Loiane Massunari

Análise da atividade antimicrobiana dos extratos de *Cordia verbenacea* DC, *Mikania laevigata* Sch. Bip. ex Baker e *Psidium Cattleianum* frente a microrganismos endodônticos.

Dissertação de mestrado apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, como parte dos requisitos para a obtenção de título de Mestre em Ciência Odontológica - Área de Concentração: Endodontia.
Orientadora: Cristiane Duque
Coorientador: Eloi Dezan Junior

Araçatuba - SP

2014

Catalogação na Publicação (CIP)

Serviço Técnico de Biblioteca e Documentação – FOA / UNESP

Massunari, Loiane.
M422a Análise da atividade antimicrobiana dos extratos de *Cordia verbenacea* DC, *Mikania laevigata* Sch. Bip. ex Baker e *Psidium Cattleianum* frente a microrganismos endodônticos / Loiane Massunari. - Araçatuba, 2014
89 f. : il. ; tab. + 1 CD-ROM

Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba
Orientadora: Profa. Cristiane Duque
Coorientador: Prof. Eloi Dezan Júnior

1. Extratos vegetais 2. Biofilmes 3. *Enterococcus faecalis* 4. *Candida albicans* I. Título

Black D24
CDD 617.67

Dados Curriculares

Loiane Massunari

Nascimento	16.03.1988
	Presidente Prudente - SP
Filiação	Jorge Masao Massunari
	Elenice Martins
2006 - 2011	Curso de Graduação em Odontologia pela Faculdade de Odontologia de Araçatuba - UNESP
2012 - 2014	Curso de pós-graduação em Ciência Odontológica - Área de concentração: Endodontia – Nível de mestrado pela Faculdade de Odontologia de Araçatuba - UNESP
Associações	CROSP - Conselho Regional de Odontologia de São Paulo
	SBPqO - Sociedade Brasileira de Pesquisa Odontológica

Dedicatória

À Deus

Meu criador

Sempre em primeiro lugar

À minha família

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

Nada teria sentido sem vocês!!!

Agradecimentos Especiais

À Deus

Por estar sempre comigo, me guiando pelos seus caminhos;

Pelo seu amor e misericórdia perante os meus erros;

Por seus ensinamentos que nunca me faltaram.

"ao único Deus, nosso Salvador, mediante Jesus Cristo, Senhor nosso, glória, magestade, império e soberania, antes de todas as eras, e agora, e por todos os séculos. Amém" Judas 1:25

Aos meus pais Jorge e Elenice

Pelo amor incondicional;

Por toda a dedicação e cuidados;

Pelas correções e conselhos sempre oportunos;

Por sempre me proporcionarem muito mais do que precisei.

Pessoas essenciais na minha formação, os responsáveis por cada conquista minha, e independente delas, sempre estarei com vocês. O amor de vocês me motiva a ser melhor!

Aos meus irmãos Nicole e Glauber

Pelo amor e cuidados;

Por serem tão presentes;

Pelos momentos únicos;

Por me ouvirem e me aconselharem.

Os maiores presentes da minha vida, juntos nós passamos por muitas experiências, aprendemos e crescemos. Nosso amor é incomparável, inesplicável e eterno. Preciso dos dois cada vez mais, vocês realmente me completam!

Ao meu namorado Márcio

Pelo amor e carinho;

Pelos conselhos e cuidados.

Você realmente entrou na minha vida para acrescentar. Sempre me encorajando e apoiando nos momentos difíceis. Quero você sempre ao meu lado!

À minha vó Mineco

Pela companhia e cuidados;

Pelos ensinamentos transmitidos.

O meu melhor exemplo, sempre disposta a enfrentar qualquer obstáculo. Dona do coração mais puro e bondoso. Estarei com você sempre que precisar!

Aos meus cunhados João e Jaqueline

Pelos momentos de alegria;

Por serem tão presentes e cuidadosos.

Vocês já fazem parte da família, conquistaram a todos. Não ganhei dois cunhados, ganhei mais dois irmãos.

À Faculdade de Odontologia de Araçatuba - Unesp

Nas pessoas dos professores Dra. Ana Maria Pires Soubhia, digníssima Diretora e Dr. Wilson Roberto Poi, digníssimo Vice-Diretor.

À minha orientadora Prof. Dra. Cristiane Duque

Por acreditar na minha competência, ser paciente frente as minhas limitações e ansiedade, pelos ensinamentos transmitidos e oportunidades dadas. Pessoa extremamente dedicada, sempre disposta a ensinar e ajudar, sem medir esforços para isso.

Ao meu coorientador Prof. Dr. Eloi Dezan Junior

Pelos ensinamentos clínicos, conselhos e disponibilidade sempre que precisei.

Às minhas amigas Annelise, Lívia, Índia, Karina e Paula

Pela amizade, conselhos e momentos únicos.

Espero tê-las sempre por perto!

À todos os meus colegas de Pós-Graduação

Pela amizade, convívio e experiências trocadas.

Aos docentes da disciplina de Endodontia da FOA - Unesp

Prof. Dr. Roberto Holland, Prof. Dr. Mauro Juvenal Nery, Prof. Dr. José Arlindo Otoboni Filho, Prof. Dr. Elói Dezan Júnior, Prof. Dr. João Eduardo Gomes Filho; Prof. Dr. Luciano Tavares Ângelo Cintra, Prof. Dr. Rogério Castilho Jacinto e Prof. Dr. Gustavo Sivieri Araújo.

Pelos ensinamentos transmitidos e contribuição na minha formação profissional.

Ao curso de pós-graduação em Ciência Odontológica da FOA

Na pessoa do coordenador Prof. Dr. Alberto Carlos Botazzo Delbem.

Aos funcionários do Departamento de Odontopediatria

Ricardo, Mário e Luisinho.

Por manterem a organização no laboratório;

Pela amizade e ajuda que nunca me foram negadas.

Aos funcionários do Departamento de Endodontia

Nelci, Claúdia, Elaine, Peterson e Grazi.

Pela amizade e ajuda sempre que precisei.

Aos funcionários da biblioteca da FOA

Ana Cláudia, Luzia, Ivone, Cláudio, Maria Cláudia, Luiz, Denise e Izamar.

Pela atenção e disponibilidade.

Aos funcionários da sessão de Pós Graduação da FOA

Valéria, Cristiane e Lilian.

Pelo profissionalismo e disponibilidade.

À coordenação de aperfeiçoamento de pessoal de nível superior - capes

Pela concessão da bolsa de estudo.

Muito Obrigado!!!

Análise da atividade antimicrobiana dos extratos de *Cordia verbenacea* DC, *Mikania laevigata* Sch. Bip. ex Baker e *Psidium Cattleianum* frente a microrganismos endodônticos.

Resumo

Na busca por fontes alternativas de antimicrobianos, diversos autores tem explorado o uso de plantas medicinais para o tratamento de várias doenças. A atividade antimicrobiana de várias espécies de plantas como, *Cordia verbenacea* DC, *Mikania laevigata* Schultz Bip. Ex Baker e *Psidium cattleianum*, popularmente chamadas de erva-baleeira, guaco e de araçá, respectivamente, tem sido relatada contra diversos microrganismos orais. O objetivo do presente estudo foi avaliar o efeito antimicrobiano dos extratos hidroetanólico bruto e as suas respectivas frações aquosa, butanol, hexano e acetato de etila de *Cordia verbenacea* (CV) e *Mikania laevigata* (ML) e extratos aquoso e hidroetanólico de *Psidium cattleianum* (PC) contra *Enterococcus faecalis*, *Pseudomonas aeruginosa, Actinomyces israelii* e *Candida albicans,* sob condições planctônicas e de biofilme. Os microrganismos foram analisados em condições planctônicas por meio de ensaios para a determinação da concentração inibitória mínima (CIM) e concentração letal mínima (CLM), pelo método de microdiluição em caldo. Posteriormente, foram selecionados somente os extratos capazes de eliminar completamente cada microrganismo (CLM 100%), para a análise da atividade antimicrobiana em ensaios de biofilme. Os resultados mostraram que os valores de CIM e CLM variaram de 250 à 4000μg/ml. O biofilme formado por *E. faecalis* foi eliminado frente à ação dos extratos aquoso e hidroetanólico de PC e à fração acetato de etila de ML. A mesma fração de ML e o extrato hidroetanólico de PC também foram capazes de eliminar biofilme de *P. aeruginosa*. O mesmo ocorreu com as frações hexânicas de CV e ML e o extrato hidroetanólico de ML frente ao biofilme de *A. israelii*. Nenhum extrato/fração foi capaz de eliminar biofilme de *C. albicans*. Este estudo demonstrou o potencial antimicrobiano de extratos ou frações de *Cordia verbenacea*, *Mikania laevigata* e *Psidium cattleianum*, sugerindo seu emprego com finalidade endodôntica devido à sua efetividade contra importantes patógenos associados às infecções endodônticas persistentes ou secundárias.

Palavras-chave: Extratos vegetais, biofilmes, *Enterococcus faecalis*, *Candida albicans.*

Antimicrobial analysis of the *Cordia verbenacea* DC, *Mikania laevigata* Sch. Bip. ex Baker e *Psidium Cattleianum* plant extracts against endodontic microorganisms.

Abstract

Seeking for alternative sources of the antimicrobials, several authors have explored the use of herbal medicines for the treatment of diseases. The antimicrobial activity of various species of plants, such as *Cordia verbenacea* DC, *Mikania laevigata* Schultz Bip. Ex Baker and *Psidium cattleianum,* known as erva-baleeira, guaco and araçá, respectively, has been reported against several oral microorganisms. The present study aimed to evaluate the antimicrobial effect of *Cordia verbenacea* (CV) and *Mikania laevigata* (ML) hydroethanolic crude extracts and their fractions aqueous, butanol, hexane and ethyl acetate and aqueous and hydroethanolic extracts of *Psidium cattleianum* (PC) against *Enterococcus faecalis*, *Pseudomonas aeruginosa, Actinomyces israelii* and *Candida albicans*, under planktonic and biofilm conditions. The microorganisms in planktonic conditions were analyzed by determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) assays using the microdilution broth method. Posteriorly, only the extracts that were able to totally eliminate each microorganism (MLC 100%) were selected for the antimicrobial activity analysis in the biofilm assay. The results showed that the MIC and MLC values ranged between 250 and 4000µg/ml. *E. faecalis* biofilm was eliminated by PC aqueous and hydroethanolic extracts and the ML ethyl acetate fraction. The same ML fraction and PC hydroethanolic extract were also able to eliminate the *P. aeruginosa* biofilm. The same occurred with the CV and ML hexane fractions and ML hydroethanolic extract against the *A. israelii* biofilm. None of the extracts/fractions were able to eliminate the *C. albicans* biofilm. This study confirmed the antimicrobial activity potential of *Cordia verbenacea*, *Mikania laevigata* and *Psidium cattleianum,* suggesting their use for endodontic purposes due to their effectiveness against pathogens associated with persistent or secondary endodontic infections.

