canals. This fungus may be present in 7–55% of infected root canals [3], being considered another possible cause of endodontic treatment failure [5,11,12].

The world literature demonstrates an increase for natural products in medical specialties, encouraging alternative therapies. *Zingiber officinale*, popularly known as ginger, has been studied because of its antimicrobial, anti-inflammatory and analgesic properties [13,14]. It presents 1–3% of essential oil, 2.5–5% of gingerol and shogaol and 60% of starch, which are able to significantly inhibit the production of E2 prostaglandins induced by the LPS of *E. coli* [13].

Considering that studies conducted on ginger have demonstrated antimicrobial effectiveness, this study evaluated, *in vitro*, the action of *Zingiber Officinale* as an auxiliary chemical substance followed by its use as intra-canal medication associated and compared with other drugs commonly used during the intra-canal medication stage.

Materials and methods

This study was approved by the Ethical Committee of São José dos Campos School of Dentistry–UNESP, protocol n. 07/2008-PH/CEP.

Preparation and contamination of specimens

The study was conducted on 72 single-rooted human teeth whose crowns were sectioned to standardize the length of specimens in 16 ± 0.5 mm. The root canals were instrumented using saline solution throughout their extent up to Kerr file n. 30 (Dentsply Ind. Com. Ltda, Petrópolis, RJ, Brazil) followed by irrigation with 3 ml saline solution after each instrument. Afterwards, the canals were filled with EDTA for 3 min and irrigated with 10 ml of saline solution. Then, the roots were sealed at the apical region with light cured composite resin Z-100 (3M, Saint Paul, MN) and externally sealed with two layers of epoxy adhesive (Brascola, São Paulo, SP, Brazil), except for the cervical opening. The specimens were randomly distributed and fixated with chemically cured acrylic resin (Dencor Artigos Odontológicos, São Paulo, SP, Brazil) on cell culture plates with 24 wells (TPP, Trasadingen, Switzerland), with 12 teeth in each. All cell culture plates and materials were sterilized by Cobalt-60 gamma irradiation (20 KGy for 6 h) to neutralize the pre-existent endotoxins [15].

The micro-organisms used were *Candida albicans* (ATCC 18804), *Enterococcus faecalis* (ATCC 29212) and *Escherichia coli* (ATCC 25922). Separate suspensions were prepared in apyrogenic saline containing 10^6 cells/ml, checked by reading in a spectrophotometer.

The root canals were contaminated with 10 µl of suspension of *E. coli* and 10 µl of BHI broth (brain-heart infusion) (Himedia Laboratories, Mumbai, India). Apyrogenic cotton pellets soaked in BHI broth were placed in the root canal openings and the specimens were kept in an oven at 37°C ± 1°C, in relative humidity, for 7 days. After this period, 5 µl of suspension of *C. albicans*, 5 µl of suspension of *E. faecalis* and 10 µl of BHI broth were added to the root canals. A new apyrogenic cotton pellet soaked in BHI broth was placed in the root canal openings and the specimens were kept in an oven at 37°C ± 1°C, in relative humidity, for 21 days, with addition of BHI broth to the root canals every 2 days [9,16].

After the contamination period (28 days), the initial sample (baseline, S1) was collected from all specimens to confirm the root canal contamination and quantify the endotoxins.

Division of study groups

After verifying the contamination, the root canals were instrumented into their full length up to Kerr file n. 50, with 20% glycolic ginger extract (Apis Flora, Ribeirão Preto, São Paulo, Brazil) as an auxiliary chemical substance during the instrumentation and using 3 ml of apyrogenic saline solution. Furthermore, at each change of instrument, the 20% glycolic ginger extract solution was renewed. In order to determine the antimicrobial activity of the *Zingiber Officinale* (20% glycolic ginger extract), a second sampling specimen was performed (S2). The teeth were filled with apyrogenic saline solution and maintained for 7 days. Throughout the study period, the specimens were kept in an oven at 37°C ± 1°C. After 7 days the third sample was collected (S3). The root canals were then filled with EDTA for 3 min, followed by root canal irrigation with 10 ml of apyrogenic saline solution and division of specimens into six experimental groups (n = 12), according to the intra-canal medication used (Table I).

Medications of 2% chlorhexidine gel (CHX), 20% glycolic ginger extract (GENG) and saline (SS; control) were applied using 3 ml syringes up to complete root canal filling. The medications were associated in a 1:1 ratio in volume and placed in the root canal using a Kerr file n. 30.

After root canal filling with the intra-canal medication, the teeth were sealed with apyrogenic cotton pellets. The teeth were kept in an oven at 37°C for 14 days. After this period, the medication was removed with a Kerr file n. 50 and 10 ml of apyrogenic saline solution, followed by collection of the fourth sample (S4). The root canals were filled with apyrogenic saline solution and the fifth sample (S5) was collected after 7 days.

Table I. Division of the experimental groups.

Groups	Intra-canal medication	Origin
CHX	2% chlorhexidine gel	Byofórmula, São José dos Campos, Brazil
$Ca(OH)_2$ + CHX	Calcium Hydroxide associated with 2% chlorhexidine gel	Biodinâmica, Ibiporã, PR; Byofórmula, São José dos Campos, Brazil
GENG	20% glycolic ginger extract	Apis Flora, Ribeirão Preto, São Paulo, Brazil
$Ca(OH)_2$ + GENG	Calcium Hydroxide associated with 20% glycolic ginger extract	Biodinâmica, Ibiporã, PR; Apis Flora, Ribeirão Preto, São Paulo, Brazil
$Ca(OH)_2$ + SS	Calcium Hydroxide associated with saline solution	Biodinâmica, Ibiporã, PR, Brazil
SS (Control)	Saline solution	Laboratório Sanobiol, Pouso Alegre, MG, Brazil

Collections of root canal content

All samples (S1, S2, S3, S4 and S5) were carried in the same standard way: the root canals were filled with apyrogenic saline solution and 100 µl of the root canal content was collected and transferred to eppendorf tubes containing 900 µl of apyrogenic saline solution. All samples were submitted to microbiological tests and quantification of endotoxins to verify the presence of micro-organisms and endotoxins in the root canals.

Evaluation of antimicrobial action (culturing procedure)

Aliquots of 100 µl of all samples were plated in duplicate in three different culture media, selective for each micro-organism: agar Sabouraud Dextrose (Himedia Laboratories, Mumbai, India) with chloramphenicol (Vixmicina, União Química Farmacêutica S/A, Embu-Guaçu, SP, Brazil) for *C. albicans*, agar Enterococcosel (Becton, Dickinson and Company, Sparks, MD, USA) for *E. faecalis* and agar MacConkey (Himedia Laboratories, Mumbai, India) for *E. coli*.

The plates were kept in an oven at 37°C for 24 h. In all collections, the antimicrobial activity of treatments and substances was analyzed by counting of colony forming units per milliliter (CFU/ml). The results were statistically analyzed by the *Kruskal-Wallis* and *Dunn* tests, at a significance level of 5%.

Table II. Percentages of reduction of CFU/ml obtained in the second (S2) and third (S3) collections in relation to the baseline (S1).

Reduction	Micro-organisms	Mean	SD	Median	HG
	C. albicans	99.99	0.01	100	A
S1 × S2	*E. faecalis*	99.87	0.58	99.99	B
	E. coli	99.99	0.04	100	A
	C. albicans	99.98	0.11	100	A
S1 × S3	*E. faecalis*	99.79	0.61	99.96	B
	E. coli	99.63	3.04	100	A

HG, Homogenous groups; indicate statistical significance.

Quantification of endotoxins (endotoxins procedures)

The neutralization of endotoxins was verified by the kinetic chromogenic method of *Limulus Amebocyte Lysate test* (LAL) (Cambrex, São Paulo, SP, Brazil), using the kinetic reader QCL (Cambrex) connected to a computer with specific software WinkQCL (Cambrex). The concentrations of standard curve (between 0.005–50 EU/ml) were performed according to the manufacturer; calculation of the parameters of standard curves and values of endotoxin samples (EU/ml) were automatically performed by the software. The present statistical analysis was based on the percentage of alteration related to a decrease or increase in the quantity of endotoxin (EU/ml) of each sample in relation to the baseline (S1).

Results were subjected to statistical analysis using *Kruskal-Wallis* and *Dunn* tests (significance level of 5%).

Results

Microbiological analysis

At the baseline (S1), micro-organisms were recovered from 100% of the contaminated-root canals. The results of the *Kruskal-Wallis* test comparing the

Table III. Percentages of reduction of CFU/ml obtained in the fourth collection (S4) in relation to the baseline (S1).

	S1 × S4					
	C. albicans		*E. faecalis*		*E. coli*	
	Median	HG	Median	HG	Median	HG
CHX	100	A	100	A	100	A
$Ca(OH)_2$ + CHX	100	A	100	A	100	A
GENG	100	A	100	A	100	A
$Ca(OH)_2$ + GENG	100	A	100	A	100	A
$Ca(OH)_2$ + SS	100	A	100	A	100	A
SS	99.98	B	99.98	B	100	A

n = 12 for each group.
HG, Homogeneus groups; indicate statistical significance.

Table IV. Percentages of reduction of CFU/ml obtained in the fifth collection (S5) in relation to the baseline (S1).

Groups	S1 × S5					
	C. albicans		*E. faecalis*		*E. coli*	
	Median	HG	Median	HG	Median	HG
CHX	100	A	100	A	100	A
$Ca(OH)_2$ + CHX	100	A	100	A	100	A
GENG	100	A	100	A	100	A
$Ca(OH)_2$ + GENG	100	A	100	A	100	A
$Ca(OH)_2$ + SS	100	A	100	A	100	A
SS	100	A	99.96	B	99.97	A

n = 12 for each group.
HG, Homogeneus groups; indicate statistical significance.

percentage of reduction of micro-organisms after instrumentation (S2) and 7 days after instrumentation (S3) compared to the baseline (S1) are expressed in Table II. It is possible to observe a reduction of nearly 100% of micro-organisms after preparation using the 20% ginger glycolic extract as an auxiliary chemical substance, demonstrating great efficacy against *C. albicans*, *E. faecalis* and *E. coli*.

The mean, standard deviation, median and homogeneous groups of sample 4 (S4; after placement of intra-canal medication) and sample 5 (S5; 7 days after removal of intra-canal medication), compared to the baseline (S1), are expressed in Tables III and IV. Concerning the SS group (control group), there was a reduction in the number of micro-organisms immediately after instrumentation (S2), re-taking the growth during the study (S4 and S5).

Analysis of endotoxins

The results of quantification of endotoxins revealed a reduction of 92.32% using the ginger glycolic extract as an auxiliary chemical substance during instrumentation (S2). Seven days after instrumentation (S3), there was a reduction of 87.44% (Table V).

The mean and median values obtained in the countings of EU/ml, as well as the homogeneous groups of samples S4 and S5, are presented in Table VI. In sample S4, $Ca(OH)_2$ + SS and Ca

Table V. Percentages related to the reduction in the quantity of endotoxin (EU/ml) obtained in the second (S2) and third (S3) collections in relation to the baseline (S1).