Keywords: Plant extracts, biofilms, *Enterococcus faecali*s, *Candida albicans.*

Sumário

Sumário

Introdução Geral		15
Artigo 1		
	Abstract	20
	Introduction	21
	Materials and Methods	22
	Results and Discussion	25
	References	30
	Tables and Figures	37
Artigo 2		
	Abstract	44
	Introduction	45
	Materials and Methods	46
	Results	48
	Discussion	49
	References	51
	Tables and Figures	55
Referências Bibliográficas Gerais		58
Anexo A		65
Anexo B		66
Anexo C		67
Anexo D		68
Anexo E		69
Anexo F		81

Introdução Geral

Diante da resistência microbiana apresentada pelos microrganismos envolvidos na patogênese de diversas doenças, muitos autores tem explorado o uso de plantas medicinais, visando obter novos compostos que apresentem propriedades terapêuticas Os estudos com extratos, frações e óleos essenciais, tem se mostrado promissores, uma vez que esses apresentam propriedades anti-inflamatória, antibacteriana, antifúngica e analgésica; além de serem misturas complexas, apresentando baixo risco de desenvolver resistência microbiana (Brighenti *et al*., 2008; Bodet *et al*., 2008; de Menezes *et al*., 2010; Brighenti *et al*., 2012; Matias *et al*., 2013a; Oh *et al*., 2014). De modo geral, as substâncias que apresentam propriedades farmacológicas são sintetizadas pelas plantas como metabólitos secundários. Esses por sua vez, podem ser utilizados como o próprio princípio ativo de medicamentos, como modelo para a síntese ou semi-síntese desses (Cowan, 1999; Rodrigues *et al*., 2012), ou ainda associados a antibióticos sintéticos exercendo efeito sinérgico (Rodrigues *et al*., 2012; Matias *et al*., 2013b). Com o intuito de isolar os compostos ativos de um determinado extrato vegetal bruto, as frações vegetais são obtidas com solventes de diferentes polaridades que apresentam afinidades com determinados compostos vegetais. Posteriormente, o efeito terapêutico desejado poderá ser melhor identificado por meio da análise dos compostos isolados, decorrente da semipurificação das substâncias (Cechinel e Yunes, 1998).

A instrumentação mecânica associada às soluções irrigadoras durante o tratamento endodôntico convencional reduzem significativamente os microrganismos presentes no interior dos canais radiculares (Siqueira *et al*., 2007). Entretanto, a permanência de microrganismos devido à complexidade do sistema de canais radiculares e a resistência destes ao tratamento químico-mecânico podem ocasionar infecções persistentes ou secundárias (Siqueira e Rôças, 2009). Muitos autores têm atribuído a manutenção ou desenvolvimento de lesões periapicais, após o tratamento endodôntico, a fatores relacionados com a presença e virulência de bactérias e fungos no sistema de canais radiculares e/ou tecidos periapicais (Sundqvist *et al*., 1998; Hancock *et al*., 2001; Nair *et al*., 1999; Estrela *et al.*, 2014).

Bactérias Gram-negativas, comuns nas infecções primárias, são geralmente eliminadas após procedimento de tratamento químico-mecânico. A maioria dos estudos tem revelado que as bactérias Gram-positivas anaeróbias facultativas são mais frequentes nesses casos, incluindo *Streptococcus* spp., *Actinomyces* spp. (*A. israelii* e *A. odontolyticus*), *Propionibacterium* spp., *Lactobacilos* spp., *Enterococcus faecalis*, entre outros (Siqueira e Rôças, 2009). Mesmo após o tratamento endodôntico, infecções refratárias podem se

desenvolver devido à persistência de microrganismos patogênicos. Sakamoto *et al*. (2008) detectaram 11 espécies comuns entre os casos de necessidade de retratamento, dentre elas, *E. faecalis, Pseudomonas aeruginosa* e o fungo *Candida albicans.*

Enterococcus faecalis é a bactéria mais isolada em casos de fracasso endodôntico associado à periapicopatias persistentes (Gomes *et al*., 2008; Cogulu *et al*., 2008; Ozbek *et al*., 2009). Trata-se de cocos Gram-positivos, anaeróbio facultativo, que possui diversos fatores de virulência. Dentre esses, resistência à fagocitose por neutrófilos, uma vez que esse microrganismo possui uma adesina microbiana (substância de agregação) que promove a adesão à essas células do hospedeiro (Paradella *et al*., 2007), adesão à dentina (Stuart et *al*., 2006; Paradella *et al*., 2007), contribui para a reabsorção óssea devido a presença do ácido lipoteicóico que estimula a liberação de mediadores inflamatórios que contribuem para o dano tecidual, além de sobreviver em níveis de pH elevados devido à sua capacidade homeostática; tornando-se dessa forma resistente à ação do hidróxido de cálcio. (Haapsalo and Orstavik, 1987; Gomes *et al*., 2003; Paradella *et al*., 2007).

Outra bactéria que tem sido relatada em infecções refratárias ao tratamento endodôntico, é a *Pseudomonas aeruginosa* (Siren *et al*., 1997; Cheung e Ho 2001; Fujii *et al*., 2009). Trata-se de um patógeno oportunista, Gram-negativo, anaeróbio facultativo, que possui alta capacidade de formar biofilme, o que o torna mais resistente à ação de diversos antibióticos como a ciprofloxacina e gentamicina (Vojtová *et al*., 2011), hipoclorito de sódio e clorexidina (Raphael *et al*., 1981; Smith e Hunter, 2008; Scannapieco *et al*., 2009)

Actinomyces israelii apresenta-se morfologicamente como bacilo Gram-positivo e é considerado anaeróbio facultativo. Apesar de ser um microrganismo com baixa patogenicidade, não sendo capaz de formar esporos ou sobreviver em pH ácido, tem sido relatado em infecções extrarradiculares (Borssén e Sundqvist, 1981; Sundqvist *et al*., 1998; Xia e Baumgartner, 2003) devido a sua capacidade de se estabilizar fora do sistema de canais radiculares (Barnard *et al*., 1996; Ferreira *et al*., 2007). Uma vez na região apical, é capaz de invadir e se propagar nos tecidos moles, e apesar de ser sensível à baixas doses de antibióticos, esses somente se tornam letais para esse microrganismo, após um período de 2 a 6 semanas de exposição (Barnard *et al*., 1996) talvez pela capacidade que esse microrganismo apresenta de se organizar em grânulos (Barnard *et al*., 1996), dificultando dessa forma que o antimicrobiano atinja o seu alvo nas concentrações necessárias para eliminá-lo. Agentes antimicrobianos utilizados durante o tratamento endodôntico, como o hipoclorito de sódio e o hidróxido de cálcio, foram eficientes na eliminação de *A. israelii*, reforçando que a presença desse microrganismo em lesões secundárias, deve-se à sua

capacidade de sobreviver fora do sistema de canais radiculares, mesmo quando confrontado pelos mecanismos de defesa do hospedeiro (Barnard *et al*., 1996).

A ocorrência de fungos em infecções endodônticas refratárias tem sido atribuída ao oportunismo desses microrganismos frente à eliminação de grande parte da população bacteriana do interior dos canais radiculares (da Silva *et al*., 2000). Apesar da baixa incidência, *Candida albicans* é o fungo mais comumente isolado em dentes com infecção após o tratamento endodôntico (Waltimo *et al*., 1997; Siqueira e Rôças, 2004; Dumani *et al*., 2012). Possui capacidade de adesão e crescimento em locais com baixo teor de nutrientes e patogenicidade quando se apresenta na forma de hifa. Nessa forma, encontra-se mais hidrofóbico e com maior capacidade de penetrar em diferentes superfícies e tecidos, além de também conseguir crescer em regiões onde há ausência de fluídos, como na dentina (da Silva *et al*., 2000).

Durante o retratamento dos canais radiculares, o hidróxido de cálcio ainda é a medicação intracanal mais utilizada, embora um tempo relativamente longo seja necessário para a sua máxima eficiência (Vaghela *et al*., 2011; Delgado *et al*., 2013). Diante da busca incessante de minimizar os casos de insucesso endodôntico, medicações alternativas vêm sendo propostas (Gomes *et al*., 2003; Gomes *et al*., 2006; de Souza-Filho *et al*., 2008). Também a busca por novos veículos e/ou associações para otimizar o hidróxido de cálcio no sentido de aumentar sua eficiência e minimizar o tempo intracanal requerido (Turk *et al*., 2009; Dezan-Junior *et al*., 2010; Badr *et al*., 2011).

A atividade antimicrobiana de algumas plantas como, *Cordia verbenacea* DC, *Mikania laevigata* Schultz Bip. Ex Baker e *Psidium cattleianum*, popularmente conhecidas como erva-baleeira, guaco e araçá, respectivamente, tem sido relatada contra diversos microrganismos orais relacionados com a cárie dentária e a doença periodontal (Yatsuda *et al*., 2005; Brighenti *et al*., 2008; de Menezes *et al*., 2010; Gaetti-Jardim *et al*., 2011; Brighenti *et al*., 2012; Pimentel *et al*., 2012) e também de interesse endodôntico (Meccia *et al*., 2009; Michielin *et al*., 2009; Rodrigues *et al*., 2012; Matias *et al*., 2013a). O presente estudo teve como objetivo analisar o efeito antimicrobiano dos extratos hidroetanólicos e as suas respectivas frações aquosa, butanol, hexano e acetato de etila de *Cordia verbenacea* DC, *Mikania laevigata* Schultz Bip. Ex Baker; e dos extratos aquoso e hidroetanólico de *Psidium cattleianum* contra *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Actinomyces israelii* e *Candida albicans*, sob condições de crescimento planctônico e em biofilme.

Artigo 1

Antimicrobial activity of the crude plant extracts and fractions of *Cordia verbenacea* DC and *Mikania laevigata* Schultz Bip. ex. Baker against pathogenic microorganisms

Loiane Massunari[1], Paula F. Kreling[2], Márcio T. Oliveira[3], Juhan A. Scardelato[4], Luis V.S. Sacramento[4], Cristiane Duque [2]*, Eloi Dezan-Junior[1].

[1]Department of Endodontics, Araçatuba Dental School, Universidade Estadual Paulista (UNESP), R. José Bonifácio, 1193, 16015-050, Araçatuba/São Paulo, Brazil.

[2]Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Universidade Estadual Paulista (UNESP), R. José Bonifácio, 1193, 16015-050, Araçatuba/São Paulo, Brazil.

[3]Federal Institute of Education, Science and Technology of Mato Grosso do Sul (IFMS), R. Antônio Estevão Leal, 790, 79641-162, Três Lagoas/Mato Grosso do Sul, Brazil.

[4]Department of Natural Products and Toxicology, School of Pharmaceutical Sciences, Universidade Estadual Paulista (UNESP), Av. Alberto Toloi – Machados, 14801-902, Araraquara/São Paulo, Brazil.

***Corresponding author:**

Cristiane Duque

Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Universidade Estadual Paulista (UNESP), R. José Bonifácio, 1193, 16015-050, Araçatuba/São Paulo, Brazil.

Tel. number: +55.18.3636.3315, Fax number: +55.18.3636.3236

e-mail address: cduque@foa.unesp.br; cristianeduque@yahoo.com.br

The manuscript is according to the guide for authors of Journal of Ethnopharmacology (Anexo E).

Abstract

Ethnopharmacological relevance: The incorrect and/or excessive use of antibiotics has played a role in the dissemination of resistance among several bacterial species and development of multidrug-resistant strains. Seeking for alternative sources against antimicrobial resistance, the use of herbal medicines has been explored worldwide. Solvents such as water, butanol, hexane and ethyl acetate have been used for the semi purification of a crude plant extract, obtaining various fractions to be studied for several pharmaceutical purposes. The anti-inflammatory, bronchodilator, anti-carcinogen and antimicrobial potentials of *Mikania laevigata* Schultz Bip. Ex. Baker have been studied and showed promising results. Another plant with therapeutic effect is *Cordia verbenacea* DC, which is widely used in popular medicine, mainly as an antimicrobial, anti-inflammatory and analgesic agent.

Material and Methods: Cordia verbenacea and *Mikania laevigata* crude hydroethanolic extracts and their aqueous, hexane, butanol and ethyl acetate fractions were investigated *in vitro* for antimicrobial activities, using a minimal inhibitory/lethal concentration and biofilm assays, against the following pathogenic microorganisms: *Enterococcus faecalis, Pseudomonas aeruginosa, Actinomyces israelii* and *Candida albicans.*

Results: All tested *Cordia verbenacea* and *Mikania laevigata* substances have inhibitory activity against *E. faecalis* and *A. israelii*, except the *Mikania laevigata* aqueous fraction for *A. israelii.* The *Mikania laevigata* ethyl acetate fraction affected all tested microorganisms. *C. albicans* and *P. aeruginosa* were not affected by any *Cordia verbenacea* extract or fraction. Both concentrations of *Mikania laevigata* ethyl acetate fraction (5 or 10x MLC) completely eliminated *E. faecalis* and *P. aeruginosa* biofilms after 24h of exposition. The same was observed for the *Mikania laevigata* crude hydroethanolic extract and its hexane fraction for *A. israelii*. The *Cordia verbenacea* hexane fraction (10x MLC) was able to eliminate *A. israelli* biofilms. None of the tested extracts or fractions eliminated the *C. albicans* biofilm.

Conclusions: The results of the present study demonstrated the antimicrobial properties of *Cordia verbenacea* and *Mikania laevigata*, providing scientific basis for the popular use of these plants and reinforce their inclusion in the arsenal of antimicrobial substances against important pathogenic microorganisms.

Keywords: Plant Extracts, Antibacterial Agents, Antifungal Agents.

1. Introduction

Polymicrobial infections have affected mankind since the earliest of times and their eradication is extremely difficult due the microbial mechanisms of resistance to both the host immune system and antimicrobial agents (Nair *et al*., 2014). For decades antibiotics have been used to eliminate pathogenic microorganisms, however, those therapies are becoming ineffective. The incorrect and/or excessive use of antibiotics has played a role in the development of multidrug-resistant strains and dissemination of resistance among several bacterial species (Yap *et al*., 2014).

Some microorganisms have received special attention by the scientific community for their known ability to grow and survive in a wide array of environments, even under the action of potent antimicrobial agents. The *Enterococcus* genus is the third most commonly isolated healthcare pathogen, and are responsible for a variety of infections, including endocarditis, sepsis, surgical wound infections, and urinary tract infections (Van Tyne *et al*., 2013). *Actinomyces israelii* usually composes the endogenous microbiota of the human body. It can be found in the mouth, gastrointestinal tract, and female genital tract (Heo *et al*., 2014). However, in certain conditions, *A. israelii* may become pathogenic and cause diseases, such as actinomycosis, characterized by the abscess formation of polymicrobial origin and common in the cervicofacial region (Uehara *et al*., 2010; Heo *et al*., 2014). *Pseudomonas aeruginosa* is an opportunistic bacterium, the major human health-care problem and the main agent of lethal infections in cystic fibrosis and multidrug-resistant infections in immune-suppressed individuals (Lyczak *et al*., 2000; Apidianakis *et al*., 2005). It has the propensity to form biofilm in urinary catheters, coronary stents, endotracheal tubes (Halebeedu *et al*., 2014) and dental root canal infections (Ranta *et al*., 1988; Cheung and Ho, 2001; Garcez *et al*., 2013). *Candida albicans* is commonly found in the oral cavity of healthy individuals as a commensal microorganism (Coronado-Castellote and Jiménez-Soriano, 2013), however, in immunosuppressive conditions, this yeast become pathogenic and may cause candidiasis, a frequent mucocutaneous mycosis of the oral cavity. *C. albicans* can cooperate with bacterial growth and also promote other diseases, such as pneumonia. *In vitro* studies showed that *C. albicans* and *P. aeruginosa* coexist within the microbial community and *C. albicans* can be a substrate for *P. aeruginosa* growth (Sands *et al*., 2014; Roux *et al*., 2013). This occurs because toxic molecules produced by *P. aeruginosa,* called phenazines, can accumulate within hyphae forms of *C. albicans* and causes their disruption (Roux *et al*.; 2013; Sands *et al*., 2014).

Seeking an alternative source against the antimicrobial resistance, the use of herbal medicines has been explored worldwide. The plants are able to synthesize substances with pharmacological properties, mainly secondary metabolites form. They can be used as active principles in drugs or as chemical models for synthesis and semi-synthesis of drugs (Cowan, 1999; Rodrigues *et al*. 2012). Solvents such as water, butanol, hexane and ethyl acetate have been used for the semi purification of the crude plant extract, obtaining various fractions to be studied for several pharmaceutical purposes (Cechinel-Filho and Yunes, 1998). *Cordia verbenacea* DC, popularly known as "Erva-baleeira" belongs to the Boraginaceae family, native of Central and South America; and in Brazil, it is commonly found in Atlantic and Amazon Forests (Matias *et al*. 2013b). *Cordia verbenacea* is widely used in popular medicine, mainly as antimicrobial (Michielin *et al*., 2009; Rodrigues *et al*., 2012; Matias *et al*., 2013b), anti-inflammatory (Sertié *et al*., 2005; de Oliveira *et al*., 2011; Pimentel *et al*., 2012), and analgesic agent (Sertié *et al*., 2005; Matias *et al*., 2013a). Another plant with therapeutic effect is *Mikania laevigata* Schultz Bip. ex Baker, popularly known as "Guaco", belongs to the Asteraceae family and is commonly found in southern Brazil. The anti-inflammatory (Suyenaga *et al*., 2002; Napimoga and Yatsuda 2010), bronchodilator (Napimoga and Yatsuda ,2010; dos Santos *et al*., 2006), anti-carcinogen (Napimoga and Yatsuda, 2010; Rufatto, 2013) and antimicrobial potential (Davino *et al*., 1989; Yatsuda *et al*., 2005) of *Mikania laevigata* have been studied and showed promising results. Thus, the aim of this study was to evaluate the antimicrobial activity of the *Cordia verbenacea* crude hydroethanolic extract (CVCHE) and *Mikania laevigata* crude hydroethanolic extract (MLCHE) and their aqueous (CVAF, MLAF), hexane (CVHF, MLHF), butanol (CVBF, MLBF) and ethyl acetate (CVEAF, MLEAF) fractions against *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Actinomyces israelii*, and *Candida albicans*, under planktonic and biofilm conditions.

2. Materials and Methods

2.1. Plant material and preparation of the extracts and fractions

The *Cordia verbenacea* DC and *Mikania laevigata* Schultz Bip. ex Baker leaves were collected at the Medicinal and Toxic Plant Garden in the School of Pharmaceutical Science, UNESP, in Araraquara, São Paulo, Brazil (21°48′51.4″S and 48°12′05.1″W) and authenticated on location by Prof. Dr. Luis V. S. Sacramento. The leaves were dried in an oven at 40^{o}C for 96 hours. The active principles were extracted of the powdered leaves by maceration for 7 days (100g of plant material in 1000mL of ethanol: water 7:3 v/v). The obtained solution was filtered and evaporated to dryness (under reduced pressure and 55–

65°C). The dried extract was stored at −20°C. A portion of 10g of dry extract was partitioned between 1:1 hexane: water, ethyl acetate and n-butanol, in sequence. Fractions were separated and evaporated to dryness. The solvents used to re-suspend the extracts and fractions were: sterile distilled water for the crude extract and aqueous, hexane and butanol fractions and 5% DMSO for the ethyl acetate fraction.

2.2. Antimicrobial properties of the plant extracts and fractions

2.2.1. Microbial conditions

The following microbial strains used in the present study were kindly provided by the Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, São Paulo, Brazil): *Enterococcus faecalis* (ATCC 51299), *Actinomyces israelii* (ATCC 12102), *Pseudomonas aeruginosa* (ATCC 15442) and *Candida albicans* (ATCC 26790). The purity of the strains was confirmed by the Gram's method. Microbial suspensions were prepared from overnight grown culture in Brain Heart Infusion broth (Ref.M210/Lot.35340, Himedia, India) for bacteria strains or Sabouraud Dextrose broth (Ref.238230/Lot.2082199, Difco, France) for *Candida albicans* and incubated at 37°C for 24 hours in a 5% CO_2 atmosphere (Incubator Ultra Safe, HF212-UV). Growth curve assays were performed for each microorganism in order to determine the optical density at the mid-log phase [approximately 0.5 (5-10x10^8 CFU/ml) for bacteria and 0.3 (5-10x10^6 CFU/ml) for *Candida albicans* at 550nm] to be used in the following experiments. The absorbance was measured using a microplate reader (Eon Microplate Spectrophotometer, BioTek Instruments, USA) to assess the cell growth.