Reduction	n	Mean	SD	Median	HG
S1 × S2	72	92.32	11.60	96.15	A
S1 × S3	72	87.44	30.68	95.67	A

HG, Homogeneus groups; indicate statistical significance.

Table VI. Percentages related of the reduction in the quantity of endotoxin (EU/ml) obtained in the fourth (S4) and fifth (S5) collections in relation to the baseline (S1).

	S1 × S4			S1 × S5		
Groups	Mean	Median	HG	Mean	Median	HG
CLX	99.65	99.79	BC	99.55	99.88	A
CLX + $(CaOH)_2$	99.75	99.97	AB	99.91	99.96	A
Geng	98.28	99.60	BC	92.55	97.16	B
Geng + $Ca(OH)_2$	99.74	99.90	B	99.64	99.83	AB
SS + $Ca(OH)_2$	99.99	99.99	A	99.98	99.99	A
SS	74.8	90.50	C	85.39	89.62	B

n = 12 for each group.
HG, Homogeneus groups; indicate statistical significance.

$(OH)_2$ + CHX presented the greatest reduction of endotoxin (99.99% and 99.74%, respectively). In sample S5, the groups $Ca(OH)_2$ + SS (99.99%), $Ca(OH)_2$ + CHX (99.96%), CHX (99.88%) and $Ca(OH)_2$ + GENG (99.83%) exhibited the greatest reduction of endotoxin levels.

Discussion

In the present study, the specimens were contaminated with standardized suspensions of *Enterococcus faecalis*, *Candida albicans* and *Escherichia coli*, already described in previous studies [17–19]. Some microbial species, such as *E. faecalis* and *C. albicans*, may present resistance to calcium hydroxide, a commonly used intra-canal medication [16,20]. This study revealed that calcium hydroxide, pure or associated with chlorhexidine and 20% glycolic ginger extract, was effective on the micro-organisms tested. The antimicrobial action of $Ca(OH)_2$ has been demonstrated in other studies [9,21]. Even though reports demonstrate that $Ca(OH)_2$ is not effective on *Enterococcus faecalis* [22], it presented 100% effectiveness in this study.

Recent studies demonstrate the action of glycolic and alcoholic ginger extract on micro-organisms as *Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli*, *Porphyromonas endodontalis* and *Prevotella intermedia* [14]. Components of essential oil of rhizomes of *Zingiber officinale* reduce the growth rate of a large variety of bacteria and fungi, including *Staphylococcus* and *Candida* [23]. It was demonstrated that ethanolic extracts of *Z. Officinale* were able to inhibit the growth of Gram-negative and Gram-positive bacteria [24], even though the effect was more pronounced on Gram-positive bacteria [25]. Also, Habsah et al. [26] observed high activity of this rhizome on *Pseudomonas aeruginosas*, a highly resistant Gram-negative bacteria. In this study, after instrumentation using ginger glycolic extract as auxiliary chemical

substance, there was complete elimination of *C. albicans* and *E. coli* and more than 99% of *E. faecalis* could also be eliminated.

Notwithstanding the favorable results in the elimination of micro-organisms, the ginger glycolic extract was unable to completely eliminate the endotoxins present in the root canal system, demonstrating similar results as those obtained with sodium hypochlorite and chlorhexidine, which are auxiliary chemicals commonly employed in the endodontic practice [7,27]. This study used 20% ginger glycolic extract, whose main active principle is gingerol, which probably presents greater antimicrobial effects [14]. Studies indicate that the volatile oils gingerol and shogaol seem to be responsible for the beneficial action of this rhizome [13]. Park et al. [14] suggest that compound isolated from ginger, as gingerol were effective on *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia*. Although the mechanism of action of ginger extract on micro-organisms is not be elucidated in the literature, studies reveal effective antimicrobial action of this extract on the micro-organisms [14]. Other investigations further demonstrated that ginger is biocompatible, being used also in the diet [28,29]. However, there are no studies on the effects of active principles of ginger. The ideal time of action of this product is also not known. The results of this substance immediately after preparation (S1) suggested that the peak action of ginger occurs at nearly 7 days, with a loss of activity after this period. However, further studies should be conducted to evaluate the period of action of this phytotherapic substance.

Concerning the use of intra-canal medications, all medications analyzed were able to completely eliminate the micro-organisms, in agreement with other studies [9,30]; however, they were unable to eliminate endotoxins present in the root canals. Among the medications used, associations with calcium hydroxide revealed the best outcomes. The antimicrobial activity of calcium hydroxide is associated with its high pH [31] by the release and diffusion of hydroxyl ions. To be effective on bacteria located in the dentinal tubules, hydroxyl ions should diffuse in the dentine with sufficient concentrations, reaching alkaline pH high enough to destroy the bacteria [32]. Similarly, OH^- ions are able to induce the lipid peroxidase, leading to destruction of phospholipids. Moreover, the maintenance of alkaline pH by $Ca(OH)_2$ causes rupture of ionic bonds and alters the structure of cell membrane proteins, causing interruption of the cell metabolism of bacteria [32].

Therefore, it was concluded that ginger glycolic extract may be a good option as an auxiliary chemical in endodontic practice and calcium hydroxide intracanal medications are the most indicated, especially to neutralize endotoxins.

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3.9 *Persea americana* glycolic extract: in vitro study of antimicrobial activity against *Candida albicans* biofilm and cytotoxicity evaluation*

ABSTRACT

This study evaluated the antifungal activity of *Persea americana* extract on *Candida albicans* biofilm and its cytotoxicity in macrophage culture (RAW 264.7). To determine the minimum inhibitory concentration (MIC), microdilution in broth (CLSI M27- S4 protocol) was performed. Thereafter, the concentrations of 12.5, 25, 50, 100, and 200 mg/mL (n = 10) with 5 min exposure were analyzed on mature biofilm in microplate wells for 48 h. Saline was used as control (n = 10). After treatment, biofilm cells were scraped off and dilutions were plated on Sabouraud dextrose agar. After incubation (37°C/48h), the values of colony forming units per milliliter (CFU/mL) were converted to $\log_{10}$ and analyzed (ANOVA and Tukey test, 5%). The cytotoxicity of the *P. americana* extract was evaluated on macrophages by MTT assay. The MIC of the extract was 6.25 mg/mL and with 12.5 mg/mL there was elimination of 100% of planktonic cultures. Regarding the biofilms, a significant reduction (P < 0.001) of the biofilm at concentrations of 50 (0.580 ± 0.209 $\log_{10}$), 100 (0.998 ± 0.508 $\log_{10}$), and 200 mg/mL (1.093 ± 0.462 $\log_{10}$) was observed. The concentrations of 200 and 100 mg/mL were cytotoxic for macrophages, while the concentrations of 50, 25, and 12.5 mg/mL showed viability higher than 55%.

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Research Article

Persea americana Glycolic Extract: *In Vitro* Study of Antimicrobial Activity against *Candida albicans* Biofilm and Cytotoxicity Evaluation

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This study evaluated the antifungal activity of *Persea americana* extract on *Candida albicans* biofilm and its cytotoxicity in macrophage culture (RAW 264.7). To determine the minimum inhibitory concentration (MIC), microdilution in broth (CLSI M27-S4 protocol) was performed. Thereafter, the concentrations of 12.5, 25, 50, 100, and 200 mg/mL ($n = 10$) with 5 min exposure were analyzed on mature biofilm in microplate wells for 48 h. Saline was used as control ($n = 10$). After treatment, biofilm cells were scraped off and dilutions were plated on Sabouraud dextrose agar. After incubation (37°C/48 h), the values of colony forming units per milliliter (CFU/mL) were converted to $\log_{10}$ and analyzed (ANOVA and Tukey test, 5%). The cytotoxicity of the *P. americana* extract was evaluated on macrophages by MTT assay. The MIC of the extract was 6.25 mg/mL and with 12.5 mg/mL there was elimination of 100% of planktonic cultures. Regarding the biofilms, a significant reduction ($P < 0.001$) of the biofilm at concentrations of 50 (0.580 ± 0.209 $\log_{10}$), 100 (0.998 ± 0.508 $\log_{10}$), and 200 mg/mL (1.093 ± 0.462 $\log_{10}$) was observed. The concentrations of 200 and 100 mg/mL were cytotoxic for macrophages, while the concentrations of 50, 25, and 12.5 mg/mL showed viability higher than 55%.

1. Introduction

According to ANVISA [1], phytotherapics are medicines in which the active raw material is vegetables, such as fresh plant, plant drug, or its secondary products as extract, tincture, oil, and others such as juice and wax, obtained by adequate techniques. They are used for prophylactic, curative, palliative, or diagnosis purposes. The traditional phytotherapics medicines are synthesized from medicinal plants used traditionally by a population, with no risk to health, confirmed by toxicological studies, and demonstration of efficacy through ethnopharmacological surveys, or data obtained from the technoscientific documentation and indexed publications [1]. The use of these medicines is recognized by the World Health Organization (WHO); however, it is recommended that scientific studies be carried out to prove its effectiveness. Recent studies have demonstrated antimicrobial and anti-inflammatory efficacy of numerous plant extracts [2–4]. In 2013, Oliveira et al. [5] evaluated by microdilution method the antimicrobial activity of glycolic extracts of *Equisetum arvense* L., *Glycyrrhiza glabra* L., *Punica granatum* L., and *Stryphnodendron barbatimam* Mart. against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Candida albicans*, *Candida tropicalis,* and *Candida glabrata*, with all these extracts being effective against the microorganisms tested. Furthermore, they evaluated the cytotoxicity of plant extracts in RAW 264.7 mouse macrophages by MTT and colorimetric method for quantification of IL-1β and TNF-α cytokines. The study found that *G. glabra* extract exhibited the lowest cytotoxicity and *E. arvense* extract was the most cytotoxic. In 2014, Oliveira et al. [6] evaluated the antimicrobial activity of the extract of *Arctium lappa* L. against *S. aureus*, *S. epidermidis, Streptococcus*

mutans, *C. albicans*, *C. tropicalis,* and *C. glabrata* in planktonic cultures and biofilm. The extract of *A. lappa* L. at the concentration of 250 mg/mL was microbicide for all tested microorganisms in the planktonic culture and significantly effective in reducing biofilms of these microorganisms.

Several plants have been the focus of studies to scientifically prove their many beneficial effects and promote better indication as an alternative therapy. *P. americana*, known as avocado tree, is an evergreen tree belonging to Lauraceae family, native of Central America, and currently distributed in tropical and subtropical regions. The plant drug of avocado tree consists of dried leaves containing at least 0.4% of total flavonoids expressed in apigenin and 0.14% of essential oil [7].

The fruit is widely exploited as food and its leaves are used in traditional medicine, fact found in ethnobotanical survey carried out by Rosas-Piñón et al. [8] in three distinct regions in Mexico, where the leaves are used in the treatment of oral infections and caries control. These authors analyzed the aqueous and alcoholic extracts of avocado leaf on *S. mutans* and *Porphyromonas gingivalis*, with satisfactory results only against *S. mutans* for both extracts. Lu et al. [9] evaluated the antimicrobial activity of methanolic extract of avocado green pulp against *Mycobacterium tuberculosis*, resulting in a minimum inhibitory concentration of 15 μg/mL, which is considered very satisfactory as opposed to the resistance of this microorganism to different types of conventional antibiotics. In 2002, Adeyemi et al. [10] evaluated the anti-inflammatory and analgesic potential of the aqueous extract of avocado leaves in mice and found inhibition of pain and inflammation in a dose-dependent manner.