2.2.2. Determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC)

MIC and MLC (minimal bactericidal/fungicidal concentration) were determined by the microdilution broth method, in 96-well microtiter plates, following the criteria previously described by the National Committee for Clinical Laboratory Standards (NCCL) (2002a) for yeast and the National Committee for Clinical Laboratory Standards (NCCL) (2002b) for bacteria. The plants extracts and fractions were serially diluted in sterile distilled water in order to obtain the concentrations ranging from 30µg/ml to 4000µg/ml. Microbial cells suspension at the mid-log phase were harvested by centrifugation (Hanil Combi centrifuge, 514R) for 10 min, at 2.686xg, the supernatant was discarded and the pellet re-suspended in 2x concentrated Mueller-Hinton broth (Ref.275730/Lot.2319300, Difco, France) for bacteria, according to NCCLS M7-A7 (USA Guidelines) or RPMI-1640 medium

(R.8755/Lot.041M8303, Sigma-Aldrich, USA), according to NCCLS M27-A2 (USA Guidelines). The final concentration of bacterial suspension in the wells was 5-10x10^{5}CFU/ml and 2.5-5x10^{3}CFU/ml for *Candida albicans*. Initially, the plant extracts and fractions were serially diluted in water and after microbial suspensions (correctly adjusted for the concentrations described above), were inoculated in each well. The plates were incubated at 37°C for 24 hours in a 5% CO_2 atmosphere. Afterwards, 15µl of 0.01% Resazurin **(**R7017 Sigma-Aldrich) was applied in each well and incubated for 4 hours to promote oxidation-reduction and determine the cell viability by color's change visually detected (Martin *et al*., 2003). After that, wells corresponding to MIC (the last blue well) and at least three previous wells were homogenized, serially diluted and plated on Mueller-Hinton agar or Sabouraud Dextrose agar to determine the MLC. The plates were incubated at 37°C for 24 hours in a 5% CO_2 atmosphere. The colonies were counted and the number of viable bacteria was determined in CFU/ml. The MLC was considered when the extracts/fractions killed (than 50% to 100%) the tested microbial culture. Chlorhexidine digluconate (0.15µg/ml to 10mg/ml) and Amphotericin B (0.007µg/ml to 0.5mg/ml) were used as positive controls for the bacteria and *C. albicans,* respectively. The negative controls were cultures without antimicrobials agents in Mueller-Hinton broth or RPMI-1640 medium and in 5% DMSO. All experiments were performed in triplicate.

2.2.3. Biofilm formation in polystyrene microplates

Biofilm assays were conducted with the extracts/fractions that showed 100% growth inhibition on the MLC tests, except for *C. albicans* that only MLEAF and MLHF had effect against this specie, because it both fractions were selected in *C. albicans* biofilm. Those assays were according to Budzyñska *et al*. (2010) with some modifications. Sterile polystyrene 96-well microplates, U-shape bottom, were pretreated with 200µl of the stimulated saliva per well for 4 hours at 37°C in a 5% CO_2 atmosphere (coating phase). Saliva was collected during masticatory stimulation with a flexible film (Parafilm® M, Sigma-Aldrich), centrifuged at 2686xg for 10 minutes, and the supernatant was filtered through a 0.22 µm membrane filter (Corning Inc., Corning, USA). After the incubation time, the saliva was removed and 20µl of each microorganism suspension (approximately 5-10x10^{6}CFU/ml) was inoculated in each well containing 180 µl of BHI broth supplemented with 0.5% glucose for bacteria and Sabouraud Dextrose broth supplemented with 0.5% glucose for *Candida*. The plates were incubated at 37° in a 5% CO_2 atmosphere. After 48 hours, the culture medium was removed and the wells were washed with sterile saline (0.9% NaCl) for subsequent addition of 200µl of plant extract or fraction solution in

each well. The concentrations used for these assays were 5 and 10 times higher than the MLC concentration. The microplates were incubated again in the same conditions for 24 hours. All wells were plated in Brain Heart Infusion agar or Sabouraud Dextrose agar and incubated for 24 hours. After this incubation period the reading of the plates were made by UFC count. Chlorhexidine (5/10xMLC) and Amphotericin B (5/10xMLC) were used as positive controls as well as biofilm in water without antimicrobial agents to assess viability of the tested microorganisms without supply nutrients or toxic agents for them. Assays were repeated three times for each microorganism, in three independent experiments.

2. 3. Statistical analysis

Data from planktonic growth under MIC and MLC conditions were converted in logarithmic scale (log10 (CFU+1) and calculated the percentage (%) of microbial reduction compared to normal growth (control group). Box-whisker plots were performed to represent the distribution of non-parametric data obtained in the biofilm assays. Mann-Whitney tests were applied to compare one group with each other and these one with the positive control for biofilm assays.

Results and discussion

Mikania laevigata antimicrobial activity

The results for the antimicrobial activities of *Mikania laevigata* plant extract and fractions performed using MIC and MLC are shown in **Table 1**. All tested *Mikania laevigata* (ML) substances presented MICs ranging 250 to 4000 µg/ml showing inhibitory activity against *E. faecalis* and *A. israelii*, except the ML aqueous fraction (MLAF) for *A. israelii*. The ML ethyl acetate fraction (MLEAF) affected the growth of all tested microorganisms and the ML hexane fraction (MLHF) affected the *E. faecalis*, *A. israelli* and *C. albicans.* The percentages of microbial reduction under MIC and MLC for *Mikania laevigata* are demonstrated in **Figures 1** and **2**, respectively. MLEAF had the best effect compared to the other MLC substances. MLEAF was lethal for all tested microorganisms, except for *A. israelii*. *A. israelii* was highly sensible to the crude extract (MLCHE) and hexane fraction (MLHF) obtained from MLCHE. The substances with the best results for MLC were chosen for the biofilm assays, considering their performance against each microorganism. **Figure 3** shows box-whisker plots of the antibiofilm activity of plant extracts and fractions. Both concentrations of MLEAF (5 or 10x MLC) completely eliminated *E. faecalis* and *P. aeruginosa* biofilms after 24h of exposition. The same was observed for MLCHE and MLHF for *A. israelii.* None of the tested extracts or fractions

eliminated the *C. albicans* biofilm. The 10x MLEAF obtained the best results for the *C. albicans* biofilms. There are few studies evaluating the antimicrobial activity of *Mikania laevigata* extracts and fractions (Davino *et al*., 1989; Yatsuda *et al*., 2005). Following a similar antimicrobial methodology and testing the hydroethanolic extracts, hexane and ethyl acetate fractions of the *Mikania laevigata*, Yatsuda *et al*. (2005) found MICs and MLCs values between 12.5 and 400 µg/ml for the streptococci strains. The authors also observed that the extracts and fractions tested were able to inhibit the adherence of mutans streptococci cells to glass surfaces at sub-MIC levels (three times lower than their MIC values). The ML hexane fraction was the most effective antibacterial agent displaying MICs and MBCs values between 12.5 and 100 µg/ml. Although in the present study other bacterial species were tested and a direct comparison is not possible, the ML hexane fraction also had strong inhibitory activity against *A. israelii* and weak activity for *E. faecalis* and *C. albicans.* However, the ML ethyl acetate fraction had the most antimicrobial effect among the tested ML substances, showing activity for all tested microorganisms, mainly *A. israelii* and *E. faecalis.* There are no standard values when considering the inhibition level of the plant materials. However, many authors classified them as strong inhibitors (MIC lower than 500 µg/ml), moderate inhibitors (MIC between 600 µg/ml and 1500 µg/ml) and weak inhibitors (MIC above 1600 µg/ml) (Duarte *et al*., 2007; Wang *et al*., 2008; Sartoratto *et al*., 2004).

Analyzing the ML composition by phytochemical screening, the major compounds isolated were coumarin, coumaric acid, terpenes, sesquiterpenes, diterpenes and organic acids (Vilegas *et al*., 1997; Limberger *et al*., 2001). Coumarin 1,2-benzopyrone is considered the main substance isolated from the ML, and it can be used as a biomarker for pharmacological formulations (Yatsuda *et al*., 2005; Graça *et al*., 2007; Passari *et al*., 2014). Secondary metabolites, such as the derivatives of cinnamic acid and kaurane diterpenes also have demonstrated synergic pharmacological effects (Napimoga and Yatsuda, 2010; Ruffato *et al*., 2012, Passari *et al*., 2014). Davino *et al*. (1989) analyzing Kaurenoic acid and Cinnamoylgrandifloric acid from ML hexane extract, not observed antimicrobial activity these substances, but noted this property in their isomer isolated of the other plant extract. The authors concluded that the antimicrobial activity is due the presence of the acetate group on carbon-15 and this effect depends of this group position.

Cordia verbenacea antimicrobial activity

The results for the antimicrobial activities of *Cordia verbenacea* plant extract and fractions performed using MIC and MLC are shown in **Table 2**. All tested *Cordia*

verbenacea (CV) substances have inhibitory activity against *E. faecalis* and *A. israelii*, with MICs ranging from 410 to 4000 µg/ml. *C. albicans* and *P. aeruginosa* were not affected by any CV extract or fraction. The percentage of microbial reduction under MIC and MLC for *Cordia verbenacea* substances are demonstrated in **Figures 1** and **2**, respectively. CVCHE had the best effect compared to the other CV substances. *A. israelii* was highly sensible to the crude extract (CVCHE) and hexane fraction (CVHF) obtained from both plants. Because of that effect on *A. israelii*, CVCHE and CVHF were chosen for the biofilm assays. **Figure 3** shows box-whisker plots of the antibiofilm activity of plant extracts and fractions. CVCHE and CVHF significantly reduced the microbial growth on the *A. israelli* biofilms, however, only 10x CVHF was able to eliminate them.

One of the first studies with *Cordia verbenacea* (CV) was developed by de Carvalho *et al*. (2004) that observed its antimicrobial activity by the agar plate diffusion method. They found that CV essential oil was able to inhibit 88.8% of the tested Gram-positive strains and 93.3% of the yeast strains, including *Candida albicans*. Despite our results that showed the CV extract/fractions effects and no essential oil, we did not observe any effect in the *C. albicans* growth in the presence of the CV substances tested, perhaps due to the difference in the methodologies to obtain the substances tested, changing the chemical composition of the essential oils and plant extracts. The authors also observed that most of the tested Gram-negative strains were resistant, inhibiting only 20% of the strains (*Proteus mirabilis* and *Proteus vulgaris*). According to Yokota and Fullii (2007), Gram-negative bacteria have structural particularities that inhibit the penetration of antimicrobial agents, such as the external layer of lipopolysaccharides. This structure is responsible for the surface properties, such as permeability and susceptibility to antibiotics. Meccia *et* al. (2009) obtained MICs of 170µg/ml for *Staphylococcus aureus* and 200 µg/ml for *Enterococcus faecalis* for the CV essential oil. Our CV crude extract and their fractions also exhibited effects in the *E. faecalis* growth, but the MIC found was 4000µg/ml. Michielin *et al*. (2009) evaluated the use of several extraction methods and solvents to obtain CV extracts by analyzing the antibacterial activity. They suggested the importance of CV as a source of antibacterial components, independent of the extraction method used, mainly against Gram-positive and Gram-negative bacteria. *Pseudomonas aeruginosa* was sensible, by the agar diffusion method, to 11 of the 23 proposed extracts, showing 21mm of inhibition zone in the CV extract containing 25% ethanol, obtained by the Soxhlet method. The CV hydroethanolic extract inhibited *Pseudomonas aeruginosa* in MIC assays at a concentration of 1000µg/ml. On the other hand, our results showed MIC>4000µg/ml for the same bacterium. This can occur due the different methods of the extraction employed and

the different ethanol concentrations used by obtainment of the crude extracts. Another influence on the difference of the results can be due to the collection site and season that the leaves were collected, which may influence the presence and concentrations of the plant metabolite in the extracts (Passari *et al*., 2014). Pimentel *et al*. (2012) evaluated the effects of CV essential oil topically administered in a rat periodontitis model and observed significantly higher bone loss values in the non-treatment group compared with the CV groups through the analysis of alveolar bone loss in mandibular molars. In the microbiological assay, the authors noted that the CV group presented a statistically lower frequency of *Porphyromonas gingivalis* detection than the control group. Rodrigues *et al*. (2012) analyzed the antibacterial and antifungal activity of the CV essential oil obtained by the hydrodistillation method. Their results showed inhibitory activity against the standard strains: MIC of 64μg/ml for *Staphylococcus aureus* ATCC12692 and *Bacillus cereus* ATCC33018 and MIC of 512 μg/ml for *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 33018 and *Escherichia coli* ATCC 25922. The multi-resistant *Escherichia coli* 27 (MIC: 64 μg/ml), *Candida albicans* and *Candida krusei* yeasts (MIC: 512 μg/ml) were also sensible to the CV essential oil.

Several studies have associated plant compounds with synthetic antibiotics. Rodrigues *et al*. (2012) studied the association of CV essential oil with aminoglycosides and observed a synergistic effect between them. The most expressive MIC reduction (256 to 64 μg/ml) was observed when the CV essential oil was associated to amikacin for all tested strains. Similar results were obtained by Matias *et al*. (2013b) that showed the antibiotic activity of norfloxacin was enhanced in the presence of sub-inhibitory concentrations (determined by MIC assays) of the CV hexane extract. There was an increase of 50 per cent in the inhibition zone compared to the antibiotic. In addition to those results, Matias *et al*. (2013b) tested the CV methanolic extract and CV methanolic fraction against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* by MIC assays (MIC ≥1024μg/ml, for all strains). The same authors evaluated the extracts as modulators of resistance to the aminoglycosides. The antibiotic MICs were determined in the presence or absence of the CV extract and fraction tested at sub-inhibitory concentrations. The results showed that the antibacterial effect of the gentamicin, amikacin, and neomycin, when associated to the extract and fraction tested, was significantly improved, except for the CV methanolic extract when combined with gentamicin against the EC27 strain. The better synergistic activity combination was demonstrated by the CV fraction associated to antibiotics against *S. aureus* and *E. coli*, and the authors suggested that it could occur because of the higher concentration of polar compounds in the fraction. According to

Coutinho *et al*. (2008, 2009), several chemical compounds, including extracts and phytoconstituents, have the capacity to enhance the activity of a specific antibiotic, modulating and reversing the natural resistance of the bacteria to specific antibiotics, causing the elimination of plasmids and inhibiting the active efflux of antibiotics through the plasma membrane (Wagner and Ulrich-Merzenich, 2009; Matias *et al*. 2013a). The mechanisms of action of the combination of plants and antibiotics have been studied by some authors. Some of them believe that the hydrophobic nature of some components from the plants induces a higher interaction with the lipid bilayer of the cell membrane, affecting the respiratory chain and production of energy (Nicolson *et al*., 1999; Matias *et al*., 2013a) and others believe that these components make cells more permeable to antibiotics, leading to the interruption of vital cellular activity (Juven *et al*., 1994, Burt, 2004; Matias *et al*., 2013a)

Several authors have studied the CV chemical composition. According to Boussaada *et al.* (2008), the antibacterial activity of the plant extracts are generally associated with the presence of the sesquiterpenes, such as bisabolol and farnesyl acetate. Santos and Mello (2004) attributed this property to the tannins and flavonoids in studies with plants. Other authors suggested that the CV antibacterial potential is related to the presence of aromatic compounds in these extracts, such as eugenol, (Lis-balchin *et al*., 1998), detected in the CV ethanolic extract composition (Michielin *et al*., 2009), or the synergistic effect between the bisabolol and eugenol (Lis-balchin *et al*., 1998; Boussada *et al*., 2008; Michelin *et al*., 2009).

In the present study, MIC, MLC and biofilm assays had as positive controls groups; chlorhexidine digluconate (CHX) for the bacteria strains and Amphotericin B (AB) for *C. albicans*. CHX is the current gold standard of antibacterial tests (Westcott *et al*., 1989; Viana *et al*., 2004; Basrani and Lemonie, 2005; Gomes *et al*., 2013). The CHX effectiveness has been related in biofilms assays (Sena et al., 2006; Spratt *et al*., 2001; Clegg *et al*., 2006). Several antifungal activity studies (Youssef *et al*., 2014; Kaur *et al*., 2013; Odonne *et al.,* 2011) have used AB as control group.

This study showed that *Mikania laevigata* and *Cordia verbenacea* presents antimicrobial properties, mainly the MLEAF, which was able to reduce the growth of all the strains tested in the planktonic form; and CVHF, wich eliminated *A. Israelii* under planktonic and biofilm conditions. The results of the present study demonstrated the antimicrobial properties of *Mikania laevigata* and *Cordia verbenacea*, providing scientific basis for the popular use of these plants and reinforce their inclusion in the arsenal of

antimicrobial substances against important pathogenic microorganisms. Subsequent biocompatibity tests should be necessary to evaluate the toxicity of these substances before clinical use.

Acknowledgements

The microbial strains were gently assigned by Oswaldo Cruz Foundation (FIOCRUZ). This study was funded by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) from Brazil.

.

References

1. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, Davis RW, Rahme LG. 2005. Profiling early infection responses: *Pseudomonas aeruginosa* eludes host defenses by suppressing antimicrobial peptide gene expression. Proc Natl Acad Sci USA, Feb 15;102(7):2573-8.
2. Basrani B, Lemonie C. 2005. Chlorhexidine gluconate. Aust Endod J;31:48-52.
3. Bolard J, Joly V, Yeni P. 1993. Mechanism of action of Amphotericin B at the cellular level. It's modulation by delivery system. J. Liposome Res., v. 3, p. 409-427.
4. Boussaada O, Ammar S, Saidana D, Chriaa J, Chraif I, Daami M, Helal AN, Mighri Z. 2008. Chemical composition and antimicrobial activity of volatile components from capitula and aerial parts of *Rhaponticum acaule* DC growing wild in Tunisia. Microbiol Res; 163(1):87-95
5. Budzyńska A1, Wieckowska-Szakiel M, Sadowska B, Kalemba D, Rózalska B. 2011. Antibiofilm activity of selected plant essential oils and their major components. Pol J Microbiol.;60(1):35-41.
6. Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. Int J Food Microbiol. Aug 1;94(3):223-53.
7. Cechinel Filho V, Yunes RA. 1998. Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais. Conceitos sobre modificação estrutural para otimização da atividade. Química Nova, 21(1):99-105.
8. Cheung GS, Ho MW. 2001. Microbial flora of root canal-treated teeth associated with asymptomatic periapical radiolucent lesions. Oral Microbiol Immunol. Dec;16(6):332-7.

9. Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. 2006. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. J Endod.;32:434-437.
10. Coronado-Castellote L, Jiménez-Soriano Y. 2013. Clinical and microbiological diagnosis of oral candidiasis. J Clin Exp Dent. Dec 1;5(5):e279-86.
11. Coutinho HD, Costa JG, Lima EO, Falcão-Silva VS, Siqueira JP Jr. 2008. Enhancement of the antibiotic activity against a multiresistant *Escherichia coli* by *Mentha arvensis L*. and chlorpromazine. Chemotherapy; 54(4):328-30.
12. Coutinho HD, Costa JG, Lima EO, Falcão-Silva VS, Siqueira JP Jr. 2009. Herbal therapy associated with antibiotic therapy: potentiation of the antibiotic activity against methicillin--resistant *Staphylococcus aureus* by *Turnera ulmifolia L*. BMC Complement Altern Med. May 8;9:13.
13. Cowan MM. 1999. Plant products as antimicrobial agents. Clin Microbiol Rev. Oct;12(4):564-82.
14. Cushnie TP, Lamb AJ. 2005. Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. Journal of Ethnopharmacology. Oct 3;101(1-3):243-8.
15. Daferera DJ, Ziogas BN, Polissiou MG. 2003. The effectiveness of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. Michiganensis. Crop Protection 22, 39–44
16. Davino SC, Giesbrecht AM, Roque NF. 1989. Antimicrobial activity of kaurenoic acid derivatives substituted on carbon-15. Braz J Med Biol Res; 22(9):1127-9.
17. de Carvalho PMJr, Rodrigues RF, Sawaya AC, Marques MO, Shimizu MT. 2004. Chemical composition and antimicrobial activity of the essential oil of *Cordia verbenacea* D.C. Journal of Ethnopharmacology 95; 297–301.
18. de Oliveira DM, Luchini AC, Seito LN, Gomes JC, Crespo-López ME, Di Stasi LC. 2011. *Cordia verbenacea* and secretion of mast cells in different animal species. Journal of Ethnopharmacology 135; 463–468.
19. dos Santos SC, Krueger CL, Steil AA, Kreuger MR, Biavatti MW, Wisniewski Junior A. 2006. LC characterisation of guaco medicinal extracts, *Mikania laevigata* and *M. glomerata*, and their effects on allergic pneumonitis. Planta Med. 2006 Jun;72(8):679-84.
20. Duarte MC, Leme EE, Delarmelina C, Soares AA, Figueira GM, Sartoratto A. 2007. Activity of essential oils from Brazilian medicinal plants on *Escherichia coli*. Journal of Ethnopharmacology May 4;111(2):197-201.