Guzmán-Rodríguez et al. [11] analyzed the antimicrobial potential of peptides derived from *P. americana* on *Escherichia coli*, *S. aureus,* and *C. albicans*. The results demonstrated antimicrobial effects for *E. coli* and *S. aureus*; however, activity on *C. albicans* was not detected. On the other hand, Leite et al. [12], analyzing the methanolic extract of *P. americana*, verified antimicrobial action in the planktonic culture strains of *Candida* spp., *Cryptococcus neoformans,* and *Malassezia pachydermatis*.

Taking into consideration the resistance of microorganisms to antibiotics, it becomes necessary to study alternative methods such as plant extracts, and with the popular therapeutic use of avocado tree and some studies that suggest its antimicrobial and anti-inflammatory effects, it is of interest to study this extract in order to expand its therapeutic indication.

Many plant extracts have different therapeutic purposes, so that the studies with natural substances represent an important research field that may bring great benefits to dental therapy, being important to study their antimicrobial effects on dental-interest microorganisms and their possible cytotoxic effects. These studies are relevant to prove the various beneficial effects of these extracts, which can be further inserted in dental use formulations such as medications and intracanal irrigators, mouth rinses, toothpastes, and so on.

Thus, the objective of this study was evaluating the *in vitro* antifungal activity of *P. americana* extract on biofilm of *C. albicans* ATCC 18804 and its cytotoxicity in macrophage culture (RAW 264.7).

2. Materials and Methods

P. americana glycolic extract was provided by company Mapric (São Paulo, SP, Brazil) at the concentration of 200 mg/mL in propylene glycol.

In order to determine the minimum inhibitory concentration (MIC), broth microdilution method was used, according to CLSI [13], in accordance with norm M27-S4 protocol. Initially, *C. albicans* was cultured on Sabouraud dextrose (Himedia) for 24 h at 37°C. A standard solution containing 1×10^6 cells/mL was prepared with spectrophotometer (Micronal B-582, São Paulo, SP, Brazil). Thereafter, this solution was diluted 1 : 50, followed by a 1 : 20 dilution to obtain a suspension of approximately 5×10^2 to 2.5×10^3 cells/mL.

Ten serial 1 : 2 dilutions were made from the extract into a 96-well plate (from 200 to 0.5 mg/mL) with 100 μL of extract in 100 μL of culture medium RPMI 1640 (Himedia) buffered with MOPS (Sigma Aldrich, St. Louis, Missouri, USA) at pH 7.0 ± 0.1. Subsequently, 100 μL of the standardized suspension of the yeast was added to each well of 96-well plate. A well for positive control (medium with inoculum) and another well for negative control (medium alone) were included. The plates were incubated for 24 h at 37°C. The MIC was determined in the well of lowest concentration, in which turbidity was not observed. Aiming to find the minimum fungicidal concentration (MFC), 100 μL of MIC, a concentration above and one below were seeded on Sabouraud dextrose plates and incubated at 37°C for 48 h.

2.1. Antimicrobial Activity in Biofilm. A standard strain of *C. albicans* (ATCC 18804) was used. The inoculum was standardized in sterile saline (NaCl 0.9%) containing 10^7 cells/mL with spectrophotometer. Two hundred microliters of the inoculum was added in each well of a 96-well microplate. The microplate was incubated for 1.5 h (37°C under agitation of 75 rpm) for the initial adherence. Thus, the saline was taken, 200 μL of YNB broth was added, and the microplate was incubated in the same conditions of initial adherence for 48 h. The medium was replaced every 24 h. After biofilm formation, the wells were divided into 6 experimental groups ($n = 10$), and from these 6 groups, 5 had contact with different concentrations of the extract, according to the results obtained in the planktonic assay: 12.5 mg/mL; 25 mg/mL; 50 mg/mL; 100 mg/mL; 200 mg/mL, during periods of 5 min. The control group was maintained in sterile saline. Posteriorly, these solutions were discarded, the biofilms were washed with sterile saline, and the samples were taken to the ultrasonic homogenizer (Sonopuls HD 2200, 50 W, Bandelin Electronic, Heinrichstraße, Berlin, Germany) for 30 s with approximately 25% of power to disaggregate the biofilm. There were four decimal dilutions of inoculum suspensions, which were seeded in duplicate (100 μL of each dilution) in Sabouraud dextrose plates. These plates were incubated for 48 h at 37°C. Then, colony forming units per milliliter (CFU/mL) were counted and the results were converted to $\log_{10}$ [14].

2.2. Cell Culture [5, 6]. Macrophages cell line RAW 264.7 was used, from the Rio de Janeiro Cell Bank, Associação

Técnico Científica Paul Ehrlich (APABCAM, Rio de Janeiro, RJ, Brazil), cultured in Dulbecco modified Eagle medium (DMEM, LGC Biotechnology, Cotia, SP, Brazil), supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, Grand Island, NY, USA), and maintained under humidified atmosphere at 37°C and 5% CO_2. An automatic cell counter (Countess, Invitrogen, Paisley, UK) was used to find the number of viable cells.

2.3. Cytotoxicity Testing (MTT Assay). In 96-well microplates (TPP), 200 μL of DMEM + 10% FBS containing 2×10^4 viable cells per well was added. After incubation at 37°C, 5% CO_2 for 24 hours for adhesion of the cells, the supernatant was discarded and *P. americana* extract diluted in DMEM + 10% FBS was added, in different concentrations, performing 11 experimental groups ($n = 8$): 0.2 mg/mL, 0.39 mg/mL, 0.78 mg/mL, 1.56 mg/mL, 3.13 mg/mL, 6.2 mg/mL, 12.5 mg/mL, 25 mg/mL, 50 mg/mL, 100 mg/mL, and 200 mg/mL. For growth control, culture medium with cell suspension was used and, for negative control, culture medium without cells was used. After 5 min of exposure, cell viability was measured by MTT assay. The supernatant was discarded, the wells were washed with sterile Phosphate Buffer Saline (PBS, Cultilab, Brazil), and it was added to the solution of MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium, Sigma Aldrich], at the concentration of 0.5 mg/mL. 12 repetitions for each group were performed. After incubation for 1 hour at 37°C, the solution was removed and 100 μL of dimethyl sulfoxide (DMSO, Sigma Aldrich) was added. The plates returned to incubator for 10 minutes protected from light and were subsequently brought to the shaker for another 10 minutes. The absorbance of the wells was read under the wavelength of 570 nm in a microplate spectrophotometer (Bio-Tek, Winooski, Vermont, USA). The values of optical densities (OD) were converted into percentage of cell viability, compared with the control group [5].

2.4. Statistical Analysis. The data obtained were statistically analyzed by ANOVA and Tukey test, with significance level of 5% ($P \leq 0.05$), through the statistical program GraphPad Prism 5.0.

3. Results

In planktonic cultures, the extract of avocado tree showed antimicrobial activity for *C. albicans* with minimum inhibitory concentration (MIC) at 6.25 mg/mL and minimum microbicidal concentration (MMC) at 12.5 mg/mL.

C. albicans biofilms exposed for 5 min to different concentrations of the extract had significant reduction ($P < 0.001$) in the CFU/mL, regarding the control group, with the concentration of 50 mg/mL of *P. americana*, which reduced by $0.580 \log_{10} \pm 0.209$; in other words, this reduction corresponds to 74% (Figure 1).

The greatest reduction in CFU/mL of *C. albicans* biofilm was obtained with the concentration of 200 mg/mL of the extract, with a reduction in the number of CFU/mL of $1.093 \log_{10} \pm 0.462$ compared to the positive control group

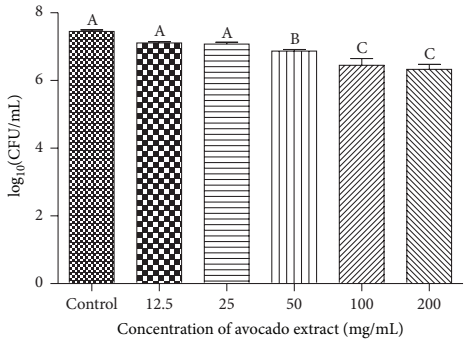

Figure 1: Number of CFU/mL ($\log_{10}$) obtained in the untreated group (control 0.9% NaCl) and treated group with different concentrations of the extract for 5 min ($n = 10$). ANOVA and Tukey test ($P < 0.05$). Different letters indicate statistically significant differences.

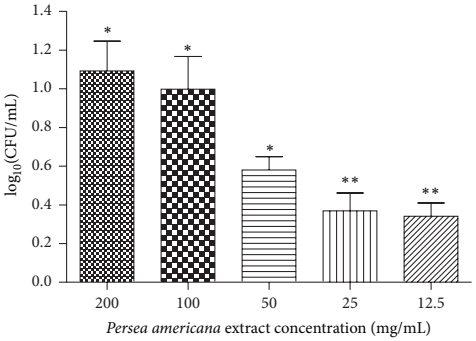

Figure 2: Reduction of *C. albicans* biofilm in the treated groups ($n = 10$). *Reduction statistically significant. **Reduction not statistically significant.

biofilm, which had no contact with the extract (Figure 2). At the concentration of 100 mg/mL the reduction of *C. albicans* was $0.998 \log_{10} \pm 0.508$ CFU/mL, which was statistically similar to the value obtained at the concentration of 200 mg/mL ($P > 0.05$). At concentrations of 25 and 12.5 mg/mL, microbial reduction was not statistically different from the control group ($P > 0.05$).

Regarding the cytotoxicity test of *P. americana* extract, it can be observed that all concentrations of the extract, except 0.1 mg/mL, promoted reduction compared to control ($P < 0.05$), and the concentrations of 200 mg/mL and 100 mg/mL were the most cytotoxic ones for macrophages. The concentration of 50 mg/mL promoted cell viability around 55% and, from 25 mg/mL of the extract, cell viability was higher than 70%. The cell viability extract concentration x values are shown in Figure 3, where the red line marks the limit of cell viability 50%.

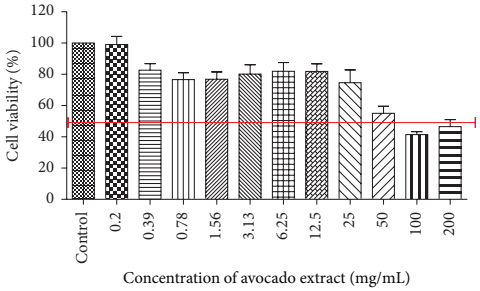

FIGURE 3: Cell viability of different concentrations of avocado comparing to control ($n = 8$).

4. Discussion

The glycolic extract of avocado *P. americana* showed effective antifungal activity against *C. albicans* with MIC of 6.25 mg/mL and CMM of 12.50 mg/mL in planktonic cultures and caused a significant reduction of the biofilm from the concentration of 50 mg/mL, which could reduce by $0.580 \log_{10} \pm 0.209$, representing a reduction of 74%. These results agree with Leite et al. [12], showing that the methanol and hexane extracts of avocado seed had significant antifungal action in five *Candida* species and two species of *Cryptococcus neoformans* and *Malassezia pachydermatis*.

The potential antifungal activity of avocado extract is relevant, since in the last 30 years there has been a significant increase in fungal infections in humans due mainly to the increased incidence of immunosuppressive diseases such as HIV, therapies affecting the immune system, such as chemotherapy, and use of broad-spectrum antibiotics [15].

We highlight the antifungal action of avocado extract on *C. albicans* since most fungal infections that affect man have as etiological agent fungi of the genus *Candida* and most isolates of human candidiasis are from *C. albicans*. Studies show that *C. albicans* resistance to antifungal drugs is due to biofilm formation [16, 17] and more precisely to the presence of the matrix, which prevents the penetration of the drugs into the biofilm and limits their action of fungus cells [18]. This scenario shows the demand for antifungal agents that have effective action on biofilms of *C. albicans*, as found in this study with glycolic extract of *P. americana*.

Several studies extracted chemical constituents from various parts of avocado, finding compounds of the classes of alkanes [19, 20], alkaloids, flavonoids, and steroids, among others. From the leaves of avocado a compound called persin [21] was extracted, with a chemical structure similar to a fatty acid, which showed antifungal activity against *Colletotrichum gloeosporioides* [22]. Furan derivatives were also isolated from avocado plant and were shown to have antibacterial, antifungal, and insecticide action [23]. Rodríguez-Carpena et al. [24] analyzed the antibacterial action of methanol extracts of peel, pulp, and seed of avocado fruit over five strains of Gram-positive and Gram-negative bacteria, finding greater action on Gram-positive bacteria. On the other hand, Guzmán-Rodríguez et al. [11] found that peptides extracted from the avocado fruit (*P. americana*) showed antimicrobial activity on *Escherichia coli* and *S. aureus* but were not effective against *C. albicans*. Even though the same plant (*P. americana*), different extracts or extracted products have different antimicrobial results, what is a remarkable point to be considered to expand the studies with a focus on its therapeutic use.

Regarding the cytotoxic test, it can be seen that the extract higher concentrations (10% and 20%) caused significant cell death compared to control; however, at the concentration of 5% that in the antimicrobial tests showed a significant reduction in the *C. albicans* biofilm, the viability of RAW 264.7 macrophages in contact for 5 min was greater than 55%. As the main focus of the study is a possible therapeutic use for the extract in mouthwashes and toothpastes (external use), the concentration of 5% promoted good results on the analysis of antimicrobial activity and cell viability. On the other hand, when the focus indication is, for example, cancer treatment, this plant has shown interesting results in cytotoxicity. Avocado fruit is rich in phytochemicals, which play an important role in inhibition of growth of cancer cells [25]. Khalifa et al. [26] examined the effect of aqueous extracts from *P. americana* fruit and from leaves of *Tabernaemontana divaricata*, *Nerium oleander,* and *Annona cherimola* (positive control) in *Vicia faba* root cells. The roots of *Vicia faba* were soaked in plant extracts dilutions of 100, 1.250, 2.500, 5.000, 10.000, and 20.000 ppm for 4 and 24 h. All treatments resulted in a significant reduction in the mitotic index in a dose-dependent manner, and *P. americana* showed the highest cytotoxic effects with the increase of the concentration. *P. americana* treatment showed the highest cytotoxic effect on cancer cells, prophase cell percentage increased linearly with the applied concentration, and no micronuclei were detected. This study shows that root tip assay of beans can be used in initial screening for new plant extracts to validate their use as candidates for containing active cytotoxic agents against malignant cells. This will greatly help in exploring new plant extracts as drugs for cancer treatment.

Thus, it can be seen that the studies with *P. americana* are still at the beginning and that many biological actions of its constituents need to be analyzed in order to expand its therapeutic indication in several areas of health, including dentistry because, with their potential antifungal effect, this extract may in a near future be used as an alternative substance/adjuvant in the treatment of oral candidiasis.

5. Conclusion

Glycolic extract of *P. americana* showed significant antifungal activity against *C. albicans* biofilm and the concentration of 5% (50 mg/mL) presented the best results on the analysis of antimicrobial activity and cell viability.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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3.10 Intracanal medications neutralize the effects of lipoteichoic acid to induce the production of proinflammatory cytokines and nitric oxide in macrophages RAW 264.7: *in vitro* study*

ABSTRACT

Gram-positive bacteria release lipoteichoic acid (LTA) as an important virulence factor. This study evaluated the in vitro ability of different intracanal medications to neutralize the effects of *Enterococcus faecalis* LTA to induce the production of pro-inflammatory cytokines (IL-1β, TNF-α, MIP-1α, IP-10, G-CSF and IL-6) and nitric oxide (NO) by macrophages (RAW 264.7). Forty eight human single-rooted teeth were standardized, sterilizated (Co60 gamma radiation) and three repeated inoculations of E. faecalis LTA were made every 24 hours. Canals instrumentation was performed with 5 rotary NiTi instruments (BioRaCe system), using pyrogen-free saline as irrigant. After EDTA, specimens were divided into 4 groups (n=12) according to the intracanal medication (MIC): HC) calcium hydroxide/saline solution; CLX) chlorhexidine gel 2%; CLX+HC) 2% chlorhexidine gel+calcium hydroxide; CONT) pyrogen-free saline. After 14 days, the samples were collected and macrophages were activated with these samples. After 24 hours, the production of pro-inflammatory cytokines was detected by ELISA and NO by Griess method. The results were statistically analyzed (ANOVA and Tukey test, 5%). The HC group showed significantly lower values of most of the evaluated cytokines (TNF-α, IL-1β, IL-6, G-CSF, MIP-1α) and nitric oxide when compared to CONT ($p<0.05$). The CLX+HC group also induced statistically lower production of most cytokines evaluated when compared to CONT ($p<0.05$), except for IL-1β and MIP-1α. Chlorhexidine had differing results according to the evaluated cytokines, so that only IL-6, G-CSF and nitric oxide showed significantly lower values compared to the CONT ($p<0.05$). It was concluded that all intracanal medications (HC, CLX and CLX+HC) neutralized different cytotoxic effects of LTA on macrophages, being HC and CLX+HC the most effective medications.

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Gram-positive bacteria release lipoteichoic acid (LTA) as an important virulence factor. This study evaluated the *in vitro* ability of different intracanal medications to neutralize the effects of *Enterococcus faecalis* LTA to induce the production of pro-inflammatory cytokines (IL-1β, TNF-α, MIP-1α, IP-10, G-CSF and IL-6) and nitric oxide (NO) by macrophages (RAW 264.7). Forty eight human single-rooted teeth were standardized, sterilizated (Co60 gamma radiation) and three repeated inoculations of *E. faecalis* LTA were made every 24 hours. Canals instrumentation was performed with 5 rotary NiTi instruments (BioRaCe system), using pyrogen-free saline as irrigant. After EDTA, specimens were divided into 4 groups (n=12) according to the intracanal medication (MIC): HC) calcium hydroxide/saline solution; CLX) chlorhexidine gel 2%; CLX+HC) 2% chlorhexidine gel+calcium hydroxide; CONT) pyrogen-free saline. After 14 days, the samples were collected and macrophages were activated with these samples. After 24 hours, the production of pro-inflammatory cytokines was detected by ELISA and NO by Griess method. The results were analyzed (ANOVA and Tukey test, 5%). The HC group showed significantly lower values of TNF-α, IL-1β, IL-6, G-CSF, MIP-1α and NO when compared to CONT ($p < 0.05$). CLX+HC group also induced statistically lower production of most cytokines when compared to CONT ($p < 0.05$), except for IL-1β and MIP-1α. Chlorhexidine had differing results according to the evaluated cytokines, only IL-6, G-CSF and NO showed significantly lower values compared to CONT ($p < 0.05$). It was concluded that all medications neutralized different cytotoxic effects of LTA on macrophages, being HC and CLX+HC the most effective medications.

Keywords: lipoteichoic acid, cytokines, nitric oxide; intracanal medication; root canal therapy

INTRODUCTION

Primary infections of the root canals are polymicrobial with a predominance of Gram-negative anaerobic bacteria, especially of genuses *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Campylobacter*, *Tannerella*, *Treponema* and *Dialister* (1,2). Gram-negative bacteria, during its multiplication or death, release endotoxin (LPS) from the cell wall, which have significant cytotoxic effects. Since 1975, the literature has been reporting the presence of endotoxins in infected root canals and their importance in the induction and maintenance of pulp and periapical changes (2,4-10). On the other hand, in the secondary and/or persistent infection of root canals, Gram-positive bacteria are predominant. Murad et al. (11) found that *Enterococcus faecium* and *Staphylococcus*

epidermidis were the most prevalent species in persistent infections of root canals, followed by *E. faecalis*, *Parvimonas microns*, *Eubacterium saburreum*, *Streptococcus sanguis*, *Staphylococcus waneri*. A recent study (12) showed *Enterococcus faecalis* as the most frequently isolated in secondary endodontic infections. Thus, secondary infections have complex and mixed profile of microorganisms belonging to the genuses *Enterococcus*, *Actinomyces*, *Streptococcus*, *Lactobacillus*, *Candida*, *Propionibacterium*, *Staphylococcus*, *Eubacterium* and *Bifidobacterium*. Species like *Pseudoramibacter alactolyticus*, *Parvimonas microns*, *Filifactor alocis* and *Olsenella uli* may also be present in these infections (1,13).

The genus *Enterococcus* is resistant to several antimicrobial substances used during endodontic treatment and may be favored by changes in root canal ecosystem and establish a persistent infection. Among the major virulence factors of *E. faecalis*, we have: lipoteichoic acid (LTA), gelatinase, hyaluronidase, cytolysin, aggregation substance, pheromones and heat shock proteins (14). Thus, the Gram-positive bacteria have a significant role in endodontic failure cases, being important to extend the studies on its virulence factors in order to develop an endodontic treatment protocols capable of destroying these microorganisms and inactivate its virulence factors, which may potentially influence the apical periodontitis progression (15).

In contrast to Gram-negative bacteria, Gram positive bacteria, do not express LPS but lipoteichoic acid (LTA), which is similar to LPS in both, structure and immunological properties (15). Anionic polymers such as LTA, are the main components of the Gram-positive cell wall, representing 50% of its dry weight (16). LTA shows amphipathic structure formed by linking the poly glycerophosphates to fatty acids and it is involved in inflammatory response, septic syndrome, biofilm formation and adhesion to tooth activity due to its adsorption to hydroxyapatite, which is a feature related to its penetration into the dentin tubules (15-17).