21. Garcez AS, Núñez SC, Azambuja N Jr, Fregnani ER, Rodriguez HM, Hamblin MR, Suzuki H, Ribeiro MS. 2013. Effects of photodynamic therapy on Gram-positive and Gram-negative bacterial biofilms by bioluminescence imaging and scanning electron microscopic analysis. Photomed Laser Surg. Nov;31(11):519-25.
22. Gomes BP, Pinheiro ET, Sousa EL, Jacinto RC, Zaia AA, Ferraz CC, de Souza-Filho FJ. 2006. *Enterococcus faecalis* in dental root canals detected by culture and by polymerase chain reaction analysis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. Aug;102(2):247-53.
23. Gomes BP, Vianna ME, Zaia AA, Almeida JF, Souza-Filho FJ, Ferraz CC. 2013. Chlorhexidine in endodontics. Braz Dent J;24(2):89-102.
24. Graça C, Baggio CH, Freitas CS, Rattmann YD, de Souza LM, Cipriani TR, Sassaki GL, Rieck L, Pontarolo R, da Silva-Santos JE, Marques MC. 2007. In vivo assessment of safety and mechanisms underlying in vitro relaxation induced by *Mikania laevigata* Schultz Bip. ex Baker in the rat trachea. Journal of Ethnopharmacology. 112(3):430-9.
25. Graça C, Freitas CS, Baggio CH, Dalsenter PR, Marques MC. 2007. *Mikania laevigata* syrup does not induce side effects on reproductive system of male Wistar rats. Journal of Ethnopharmacology. Apr 20;111(1):29-32.
26. Halebeedu PP, Kumar GS, Gopal S. 2014. Revamping the role of biofilm regulating operons in device-associated Staphylococci and *Pseudomonas aeruginosa*. Indian J Med Microbiol. Apr-Jun;32(2):112-23.
27. Heo SH, Shin SS, Kim JW, Lim HS, Seon HJ, Jung SI, Jeong YY, Kang HK. 2014. Imaging of actinomycosis in various organs: a comprehensive review. Radiographics. Jan-Feb;34(1):19-33.
28. Juven BJ, Kanner J, Schved F, Weisslowicz H. 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Appl Bacteriol. Jun;76(6):626-31. Kaur R, Jaiswal ML, Jain V. 2013. Protective effect of *Lannea coromandelica* Houtt. Merrill. against three common pathogens. J Ayurveda Integr Med. Oct;4(4):224-8.
29. Limberger RP, Aboy AL, Bassani VL, Moreno PRH, Ritter, MR, Henriques, AT 2001. Essential oils from four Mikania species (Asteraceae). Journal of Essential Oil Research 13, 225–228.
30. Lis-Balchin M, Deans SG, Eaglesham E; 1998. Relationship between bioactivity and chemical composition of commercial essential oils. Flavour and Fragrance Journal.V.13, Issue 2, 98–104.

31. Pauli A. 2001. Antimicrobial properties of essential oil constituents. International Journal of Aromatherapy. V.11, Issue 3, 126–133.
32. Lyczak JB, Cannon CL, Pier GB. 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. Jul;2(9):1051-60.
33. Matias EF, Alves EF, Santos BS, Sobral de Souza CE, de Alencar Ferreira JV, Santos de Lavor AK, Figueredo FG, Ferreira de Lima L, Vieira Dos Santos FA, Neves Peixoto FS, Viana Colares A, Augusti Boligon A, Saraiva Rde A, Athayde ML, da Rocha JB, Alencar Menezes IR, Douglas Melo Coutinho H, da Costa JG. 2013a. Biological Activities and Chemical Characterization of *Cordia verbenacea* DC. as Tool to Validate the Ethnobiological Usage. Evidence-Based Complementary and Alternative Medicine 2013:164215.
34. Matias EF, Santos KK, Falcão-Silva VS, Siqueira-Junior JP, Costa JG, Coutinho HD. 2013b. Modulation of the norfloxacin resistance in *Staphylococcus aureus* by *Cordia verbenacea* DC. Indian J Med Res. Jan;137(1):178-82.
35. Meccia G, Rojas LB, Velasco J, Díaz T, Usubillaga A, Arzola JC, Ramos S. 2009. Chemical composition and antibacterial activity of the essential oil of Cordia verbenacea from the Venezuelan Andes. Nat Prod Commun. Aug;4(8):1119-22.
36. Michielin EM, Salvador AA, Riehl CA, Smânia A Jr, Smânia EF, Ferreira SR. 2009. Chemical composition and antibacterial activity of *Cordia verbenacea* extracts obtained by different methods. Bioresource Technology 100: 6615–6623.
37. Nair N, Biswas R, Götz F, Biswas L.2014. Impact of Staphylococcus aureus on pathogenesis in polymicrobial infections. Infect Immun. Jun;82(6):2162-9.
38. Napimoga MH, Yatsuda R. 2010. Scientific evidence for *Mikania laevigata* and *Mikania glomerata* as a pharmacological tool. J Pharm Pharmacol. Jul;62(7):809-20.
39. NCCLS: National Committee for Clinical Laboratory Standards, 2002a. Reference method for broth dilution antifungal susceptibility testing of yeast: approved standard. National Committee for Clinical Laboratory Standards, Wayne, PA, CLSI document M27-A2.
40. NCCLS: National Committee for Clinical Laboratory Standards, 2002b. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 5th ed., Wayne, PA, National Committee for Clinical Laboratory Standards, CLSI document M7-A5.

41. Nicolson K, Evans G, O'Toole PW. 1999. Potentiation of methicillin activity against methicillin-resistant *Staphylococcus aureus* by diterpenes. FEMS Microbiol Lett. Oct 15;179(2):233-9.
42. Odonne G, Herbette G, Eparvier V, Bourdy G, Rojas R, Sauvain M, Stien D. 2011. Antileishmanial sesquiterpene lactones from Pseudelephantopus spicatus, a traditional remedy from the Chayahuita Amerindians (Peru). Part III. Journal of Ethnopharmacology; Sep 1;137(1):875-9.
43. Passari LM, Scarminio IS, Bruns RE. 2014. Experimental designs characterizing seasonal variations and solvent effects on the quantities of coumarin and related metabolites from *Mikania laevigata*. Anal Chim Acta. Apr 22;821:89-96.
44. Pimentel SP, Barrella GE, Casarin RC, Cirano FR, Casati MZ, Foglio MA, Figueira GM, Ribeiro FV. 2012. Protective effect of topical *Cordia verbenacea* in a rat periodontitis model: immune-inflammatory, antibacterial and morphometric assays. BMC Complementary and Alternative Medicine Nov 21;12:224.
45. Ranta K, Haapasalo M, Ranta H. 1988. Monoinfection of root canal with *Pseudomonas aeruginosa*. Endod Dent Traumatol. Dec;4(6):269-72.
46. Rodrigues FF, Oliveira LG, Rodrigues FF, Saraiva ME, Almeida SC, Cabral ME, Campos AR, Costa JG. 2012. Chemical composition, antibacterial and antifungal activities of essential oil from *Cordia verbenacea* DC leaves. Pharmacognosy Res. Jul;4(3):161-5.
47. Roux D, Gaudry S, Khoy-Ear L, Aloulou M, Phillips-Houlbracq M, Bex J, Skurnik D, Denamur E, Monteiro RC, Dreyfuss D, Ricard JD. 2013. Airway fungal colonization compromises the immune system allowing bacterial pneumonia to prevail. Crit Care Med. Sep;41(9):e191-9.
48. Rufatto LC, Finimundy TC, Roesch-Ely M, Moura S. 2013. *Mikania laevigata*: chemical characterization and selective cytotoxic activity of extracts on tumor cell lines. Phytomedicine. Jul 15;20(10):883-9.
49. Rufatto LC, Gower A, Schwambach J, Moura S. 2012. Genus Mikania: chemical composition and phytotherapeutical activity. Revista Brasileira de Farmacognosia. 22(6):1384–1403.
50. Sands KM, Williams DW, Wilson MJ, Lewis MA, Marsh LL, Wise MP. 2014. Mechanisms of augmented growth of *Pseudomonas aeruginosa* mediated by *Candida albicans*. Crit Care Med. Mar;42(3):e256.

51. Santos SC, Mello JCP. 2004. Taninos. In: Simões CMO et al. Farmacognosia: da planta ao medicamento. 5. ed. rev. ampl. Florianópolis: Ed. Da UFSC; Porto Alegre: Ed. da UFRGS, p. 615-656.
52. Sarker SD, Nahar L, Kumarasamy Y. 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods. Aug;42(4):321-4.
53. Sartoratto A, Machado ALM, Delarmelina C, Figueira GM, Duarte MCT, Rehder VLG. 2004. Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. Brazilian Journal of Microbiology 35 (4),1517–8382.
54. Sena NT, Gomes BP, Vianna ME, Berber VB, Zaia AA, Ferraz CC, Souza-Filho FJ. 2006. In vitro antimicrobial activity of sodium hypochlorite and chlorhexidine against selected single-species biofilms. Int Endod J;39:878-885.
55. Sertié JAA, Woisky RG, Wiezel G, Rodrigues M. 2005. Pharmacological assay of *Cordia verbenacea* V: oral and topical anti-inflammatory activity, analgesic effect and fetus toxicity of a crude leaf extract. Phytomedicine 12; 338–344.
56. Spratt DA, Pratten J, Wilson M, Gulabivala K. 2001. An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. Int Endod J;34:300-307.
57. Suyenaga ES, Reche E, Farias FM, Schapoval EE, Chaves CG, Henriques AT. 2002. Antiinflammatory investigation of some species of Mikania. Phytotherapy Research 16; 519–523.
58. Uehara Y, Takahashi T, Yagoshi M, Shimoguchi K, Yanai M, Kumasaka K, Kikuchi K. 2010. Liver abscess of *Actinomyces israelii* in a hemodialysis patient: case report and review of the literature.Intern Med. 2010;49(18):2017-20.
59. Van Tyne D, Martin MJ, Gilmore MS. 2013. Structure, function, and biology of the *Enterococcus faecalis* cytolysin. Toxins (Basel) Apr 29;5(5):895-911.
60. Vianna ME, Gomes BP, Berber VB, Zaia AA, Ferraz CC, de Souza Filho FJ. 2004. In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite. Oral Surg Oral Med Oral Pathol Oral Radiol Endod; 97:79-84.
61. Vilegas JHY, de Marchi E, Lancas FM. 1997. Extraction of low-polarity compounds (with emphasis on coumarin and kaurenoic acid) from *Mikania glomerata* (Guaco) leaves. Phytochemical Analysis 8,266–270.
62. Wagner H, Ulrich-Merzenich G. 2009. Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine. Mar;16(2-3):97-110.