Studies demonstrated that LTA can activate macrophages and induce the release of nitric oxide and proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, and suggested that the ability of LTA to influence immune responses is species dependent and that the cell activation occurs by signaling via TLR-2 receptor (16,18-20), differing from LPS, that promotes cell activation via TLR-4 receptor (2,19), so that LPS and LTA induced different patterns of release of cytokines. Von Aulock von et al. (21) demonstrated that lipoteichoic acid induced greater release of IL-8, macrophage chemotactic protein-1 (MCP-1), leukotriene B4 (LTB4) and granulocyte colony-

stimulating factor (G-CSF) than LPS. However, compared to LPS, knowledge about the actions of LTA *in vivo* and *in vitro* is relatively small. As reported by van Amersfoort et al. (18), both LTA and peptidoglycan cause *in vivo* release of nitric oxide, TNF-α and IFN-γ and induce circulatory failure that indicates that components of Gram-positive bacteria induce LPS-like effects. With that, due to LTA similarity to LPS in various biological effects, it has been considered as its Gram-positive corresponding (16).

LTA can accumulate in the tissues in which it resides, eg *E. faecalis*, as in periapical lesions and, due to its high affinity to hydroxyapatite, when released, it can accumulate and persistently reside in the dentinal tubules, promoting continuous stimulation in tissue (15). Thus, further studies *in vitro* and *in vivo* are necessary to understand the role of LTA in the residual pulp tissues and progression of periapical lesions and to know how to eliminate them effectively from the root canal trough endodontic therapy. Oliveira & Barbosa (22) evaluated the effects of *Escherichia coli* LPS and *Enterococcus faecalis* LTA on the dental pulp of dogs and found that both LPS and LTA promoted the destruction of the pulp tissue. Baik et al. (15) showed that pre-treatment of LTA with calcium hydroxide annulled its ability to induce release of TNF-α, and also that LTA treated with calcium hydroxide was not capable of stimulating TLR-2 receptor. Lee et al. (17) found that pre-treatment of LTA with 2% chlorhexidine for 6 hours or with 0.2% chlorhexidine for 24 hours significantly reduced the ability to induce the production of TNF-α in macrophages (RAW 264.7), and also that chlorhexidine annulled the ability of LTA to stimulate TLR-2 receptor. Baik et al. (23) demonstrated that treatment of LTA with calcium hydroxide for 24 hours significantly inhibited the production of nitric oxide, MIP-1α (macrophage inflammatory protein-1α) and IP-10 (interferon gamma -inducible protein 10) in macrophages culture (RAW 264.7) and, also, free fatty acids released from LTA treated with calcium hydroxide were detected.

Regarding LPS, it is well established the effect of calcium hydroxide to promote the hydrolysis of its lipid portion releasing a large amount of free fatty acids, that inactivates the toxic effects of LPS (24). In root canals, several studies have demonstrated the effects of calcium hydroxide or association of chlorhexidine gel with calcium hydroxide as intracanal medication to neutralize LPS (6,8,25-30), however, researches in literature, evaluating the action of these medications on LTA in root canals are not found. Once known that LTA has important immunostimulatory activity, inducing the production and release of chemical mediators and different cytokines by

macrophages and that it may participate in the development and maintenance of periapical lesions, as well as LPS, studies of instrumentation techniques associated with the auxiliary chemicals such as the use of intracanal medications on LTA in root canals should be expanded to improve endodontic therapy and establish more efficient protocols that provide a higher success rate to the treatment. On the basis of these evidences currently available and focusing on the lack of information and studies about LTA biological activities in root canals, the aim of this study was to evaluate *in vitro* ability of different intracanal medications (calcium hydroxide, 2% chlorhexidine gel and calcium hydroxide associated with chlorhexidine gel 2%) to neutralize the effects of *E. faecalis* LTA to induce the production of pro-inflammatory cytokines (IL-1β, TNF-α, MIP-1α, IP-10, G-CSF and IL-6) and nitric oxide by macrophages (RAW 264.7).

MATERIAL AND METHODS

Preparation of Specimens

Forty eight human single-rooted teeth, recently extracted for various clinical indications and prior authorization of patients through free and informed consent, were used. This study was approved by the Ethics Committee in Research of the Institute of Science and Technology - UNESP. The selection of teeth was made based on the dimensions and morphological similarity of the root with the crowns sectioned, standardized at the size of 16 mm. The root canals were previously prepared, in order to standardize the diameter, with nickel-titanium instruments (NiTi) of BioRaCe system (FKG Dentaire - Swiss Dental Products - Switzerland) coupled to an electric motor with VDW.Silver contra angle handpiece, in the following sequence: BR0 (25/12:08), for enlargement of the cervical and middle thirds of the preparation; BR1 (15/12:04) up to work length (15 mm); BR2 (25/12:04) up to work length (15 mm), always using 5 mL of saline solution at each change of instrument.

Following, the root canals were filled with ethylenediaminetetraacetic acid (EDTA) 17% for 3 min and irrigated with 10 mL of saline solution. The root canals were dried with the aid of paper points number 30 and the apical region was sealed using light cured composite resin Z-100 (3M Dental Products, St Paul, USA), and the roots were coated externally with two layers of adhesive epoxy, except the region of the cervical gap (28,29). Then the specimens were randomly distributed and fixed with acrylic resin chemically activated in 4 microplates (n=12) of 24 wells (TPP). Specimens and all materials (files, tweezers, spatulas, scissors, gloves, etc.) were sterilized by

gamma irradiation with cobalt 60 (20 kGy for 6 hours) to neutralize preexisting endotoxins (6,8,9,26,28,29).

Experimental Groups

It was used *E. faecalis* LTA (Sigma L4015), prepared at a concentration of 250 µg/mL with bidistilled and pyrogen-free water. Three aliquots of 10 µL of the LTA solution were inoculated within the root canals every 24 h and during the interval of each inoculation, the specimens were kept at 37°C, with relative humidity, for 24 h.

Twenty four hours after the third inoculation of LTA, the instrumentation of root canals were performed with 5 NiTi rotary instruments (BioRaCe system) in the following sequence: BR3 (25/00:06), BR4 (35/00:04), BR5 (40/0:04) BR6 (50/12:04) and BR7 (60/0:02). The instruments were mounted on the contra angle handpiece, connected to the electric motor VDW.Silver, according to the manufacturer's specifications, and the speed of 500 rpm and torque of 1 Ncm. At each exchange of instrument, the canals were irrigated with 5 mL of pyrogen-free saline. Ordering to perform the irrigation, tips of Navitip system (Ultradent – Products – Inc - USA) attached to pyrogen-free syringes of 5 mL (Injex) and for aspiration White Mac Tips (Ultradent) attached to a vacuum pump cannula were used.

After instrumentation, the canals were flooded with EDTA 17% for 3 min and washed with 5 mL of sterile and pyrogen-free saline (Aster). The canals were dried with paper points and filled with intracanal medication (MIC). According to the MIC, the specimens were divided into four experimental groups (n=12): HC) calcium hydroxide paste with sterile and pyrogen-free physiological solution; CLX) chlorhexidine gel 2%; CLX+HC) chlorhexidine gel 2% paste + calcium hydroxide PA; CONT) pyrogen-free saline (control). Medication was placed into the root canals with spiral Lentulo. After 14 days at 37°C and relative humidity of 100%, MIC was removed with irrigation of 10 mL of pyrogen-free saline.

Three collections of root canals were performed: S1) immediately after instrumentation; S2) after EDTA; S3) after MIC. For this pourpose, the root canals were flooded with sterile and pyrogen-free saline, which was stirred and aspirated with the aid of an insulin-like syringe and needle. This procedure was repeated until completion of the final sample volume of 100 µL. These samples were used to verify if the intracanal medications used in the treatments described above have the ability to

neutralize the cytotoxic effects of lipoteichoic acid in macrophages culture (RAW 264.7).

Activation of Cell Culture

The Murine macrophage cell line RAW264.7 was obtained from the Technical-Scientific Association Paul Ehrlich cell bank (APABCAM, Rio de Janeiro, RJ), maintained in DMEM (LGCBio, São Paulo) supplemented with 10% fetal bovine serum (Invitrogen) and incubated at 37ºC in a 5% CO_2 humidified incubator. After counting viable cells in a Neubauer chamber, $5x10^5$ cells/mL of DMEM/well were distributed in a 24-well microplate (TPP). Cells were maintained at 37/5% CO_2 for 24 h. These cells were activated with 30 µl of each sample collected from root canals (6,8,2) and incubated at 37°C/5% CO_2. After 24 hours, supernatants were removed and frozen (-20ºC) for subsequent detection and quantification of cytokines (IL-1β, TNF-α, MIP-1α, IP-10, G-CSF and IL-6) by immunosorbent assay (ELISA) and nitric oxide by the Griess method (23).

Quantification of cytokines and nitric oxide

Ordering to perform ELISA DuoSet kits anti-IL-1β, anti-TNF-α, anti-MIP-1α anti-IP-10, anti-G-CSF and anti-IL-6 (R & D Systems, NS) were used, according to the manufacturer recommendations. After obtaining the optical densities, the levels of cytokines (pg/mL) present in the culture supernatants of macrophages was determined using GraphPad Prism 5.0.

Nitric oxide production in the culture supernatants of macrophages was determined indirectly by the nitrite concentration detected by the Griess reagent (Sigma) in comparison with the standard curve nitrite (Sigma). Optical densities were read on a microplate reader with a wavelength of 520 nm reader.

All results were statistically analyzed by ANOVA, with a significance level of 5%, and Tukey test.

Results

TNF-α

Comparing the three samples of the same group, it can be observed that all groups showed a significant increase in the production of TNF-α in S3, when compared to the samples S1 and S2 ($p<0.05$). The mean values of TNF-α (pg/mL) in the group HC

were: S1) 3.67 ± 3.32 pg/mL, S2) 1.44 ± 2.39 pg/mL, S3) 198.68 ± 152 , 55 pg/ml; group CLX: S1) 2.64 ± 3.20 pg/ml; S2) 2.37 ± 2.20 pg/ml; S3) 515.36 ± 427.13 pg/mL; group CLX+HC: S1) 3.25 ± 7.25 pg/ml; S2) 0.00 ± 0.00 pg/ml; S3) 59.36 ± 22.56 pg/mL; group CONT: S1) 2.13 ± 2.67 pg/ml; S2) 0.00 ± 0.00 pg/ml; S3) 722.21 ± 379.95 pg/mL. When comparing different intracanal medications (MIC), there were significant differences between groups in S3 collection ($p<0.05$). Mean values, standard deviations and formation of homogeneous groups are presented in Table 1. The group CLX+HC showed a lower production of TNF-α, being similar to HC ($p>0.05$) and different from other groups ($p<0.05$). The group CONT had the highest production, being different from groups CLX+HC and HC ($p<0.05$) and similar to the CLX group ($p>0.05$).

IL-1β

Comparing the three samples of the same group, it was observed that in the group HC, all samples showed similar values ($p>0.05$) of IL-1β: S1) 0.40 ± 1.27 pg/mL, S2) 1.72 ± 2.18 pg/ml and S3) 0.64 ± 1.69 pg/ml. In the group CLX, the collection S3 (10.02 ± 3.52 pg/ml) induced significantly higher production of IL-1β ($p<0.05$) compared to samples S1 (1.56 ± 1.96 pg/mL) and S2 (2.50 ± 2.58 pg/ml). In the group CLX+HC, S1 and S2 samples showed undetectable levels of IL-1β, being different ($p<0.05$) from S3 (2.68 ± 2.51 pg/ml). In the control group CONT, the collection S3 induced higher production of IL-1β (S3: 4.65 ± 2.43 pg/mL), which was statistically similar to S1 (2.18 ± 2.27 pg/mL) and different from S2 (0.63 ± 1.29 pg/ml). Regarding the MIC, there were significant differences between groups after collection S3 ($p<0.05$). Mean values, standard deviations and formation of homogeneous groups are presented in Table 1. The group HC showed a lower production of IL-1β, being similar to the group HC+CLX ($p>0.05$) and different from other groups ($p<0.05$). The group CLX had the highest production of IL-1β, unlike the other groups ($p<0.05$). The group CONT showed intermediate values, similar to the group HC+CLX ($p>0.05$) and different from the others ($p<0.05$).

IL-6

When comparing 3 collections of the same group, it can be observed that all groups showed a significant increase of IL-6 in the collecting S3 compared to

collections S1 and S2 ($p < 0.05$), except in the group HC, that reported similar results ($p > 0.05$) in the three collections. The mean values of IL-6 (pg/ml) in the group HC were: S1) 0.81 ± 1.11 pg/ml, S2) 0.48 ± 0.55 pg/ml, S3) 1.93 ± 2 , 06 pg/ml; group CLX: S1) 1.85 ± 1.40 pg/ml, S2) 2.12 ± 0.72 pg/ml, S3) 17.11 ± 11.68 pg/mL; group CLX+HC: S1) 0.17 ± 0.49 pg/ml, S2) 0.01 ± 0.06 pg/ml, S3) 3.91 ± 1.81 pg/ml; group CONT: S1) 1.33 ± 1.36 pg/ml, S2) 0.14 ± 0.45 pg/ml, S3) 76.91 ± 83.76 pg/mL. Comparing the different MIC, it was found significant differences between groups at the collection S3. Mean values, standard deviations and formation of homogeneous groups are presented in Table 1. The groups HC and HC+CLX showed the lowest levels of IL-6 being similar between themselves ($p > 0.05$) and to the group CLX ($p > 0.05$) but different from the group CONT ($p < 0.05$). The group CONT showed higher levels of IL-6, being different from all groups evaluated ($p < 0.05$).

Granulocyte colony stimulating factor (G-CSF)

In a comparison among the three collections in the same group, it was observed that in all groups, except for the group CONT, the values of G-CSF (pg/mL) in the three collections were statistically similar ($p > 0.05$). In the group CONT, the collecting S3 induced statistically greater production of G-CSF compared to collections S1 and S2 ($p < 0.05$). The average values of G-CSF (pg/mL) for group HC were: S1) 2.01 ± 4.75 pg/mL, S2) 7.39 ± 6.54 pg/mL, S3) 8.48 ± 12.61 pg/mL; group CLX: S1) 8.45 ± 5.81 pg/mL, S2) 12.30 ± 12.28 pg/mL, S3) 48.29 ± 88.38 pg/mL; group CLX+HC: S1) 8.10 ± 1.06 pg/mL, S2) 9.76 ± 2.96 pg/mL, S3) 10.89 ± 3.20 pg/mL; group CONT: S1) 25.58 ± 14.87 pg/mL; S2) 14.21 ± 12.77 pg/mL; S3) 1768.69 ± 640.36 pg/mL. Comparing the different MIC, it was observed statistical difference among groups, being similar among themselves the groups HC, CLX and CLX+HC ($p > 0.05$) once they had significantly lower G-CSF values when related to the group CONT ($p < 0.05$). Mean values, standard deviations and the formation of homogeneous groups are shown in Table 1.

MIP-1α (macrophage inflammatory protein-1α)

Comparing the 3 collections in the same group, it can be observed that in all groups, except for the group HC, there was significant increase in the production of MIP-1α in the collecting S3 compared to the collections S1 and S2 ($p < 0, 05$). The group HC, the collection S3 induced statistically lower production of MIP-1α in relation to the collections S1 and S2 ($p > 0.05$). The mean values of production of MIP-1α (pg/mL)

obtained with each collection for the group HC were: S1) 21.43 ± 7.7 pg/mL, S2) 25.90 ± 6.7 pg/mL, S3) 6.14 ± 2.37 pg/mL; group CLX: S1) 14.36 ± 4.7 pg/mL, S2) 13.03 ± 9.3 pg/mL, S3) 110.38 ± 11.3 pg/mL; group CLX+HC: S1) 15.48 ± 9.1 pg/mL, S2) 14.63 ± 2.84 pg/mL, S3) 103.09 ± 21.0 pg/mL; group CONT S1) 10.64 ± 1.8 pg/mL; S2) 29.46 ± 20.3 pg/mL; S3) 91.92 ± 25.1 pg/mL. Comparing the MIC, it can be found an important difference after the collection S3. Mean values, standard deviations and formation of homogeneous groups are presented in Table 1. The group HC showed the lowest production of MIP-1α, being different from all the other groups ($p < 0.05$).

Nitric oxide

Comparing the 3 collections in the same group, it was found in the groups CLX and CONT significant increase in nitric oxide release in the collection S3 compared to S1 and S2 ($p<0.05$). In the group HC, 3 collections showed similar induction of nitric oxide production ($p<0.05$) and in the group CLX+HC, the collection S3 was similar to S1 and different from S2. Mean values for nitric oxide production (pg/mL) in the group HC were: S1) 0.25 ± 0.14 pg/mL, S2) 0.13 ± 0.14 pg/mL, S3) 0.18 ± 0.11 pg/mL; group CLX: S1) 0.24 ± 0.12 pg/mL, S2) 0.22 ± 0.05 pg/mL, S3) 0.37 ± 0.12 pg/mL; group CLX+HC: S1) 0.24 ± 0.08 pg/mL, S2) 0.18 ± 0.07 pg/mL, S3) 0.33 ± 0.11 pg/mL; group CONT: S1) 0.36 ± 0.12 pg/mL, S2) 0.22 ± 0.10 pg/mL, S3) 0.54 ± 0.19 pg/mL. Comparing the MIC, it was noted that the group HC showed the lowest production of nitric oxide, similar to the group CLX+HC ($p>0.05$) and different from the other groups ($p<0.05$). The group CONT had the highest production of nitric oxide, different from all groups ($p<0.05$). Mean values, standard deviations and the formation of homogeneous groups are shown in Table 1.

Table 1 - Mean values, standard deviation and formation of homogeneous groups of the production of cytokines (pg/mL), obtained with the third collection (S3) of each group after intracanal medication (MIC).

Groups	Mean values ± Standard deviation (pg/mL)					
	TNF-α	IL-1β	IL-6	G-CSF	MIP-1α	NO
HC	**198,68 ± 152,55^{AB}**	**0,64 ± 1,69^{A}**	**1,93 ± 2,06^{A}**	**8,48 ± 12,6^{A}**	**6,14 ± 2,37^{A}**	**0,18 ± 0,11^{A}**
CLX	**515,36 ± 427,13^{BC}**	**10,02 ± 3,52^{C}**	**17,11 ± 11,68^{A}**	**48,29 ± 88,3^{A}**	**110,38 ± 11,3^{B}**	**0,37 ± 0,12^{B}**
CLX+HC	**59,36 ± 22,56^{A}**	**2,68 ± 2,51^{AB}**	**3,91 ± 1,81^{A}**	**10,89 ± 3,2^{A}**	**103,09 ± 21,0^{B}**	**0,33 ± 0,11^{AB}**
CONT	**722,21 ± 379,95^{C}**	**4,65 ± 2,43^{B}**	**76,91 ± 83,76^{B}**	**1768,69 ± 640,3^{B}**	**91,92 ± 25,1^{B}**	**0,54 ± 0,19^{C}**

NO: nitric oxide; HC: calcium hydroxide; CLX: 2% chlorhexidine gel; CLX+HC: chlorhexidine gel + calcium hydroxide; CONT: pyrogen-free saline.

IP-10 (interferon-gamma inducible protein 10)

Comparing the 3 collections of the same group, it was found that all groups showed significant increases in production of IP-10 in the collecting S3, when compared to S1 and S2 ($p<0.05$). The average values of IP-10 (in optical density, OD) in the group HC were: S1) 0.041 ± 0.01, S2) 0.028 ± 0.01, S3) 0.196 ± 0.11; group CLX: S1) 0.036 ± 0.01; S2) 0.029 ± 0.02; S3) 0.209 ± 0.06; group CLX+CH: S1) 0.03 ± 0.060, S2) 0.015 ± 0.01, S3) 0.157 ± 0.07; group CONT: S1) 0.022 ± 0.02, S2) 0.060 ± 0.03, S3) 0.278 ± 0.09. Comparing the different MIC, it was found differences with the collection S3, the group CONT had the highest production of IP-10, however, it was similar to the groups HC and CLX ($p>0.05$). The group CLX+HC showed the lowest production of IP-10, similar to the groups CLX and HC ($p> 0.05$) and different from CONT ($p <0.05$). Mean values, standard deviations and the formation of homogeneous groups are shown in Table 2.

Table 2 - Mean values, standard deviation and formation of homogeneous groups of the production of IP-10 (optical density, OD), obtained with the third collection (S3) of each group after intracanal medication (MIC).

Groups	Mean values ± Standard deviation (OD)
HC	**0,196 ± 0,11^{AB}**
CLX	**0,209 ± 0,06^{AB}**
CLX+HC	**0,157 ± 0,07^{A}**
CONT	**0,278 ± 0,09^{B}**

HC: calcium hydroxide; CLX: 2% chlorhexidine gel; CLX+HC: chlorhexidine gel + calcium hydroxide; CONT: pyrogen-free saline

Discussion

In the present study, it was found that the instrumentation with NiTi rotary instruments associated with pyrogen-free physiological solution as irrigating for the root canal preparation caused a significant reduction in the LTA cytotoxic effects in macrophages (RAW 264.7), which indicates success of this instrumentation protocol to eliminate the present LTA in the the root canals. Low or even undetectable amounts of TNF-α, IL-1β, IL-6, G-CSF, nitric oxide, MIP-1α and IP-10 were observed in the collection immediately after instrumentation (S1), but these results are difficult to discuss, once there are no studies observed in the literature that evaluate the effectiveness of rotary instrumentation on LTA, but only on LPS. Although using a different methodology, Martinho et al. (31) found that rotary instrumentation with NiTi instruments significantly reduced the amount of endotoxin in the root canals, in agreement with the present study. As there are no commercial kits for LTA quantification, like those that exist for endotoxin (LPS), the way found to evaluate the effects of different treatment protocols was trhough cell activation, once LTA induces the production of several pro-inflammatory cytokines and nitric oxide by macrophages (19,20,32).