63. Wang, Yun-Song., He, Hong-Pin, Yang, Jing-Hua, Di, Ying-Tong, Hao, Xiao-Jiang. 2008. New Monoterpenoid Coumarins from Clausena anisum-olens. Molecules, 13, 931–937. ISSN:1420-3049.
64. Westcott WW, Martindale W. Martindale, The Extra Pharmacopoeia. Reynolds JEF (Editor). 29th ed London. The Pharmaceutical Press, 1989.
65. Yap PS, Yiap BC, Ping HC, Lim SH. 2014. Essential oils, a new horizon in combating bacterial antibiotic resistance. Open Microbiol J. Feb 7;8:6-14.
66. Yatsuda R, Rosalen PL, Cury JA, Murata RM, Rehder VL, Melo LV, Koo H. 2005. Effects of Mikania genus plants on growth and cell adherence of mutans streptococci. Journal of Ethnopharmacology. Feb 28;97(2):183-9.
67. Yokota S, Fujii N. 2007. Contributions of the lipopolysaccharide outer core oligosaccharide region on the cell surface properties of *Pseudomonas aeruginosa*. Comp Immunol Microbiol Infect Dis. Mar;30(2):97-109.
68. Youssef DT, Shaala LA, Mohamed GA, Badr JM, Bamanie FH, Ibrahim SR. 2014. Theonellamide G, a potent antifungal and cytotoxic bicyclic glycopeptide from the Red Sea marine sponge Theonella swinhoei. Mar Drugs; Apr 1;12(4):1911-23.

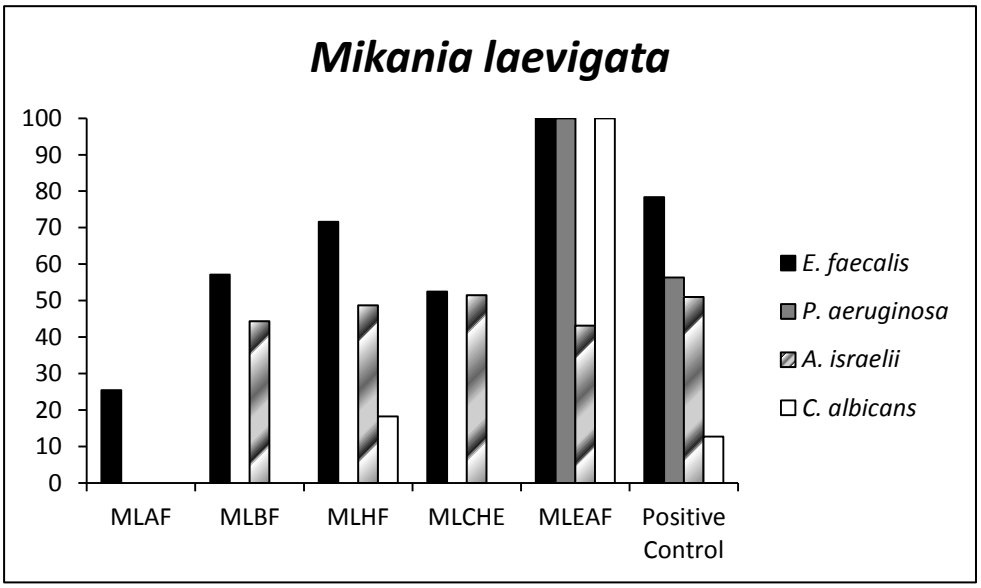
Table 1. Minimum inhibitory concentration (MIC) and minimal lethal concentration (MLC) of *Mikania laevigata* extract and fractions.

Antimicrobial Tested	**Microorganism**	**MIC (µg/ml)**	**MLC 50-99,99% (µg/ml)**	**MLC 100% (µg/ml)**
ML crude hydroethanolic extract (MLCHE)	*E.faecalis*	4000	4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	330	-	2000
	C.albicans	>4000	>4000	>4000
ML Aqueous fraction (MLAF)	*E.faecalis*	4000	>4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	>4000	>4000	>4000
	C.albicans	>4000	>4000	>4000
ML Hexane fraction (MLHF)	*E.faecalis*	4000	4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	330	-	1000
	C.albicans	4000	>4000	>4000
ML Butanol fraction (MLBF)	*E.faecalis*	4000	4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	2000	>4000	>4000
	C.albicans	>4000	>4000	>4000
ML Ethyl acetate fraction (MLEAF)	*E.faecalis*	1000	-	1000
	P.aeruginosa	4000	-	4000
	A.israelii	250	4000	>4000
	C.albicans	4000	-	4000
Chlorhexidine digluconate (CHX)	*E.faecalis*	6,5	-	9,8
	P.aeruginosa	39	-	78
	A.israelii	1,2	-	1,2
Amphotericin B (AB)	*C.albicans*	0,16	-	0,49

Table 2. Minimum inhibitory concentration (MIC) and minimal lethal concentration (MLC) of *Cordia verbenacea* extract and fractions.

Antimicrobial Tested	**Microorganism**	**MIC (µg/ml)**	**MLC 50-99,99% (µg/ml)**	**MLC 100% (µg/ml)**
CV crude hydroethanolic extract (CVCHE)	*E.faecalis*	4000	4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	500	-	500
	C.albicans	>4000	>4000	>4000
CV Aqueous fraction (CVAF)	*E.faecalis*	4000	4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	4000	4000	>4000
	C.albicans	>4000	>4000	>4000
CV Hexane fraction (CVHF)	*E.faecalis*	4000	>4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	500	-	2000
	C.albicans	>4000	>4000	>4000
CV Butanol fraction (CVBF)	*E.faecalis*	4000	4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	2600	>4000	>4000
	C.albicans	>4000	>4000	>4000
CV Ethyl acetate fraction (CVEAF)	*E.faecalis*	4000	>4000	>4000
	P.aeruginosa	>4000	>4000	>4000
	A.israelii	410	4000	>4000
	C.albicans	>4000	>4000	>4000
Chlorhexidine digluconate (CHX)	*E.faecalis*	6,5	-	9,8
	P.aeruginosa	39	-	78
	A.israelii	1,2	-	1,2
Amphotericin B (AB)	*C.albicans*	0,16	-	0,49

Figure 1. Percentage (%) of microbial reduction compared to normal growth under minimum inhibitory concentration **(MIC)** of *Mikania laevigata* and *Cordia verbenacea* extract and fractions. Data were expressed in mean of log (CFU+1).



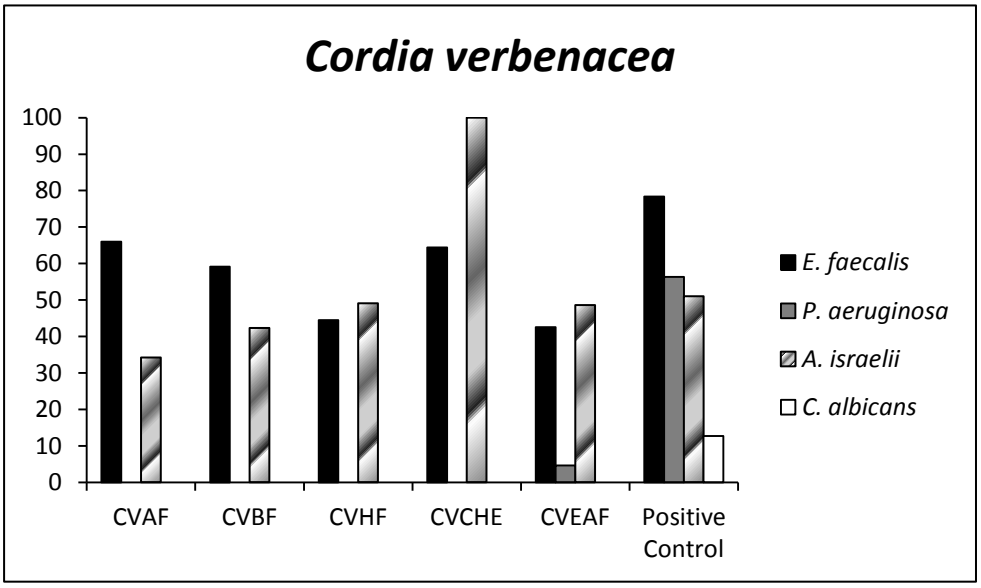
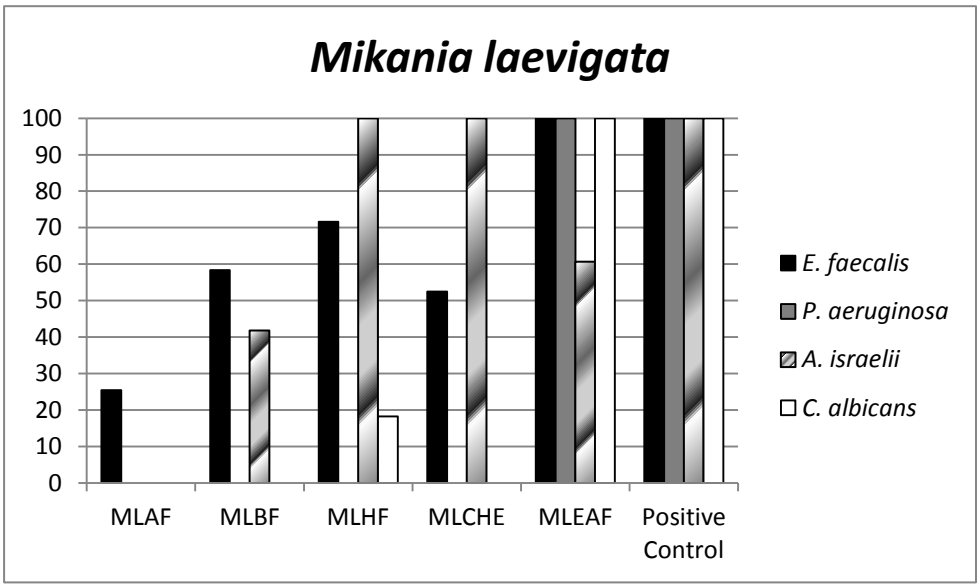


Figure 2. Percentage (%) of microbial reduction compared to normal growth under minimum lethal concentration **(MLC)** of *Mikania laevigata* and *Cordia verbenacea* extract and fractions. Data were expressed in mean of log (CFU+1).