Despite the collections immediately after instrumentation have induced low production of cytokines, demonstrating effectiveness of the instrumentation protocol, it was found that in the group CONT, which received only saline during the period of 14 days of the medication, there was a significant increase in the production of all the evaluated cytokines (TNF-α, IL-1β, IL-6, G-CSF, MIP-1α and IP-10) and nitric oxide ($p<0.5$) in the collection S3. With this, it was verified that LTA was also present inside the root canals system, requiring its neutralization with the use of effective intracanal medications. Along similar lines, these results agree with Baik et al. (2008), who reported that LTA has a high affinity to hydroxyapatite, which can accumulate and reside persistently inside the dentin tubules.

Regarding intracanal medications evaluated, the best results were obtained with calcium hydroxide, which was able to neutralize the cytotoxic effects of LTA to induce the production of most of the evaluated cytokines (TNF-α, IL-1β, IL-6, G-CSF, MIP-1α) and nitric oxide by macrophages (RAW 264.7), presenting, in the collection S3, significantly lower values when compared to the group CONT (saline), except for the chemokine IP-10. These results agree with Baik et al. (15,23), that demonstrated that the pretreatment of LTA with calcium hydroxide inhibits its ability to induce the release of

TNF-α, nitric oxide and MIP-1α in macrophages (RAW 264.7), because deacylated LTA can not stimulate the receptor TLR2, and for this reason it is unable to stimulate the expression of pro-inflammatory cytokines. Ryu et al. (33) also found that the ability of LTA to induce nitric oxide and TNF-α was abolished when LTA was treated with 0.2 N sodium hydroxide, demonstrating that the alkaline treatment can inactivate LTA. As it happens on LPS, calcium hydroxide promotes deacylation of LTA, resulting in the release of free fatty acids and the loss of their immunostimulatory activity (20,23). Therefore, the available evidence seems to point that, when used as intracanal medication for 14 days, calcium hydroxide was effective in reducing the cytotoxic effects of LTA in macrophages.

Chlorhexidine showed conflicting results according to the evaluated cytokine, so that, only IL-6, G-CSF and nitric oxide only chlorhexidine showed significantly lower values compared to control. Lee et al. (17) showed that pretreatment of LTA with 2% chlorhexidine for 6 hours or with 0.2% chlorhexidine for 24 hours caused a significant decrease in its ability to induce the production of TNF-α in macrophages (RAW 264.7), via receptor TLR-2. According to Zorko & Jerala (34), chlorhexidine can connect both, LTA (by electrostatic interactions) and LPS (hydrophobic interactions), preventing cellular activation via TLR2 and TLR4, respectively. However, according to the results of this study, it was observed that chlorhexidine can neutralize some of the cytotoxic effects of LTA in macrophages, such as the ability of induction of the production of IL-6, G-CSF and nitric oxide, but not TNF-α, IL-1β, IP-10 and MIP-1α. Thus, it can be observed that the chlorhexidine, when used as an intracanal medication, was unable to promote effective neutralization of LTA at all the pro-inflammatory cytokines, with the need to expand the studies on the role of chlorhexidine on LTA and intracellular signaling pathways involved in the production of these cytokines and chemical mediators.

Regarding the association of chlorhexidine and calcium hydroxide as an intracanal medication, the results provide confirmatory evidence that there was an effective neutralization of the cytotoxic effects of LTA, leading to a statistically lower production of most of the evaluated cytokines (TNF-α, IL-6, G-CSF, IP -10) and nitric oxide compared to the control, except for IL-1β and MIP-1α. Studies in the literature that evaluated this association on LTA were not found, however, about LPS, Oliveira et al. (8) found that in *in vivo* assays, this intracanal medication (CLX+HC) produced significant neutralization of endotoxin, with reduction in the root canals that range from

99.2 to 100% and neutralization of their cytotoxic effects (TNF-α and IL-1β). Valera et al. (28) also observed *in vitro* reduction of LPS in root canals that received HC or HC+CLX as intracanal medications. Thus, knowing that LPS and LTA induce release of several pro-inflammatory cytokines and chemical mediators that activate different cell receptors (34,35), it becomes important to use intracanal medications that have the ability to neutralize LPS and LTA. According to the results of this study, both HC and HC+CLX promoted significant reduction in the cytotoxic potential of LTA in macrophages, being these results the pioneer in the endodontic literature.

Conclusion

On the basis of the evidence obtained in this study, it was concluded that all the medications were able to neutralize the ability of LTA of inducing the production of IL-6, G-CSF and nitric oxide by macrophages, when compared to the control group. Nonetheless, the calcium hydroxide was the most effective medication, once it reduced the ability of LTA to induce the production of most of the evaluated cytokines and nitric oxide, followed by the association of chlorhexidine and calcium hydroxide.

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4 CONSIDERAÇÕES FINAIS

A Odontologia passa por inúmeros avanços tecnológicos, no entanto, na parte terapêutica, são poucos os avanços obtidos que proporcionam mudanças na prática clínica. Na Endodontia, em particular, vemos uma evolução nos sistemas de preparo dos canais radiculares, com diferentes instrumentos oscilatórios, rotatórios, reciprocantes e muitos deles, em associação com as substâncias químicas auxiliares tradicionais (hipoclorito de sódio e clorexidina), têm proporcionado redução significativa de micro-organismos e endotoxinas nos canais (Neves et al., 2015; Marinho et al., 2015a; Marinho et al., 2015b; Marinho et al., 2014; Martinho et al., 2014). No entanto, em especial a endotoxinas, esta redução se deve principalmente à técnica de instrumentação e ao ato de irrigar e aspirar, independente da utilização de hipoclorito de sódio ou clorexidina (Marinho et al., 2014). Porém, quando medicações intracanais a base de hidróxido de cálcio são utilizadas, observamos significativa redução/neutralização de endotoxinas, com diminuição do potencial citotóxico do conteúdo coletado dos canais radiculares, como demonstrado nos artigos apresentados nos Capítulos 3.1, 3.2 e 3.4.

Há mais de 20 anos, Safavi & Nichols (1993, 1994) demonstraram a capacidade do hidróxido de cálcio em romper as ligações ester da porção lipíca de LPS liberando ácidos graxos livres e, com isso, promovendo detoxificação/neutralização de LPS, inclusive inibindo a produção de prostaglandina E_2 em cultura de monócitos estimulados com LPS tratado com hidróxido de cálcio. Na sequência, vários autores demonstraram o potencial do hidróxido de cálcio em reduzir ou neutralizar

os efeitos citotóxicos de LPS (Barthel et al., 1997; Nelson-Filho et al., 2002; Silva et al., 2002; Tanomaru et al., 2003). No artigo do capítulo 3.1, verificamos *in vitro* que o hidróxido de cálcio, quando usado como medicação intracanal por 7 dias, alterou a habilidade da endotoxina de estimular a produção de anticorpos em cultura de linfócitos B. No artigo do capítulo 3.4, em estudo *in vivo* também verificamos que quando hidróxido de cálcio em associação a clorexidina gel foi utilizado como medicação intracanal por 14 dias houve importante neutralização dos efeitos citotóxicos, com diminuição significativa da produção de TNF-α por macrófagos estimulados pelo conteúdo do canal. Estes resultados concordam com diferentes autores que utilizaram hidróxido de cálcio (sozinho ou associado a clorexidina) como medicação intracanal e verificaram redução do potencial citotóxico do conteúdo coletado dos canais (Marinho et al., 2015; Sousa et al., 2014; Marinho et al., 2014; Xavier et al., 2013). Assim, já está bem estabelecido que o hidróxido de cálcio, como medicação intracanal, consegue neutralizar endotoxinas.

Considerando esta importante ação do hidróxido de cálcio, no artigo do Capítulo 3.2 analisamos *in vitro* seu potencial como agente irrigante, utilizando água de cal [$(Ca)OH_2$ 0,14%], em comparação com hipoclorito de sódio 2,5% e 5,25% e clorexidina 2%. Os resultados demonstraram que hipoclorito de sódio e clorexidina não detoxificaram LPS, porém, o hidróxido de cálcio foi efetivo como irrigante. Com este resultado, propomos num estudo *in vivo* (Capítulo 3.4) associar a água de cal no final da instrumentação endodôntica, utilizando clorexidina como substância química auxiliar. Os resultados demonstraram que o uso combinado de clorexidina e água de cal promoveu redução significativamente maior de endotoxinas nos canais em comparação com o grupo que só utilizou clorexidina. No entanto, o potencial citotóxico só foi significativamente diminuído quando foi utilizada medicação intracanal (pasta de hidróxido de cálcio e clorexidina) por 14 dias.

Na tentativa de ampliar a terapia endodôntica, e analisando a literatura, verificamos que o extrato de própolis apresenta potencial de uso na Odontologia, pois estudos demonstraram sua importante ação antimicrobiana e anti-inflamatória (Awawdeh et al., 2009; Ozan et al., 2007a; Ozan et al., 2007b; Santos et al., 2002). Em 2010 (Capítulo 3.3), avaliamos *in vitro* a ação do extrato glicólico de própolis 12% como agente irrigante sobre *E. coli* e endotoxinas em canais radiculares. Os resultados demonstraram importante ação antimicrobiana do extrato de própolis sobre *E. coli*, o qual promoveu ausência de crescimento microbiano após a instrumentação, com efeito residual após 7 dias. No entanto, com a morte das bactérias houve grande liberação de endotoxinas e o extrato de própolis não foi efetivo na sua neutralização, embora tenha promovido redução significativa em relação ao controle (solução salina). Somente após o uso das medicacões contendo hidróxido de cálcio, os níveis de endotoxinas ficaram baixos. Contudo, desde 2010 mais de 100 artigos foram publicados no *PubMed* com diferentes tipos de própolis, demonstrando seu potencial uso terapêutico na Odontologia em diversas finalidades, tais como medicação intracanal (Carbajal et al., 2014), dentifrício (Bhat et al., 2015), enxaguatório bucal (Ercan et al., 2015), meio de estoque para dentes avulsionados (Ahangari et al., 2013), desinfetante cavitário (Prabhakar et al., 2015), dessensibilizante dentinário (Chen et al., 2015), entre outros, sendo um importante agente antimicrobiano alternativo, que deveria ter seu uso clínico odontológico mais difundido.

Continuando a busca por substâncias antimicrobianas alternativas para uso odontológico, houve grande interesse em estudar extratos de plantas, uma vez que a população mundial utiliza extratos, óleos e chás desde tempos remotos, com sucesso clínico em inúmeras enfermidades, entretanto, com poucos estudos científicos que comprovam sua eficácia e segurança clínica. Diante da grande diversidade de plantas com potencial medicinal, especialmente nos países tropicais e

subtropicais como o Brasil, a escolha de qual planta estudar foi baseada em seu uso popular. Assim, em 2013 (Capítulo 3.5), avaliamos *in vitro* a atividade antimicrobiana dos extratos glicólicos de gengibre (*Zingiber officinale*), *Aloe vera* e óleo essencial de mamona (*Ricinus communis*) sobre *C. albicans* e *E. faecalis* em canais radiculares, em comparação com hipoclorito de sódio 2,5% e clorexidina 2%. Concluiu-se que como substâncias químicas auxiliares do preparo dos canais radiculares, hipoclorito de sódio e clorexidina foram as mais efetivas na eliminação de *C. albicans* e *E. faecalis*, sendo seguidos pelo extrato de gengibre e óleo de mamona, já o extrato de *Aloe vera* não apresentou atividade antimicrobiana. Leite et al. (2014) demonstraram ação antimicrobiana de dentifrícios experimentais contendo óleo de *Ricinus comunis* sobre *E. faecalis, S. mutans, S. aureus*, no entanto, sem ação sobre *E. coli, C. albicans* e *C. glabrata*. Zarai et al. (2012) já tinham demonstrado maior suscetibilidade de bactérias Gram-positivas, como *Bacillus subtilis* e *S. aureus* ao óleo de *R. comunis*, enquanto *P. aeruginosa* e *E. coli* foram as mais resistentes ao óleo. Esta maior resistência de bactérias Gram-negativas aos óleos essenciais têm sido atribuída a presença da membrana externa hidrofílica que contém polissacarídeos hidrófilos que atuam como uma barreira ao óleo essencial hidrofóbico (Kordali et al., 2005; Harai et al., 2012). Assim, o extrato de *Z. officinale* apresentou maior potencial de uso intracanal, tendo seu estudo ampliado.

Ainda em 2013, Maekawa et al. analisaram os efeitos dos extratos glicólicos de *Zingiber officinale* e própolis, como medicação intracanal por 14 dias (associados ou não ao hidróxido de cálcio), sobre *E. faecalis, C. albicans, E. coli* e endotoxinas e os resultados demonstraram que todas as medicações foram capazes de eliminar os micro-organismos e reduzir a quantidade de endotoxinas, entretanto, as preparações contendo hidróxido de cálcio foram mais efetivas na neutralização de endotoxinas, sugerindo o uso combinado de medicações para obter maiores índices de sucesso, uma vez que o hidróxido de cálcio

sozinho não foi tão efetivo sobre *E. faecalis* e *C. albicans*, como os extratos de gengibre e própolis. Considerando os estudos anteriores que demonstraram ação antimicrobiana do extrato de gengibre, Valera et al. (2015), apresentado no Capítulo 3.8, analisaram *in vitro* alguns protocolos de terapia endodôntica utilizando extrato glicólico de *Z. officinale* 20% como substância química auxiliar em associação com diferentes medicações intracanais contendo hidróxido de cálcio. O extrato de gengibre demonstrou efetiva ação antimicrobiana sobre *E. faecalis, C. albicans* e *E. coli*, entretanto, as maiores reduções de endotoxinas só foram obtidas com as medicações contendo hidróxido de cálcio. Com isso, o uso terapêutico do extrato de gengibre na prática clínica deve ser ampliado, analisando seus efeitos sobre outros micro-organismos, incluindo bactérias anaeróbias estritas, um vez que os estudos demonstram seu grande potencial antimicrobiano.

Chakraborty et al. (2014) verificaram importante ação antimicrobiana do extrato de *Z. officinale* sobre cepas clínicas de *S. aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, E. coli, B. subtilis* e *Proteus mirabilis* resistentes a multi-antibióticos. Jain et al. (2015) verificaram ação do extrato de gengibre sobre *S. mutans*. Como as pesquisas utilizam diferentes preparações dos extratos (glicólico, aquoso, etanólico), torna difícil a discussão sobre possíveis mecanismos de ação, sendo este outro campo de estudo que precisa ser explorado.

A compreensão do mecanismo de ação antimicrobiano dos extratos de plantas, bem como os constituintes responsáveis por tais efeitos, certamente é de grande interesse para evolução da pesquisa e desenvolvimento de novos fármacos, no entanto, no momento estamos ainda realizando a triagem dos extratos, a fim de selecionar os que apresentam maior interesse para Odontologia e, posteriormente, aprofundar suas análises. Dessa forma, em 2013 (Capítulo 3.6), avaliamos a atividade antimicrobiana dos extratos de *Equisetum arvense, Glycyrrhiza glabra, Punica granatum* e *Stryphnodendron barbatiman* sobre

cepas ATCC e clínicas de *S. aureus, S. epidermidis, S. mutans, C. albicans, C. tropicalis e C. glabrata*, bem como analisamos sua citotoxicidade em macrófagos (RAW 264.7). Todos os extratos foram microbicidas para as cepas avaliadas, em diferentes concentrações (MMC), no entanto, o extrato de *E. arvense* foi citotóxico para macrófagos.

Estes resultados concordam com estudos mais atuais, como de Pagliarulo et al. (2016), que verificaram ação antimicrobiana de polifenóis de extrato hidroalcoolico de *P. granatum* sobre *S. aureus* e *E. coli*. Bernardo et al. (2015) demonstraram atividade antimicrobiana do extrato etanólico de *P. granatum* sobre *S. aureus* e *S. epidermidis*, e ainda, a aplicação *in vivo* (ratos Wistar) por 5 min demonstrou que o extrato foi um antisséptico alternativo sobre *S. epidermidis* comparado a clorexidina. Já com relação a *G. glabra*, Snowden et al. (2014), analisando diversos extratos de plantas sobre *S. aureus*, verificaram que o extrato de *G. glabra* foi moderadamente efetivo. Sedighinia et al. (2012) também demonstraram que o extrato de *G. glabra* apresentou boa atividade antibacteriana sobre *S. mutans*, *Streptococcus sanguis*, *Actinomyces viscosus*, *E. faecalis*, *S. aureus* e *E. coli*. Este mesmo grupo de autores (Soliemanpour et al., 2015) analisaram o efeito sinérgico entre extrato etanólico de *Tribulus terrestre* misturado com extrato de *G. glabra* e de *Capsella bursa-pastoris* sobre estes 6 micro-organismos. De acordo com os autores, este estudo *in vitro* foi uma avaliação preliminar da atividade antibacteriana das plantas e seus resultados suportam o uso do extrato de *T. terrestre* e da mistura com *G. glabra* e *C. bursa-pastoris* no tratamento de infecções orais, de modo que estudos *in vivo* são necessários para melhor avaliação de seus efeitos. Assim, os resultados de nosso estudo (capítulo 3.6) são de grande importância para selecionarmos os extratos que demonstram ação antimicrobiana sobre micro-organismos de interesse odontológico e baixa

citotoxicidade, de modo que estes resultados irão nortear as pesquisas futuras.

Outra preocupação é verificar a ação antimicrobiana dos extratos quando os micro-organismos estão organizados em biofilme e não apenas sobre cultura planctônica, assim, no artigo apresentado no Capítulo 3.7, em 2014, verificamos importante atividade antimicrobiana do extrato glicólico de *Arctium lappa* (bardana) sobre biofilmes monotípicos de *S. aureus, S. epidermidis, S. mutans, C. albicans, C. tropicalis* e *C. glabrata* em discos de resina acrílica. O extrato de bardana apresentou ainda a vantagem de não ser citotóxico para macrófagos no teste de MTT e da produção de citocinas pró-inflamatórias. Estes resultados são promissores e inovadores, pois não são verificados na literatura estudos atuais sobre a atividade antimicrobiana de *A. lappa*. Estudos anteriores (Gentil et al., 2006 e Pereira et al., 2005) verificaram potente ação antimicrobiana de constituintes de *A. lappa,* como medicação intracanal, sobre patógenos endodônticos *(P. aeruginosa, E. coli, Lactobacillus acidophilus, S. mutans* e *C. albicans)*. Desta forma, o extrato de *A. lappa* é um candidato potencial, que terá seu estudo aprofundado por nosso grupo de pesquisa.

Continuando nossa triagem pelos extratos de plantas, no artigo apresentado no Capítulo 3.9, avaliamos o extrato glicólico de *Persea americana* (avocado) sobre biofilme de *C. albicans* e verificamos que em determinadas concentrações (50, 100 e 200 mg/mL) o extrato conseguiu reduzir o biofilme de forma significativa, no tempo de contato do extrato de apenas 5 min. Contudo, nas concentrações de 100 e 200 mg/mL o extrato foi citotóxico para macrófagos (RAW 264.7). Em cultura planctônica, a concentração fungicida mínima foi 12,5 mg/mL. Estes resultados demonstram que em biofilme há maior resistência microbiana, sendo necessário concentrações maiores dos extratos. Kouidhi et al. (2015) relataram que os micro-organismos em biofilme são até 1000 vezes mais resistentes aos antibióticos comparados com suas culturas

planctônicas. A ação antifúngica apresentada pelo extrato glicólico de *P. americana* precisa ser explorada para outras espécies de *Candida* a fim de verificar seu real potencial antifúngico, bem como sobre bactérias. Em 2014, Falodun et al. verificaram que os extratos metanólicos e clorofórmicos de *P. americana* demonstraram forte atividade antifúngica sobre *C. krusei*, em cultura planctônica, bem como sobre *Cryptococcus neoformans, S. aureus* meticilina resistente e *E. coli*.

Diante do exposto, é possível verificar que com a grande diversidade de plantas com potencial antimicrobiano, a triagem inicial é muito importante para direcionar os estudos posteriores e a aplicação terapêutica destes extratos. Com os resultados já obtidos até aqui, pretendemos selecionar os melhores extratos e ampliar os estudos sobre demais bactérias e leveduras, incluindo anaeróbios estritos, e também sobre os produtos microbianos como LPS e LTA (ácido lipoteicóico).

Ao contrário das bactérias Gram-negativas, as bactérias Gram-positivas não expressam LPS e sim LTA, o qual é semelhante a LPS tanto na estrutura como nas propriedades imunológicas (Baik et al. 2008), sendo considerado seu correspondente Gram-positivo (Su et al., 2006; Siqueira & Rocas Jr, 2007). Sabendo que LTA apresenta importante atividade imunoestimuladora, induzindo a produção e liberação de mediadores pró-inflamatórios, podendo participar do desenvolvimento e manutenção das lesões periapicais, e verificando na literatura a escassez de estudos que avaliam medicações intracanais sobre LTA, no artigo do capítulo 3.10 avaliamos a capacidade do hidróxido de cálcio, clorexidina gel e associacão de hidróxido de cálcio com clorexidina em neutralizar os efeitos de LTA de induzir a produção de citocinas pró-inflamatórias (IL-1β, TNF-α, MIP-1α, IP-10, G-CSF and IL-6) e óxido nítrico sobre macrófagos (RAW 264.7). E, semelhante aos estudos anteriores sobre LPS, as medicações contendo hidróxido de cálcio apresentaram os melhores resultados na neutralização dos efeitos citotóxicos de LTA. Com a intenção de ampliar a terapia endodôntica, os

estudos envolvendo extratos de plantas devem também focar na neutralização dos produtos microbianos, que são potentes agentes inflamatórios.

Além dos estudos aqui apresentados, nosso grupo de pesquisa tem avaliado a ação antimicrobiana e anti-inflamatória de diferentes extratos de plantas (aquiléia, gimena, tomilho, chá verde, alcachofra, macela, hamamélis, pffafia, nogueira, erva baleeira, barbatimão), os quais têm apresentado resultados promissores para a Odontologia, de modo que esperamos num futuro próximo que os cirurgiões-dentistas vejam as vantagens do uso de substâncias alternativas no tratamento de diferentes enfermidades e ampliem suas opções terapêuticas, buscando sempre o melhor tratamento para o paciente.