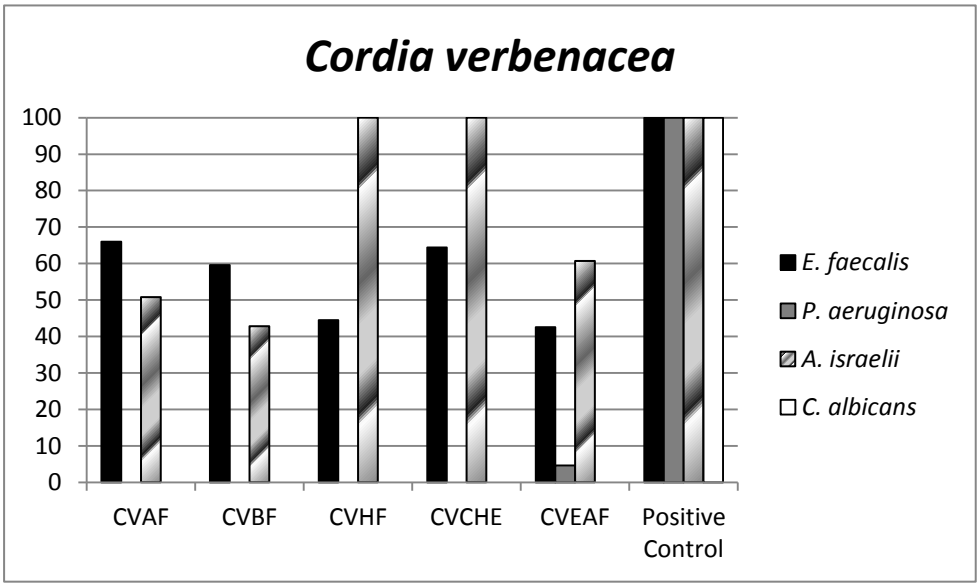
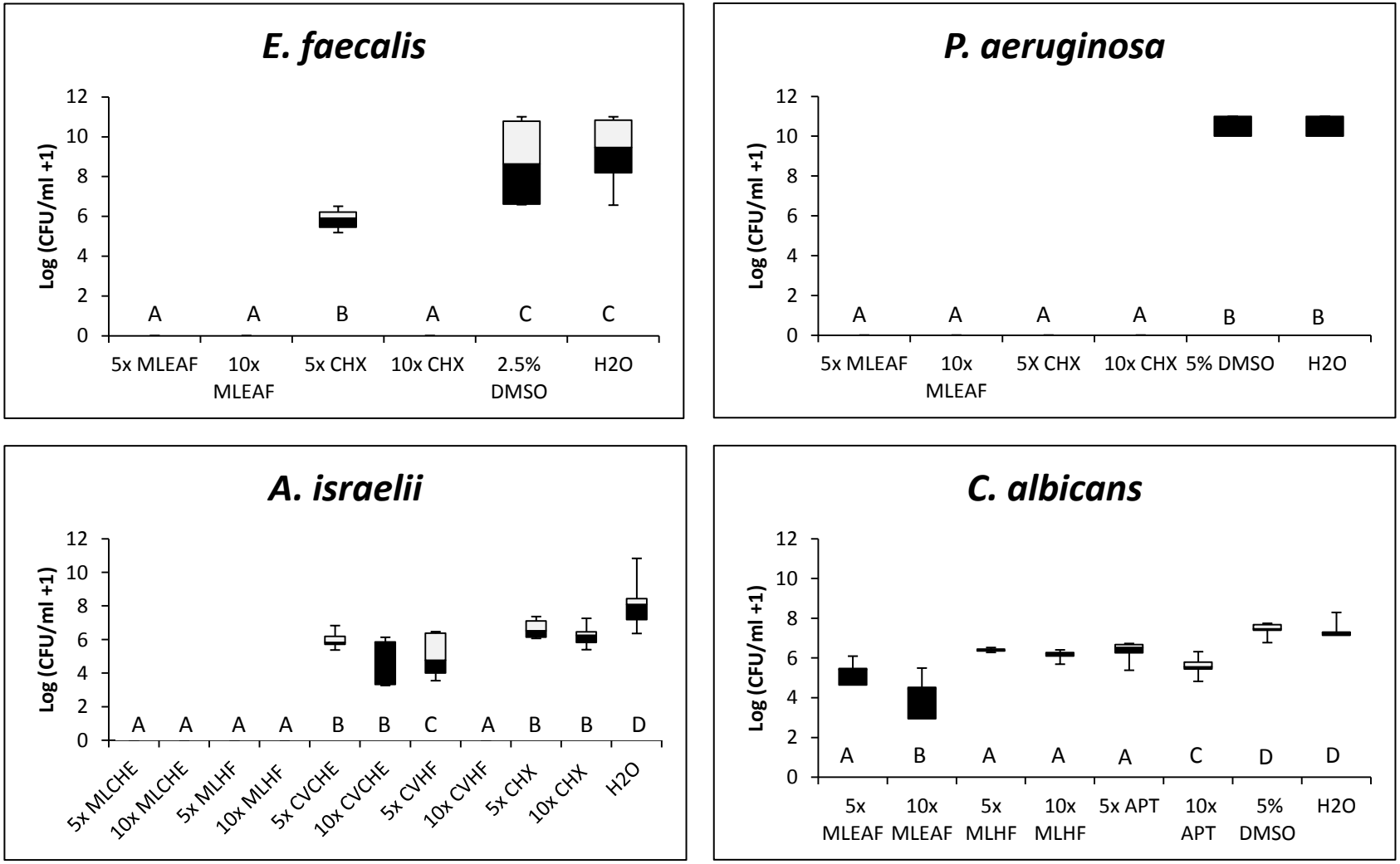


Figure 3. Box-whisker plots of the anti-biofilm activity of the extracts and fractions of *Mikania laevigata* and *Cordia verbenacea* against pathogenic microorganisms. Different capital letters show statistical difference among the groups and controls (DMSO and H_2O), according to Mann-Whitney tests. Bars indicate minimum and maximum values. Black and white boxes indicate lower and upper quartiles, respectively. Line in the middle of boxes is median.



Artigo 2

Antimicrobial activity of *Psidium cattleianum* leaf extracts on pathogens associated with endondontic infections.

Massunari L[1], Oliveira MT[2], Valentim D[1], Dezan-Junior E[1], Duque C[3].

[1]Department of Endodontics, Araçatuba Dental School, Universidade Estadual Paulista (UNESP), R. José Bonifácio, 1193, 16015-050, Araçatuba/São Paulo, Brazil.

[2]Federal Institute of Education, Science and Technology of Mato Grosso do Sul (IFMS), R. Antônio Estevão Leal, 790, 79641-162, Três Lagoas/Mato Grosso do Sul, Brazil.

[3]Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Universidade Estadual Paulista (UNESP), R. José Bonifácio, 1193, 16015-050, Araçatuba/São Paulo, Brazil.

***Corresponding author:**

Cristiane Duque

Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Universidade Estadual Paulista (UNESP), R. José Bonifácio, 1193, 16015-050, Araçatuba/São Paulo, Brazil.

Tel. number: +55.18.3636.3315, Fax number: +55.18.3636.3236

e-mail address: cduque@foa.unesp.br; cristianeduque@yahoo.com.br

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

Abstract

Persistant or secondary endodontic infection occurs when the bacteria are not efficiently controlled or new infection is introduced into the root canal and periapical tissues during or after the initial treatment. The complexity of root canals, the limitations of the chemomechanical preparation and the vulnerability of each species involved in the infection are well-recognized factors related to the failure of an endodontic treatment. The antimicrobial potential of the *Psidium cattleianum* (PC) herbal extracts have been displayed for a variety of microorganisms, however, it has not yet been tested against endodontic pathogens. Thus, the aim of this study was to evaluate the antimicrobial activity of the aqueous (PCAE) and hydroethanolic (PCHE) extracts from PC leaves against *Enterococcus faecalis, Pseudomonas aeruginosa, Actinomyces israelii* and *Candida albicans,* under planktonic and biofilm growth conditions. Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) were determined using the microdilution broth method in order to analyze the antimicrobial effect against the microorganisms in planktonic conditions. Biofilm assays were performed with the microorganisms that were completely eliminated (MLC 100%) in planktonic conditions, considering each PC extract. The results showed that the MIC and MLC values ranged between 250 and 4000µg/ml. *C. albicans* was the most resistant strain (MIC>4000µg/ml) of all tested microorganisms. Both PC extracts were able to eliminate *E. faecalis* biofilms, and only the PCHE eliminated *P. aeruginosa* biofilms. In conclusion, PCAE and PCHE are potential antibacterial agents and could be employed for endodontic purposes due to their effectiveness against important pathogens associated with persistent or secondary infections.

Keywords: Plant Extract, Antibacterial Agent, Endodontic Infection.

Introduction

The remaining microbiota after conventional endodontic treatment has been associated with the maintenance or development of periapical diseases. Resistant microorganisms associated with the complex anatomy of the root canal system require root canal irrigants and dressings with a large spectrum of antimicrobial action (1). The most used antimicrobial agents for intracanal descontamination are calcium hydroxide (CH) and chlorhexidine (CHX). The mechanism of CH is linked to the diffusion of hydroxyl ions through the infected dentinal tubules and accessory canals. CH requires a minimum period of seven days to alkalinize these sites and its effectiveness is limited against microorganisms in the root canal (2). Although CH effectively eliminates most endodontic pathogens, *Enterococcus faecalis* and *Candida albicans* can be resistant to CH, depending on the vehicles (3). Chlorhexidine has a reasonably wide range of activity against aerobic and anaerobic organisms, as well as *Candida* species. When used as an endodontic dressing, several studies reported a better antimicrobial activity for the CHX alone, compared with CH and the association of CHX and CH (4, 5, 6). Although CHX is also a potent antimicrobial irrigant, its capacity of tissue dissolution is reduced and when in contact with a common endodontic irrigant, sodium hypochlorite, it results in a "potentially toxic precipitate" (7).

Persistent or secondary endodontic infection occurs when the bacteria are not efficiently controlled or new infection is introduced into the root canal and periapical tissues during or after the initial treatment (8). Some microorganisms are commonly isolated from the root canals in persistent endodontic infections, such as *Enterococcus faecalis* and *Candida albicans* in cases of endodontic treatment failures (9, 10) and *Actinomyces israelii* and *Pseudomonas aeruginosa* in refractory periapical lesions (9, 11). The ability to produce exopolysaccharides could contribute to biofilm formation and consequently, to persistence and antibiotic tolerance of these organisms (12).

The pharmacological effects of herbal medicines have been studied and potential antioxidant and antimicrobial agents for the treatment of human diseases (13-15) included in the oral cavity (16, 17) are emerging. *Psidium* spp. have been largely studied due its antimicrobial (13, 14, 16-19), anti-inflammatory (19), antioxidant (15) and antiproliferative (14) properties. *Psidium cattleianum* (PC), commonly known as Araçá or strawberry guava, belongs to the Myrtaceae family and is native to tropical America, though it can be cultivated in other countries because it tolerates a large diversity of climates (15). PC extracts have displayed inhibitory activity against a wide range of oral microorganisms,

such as *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis* (18) *Streptococcus mutans* (16, 17) and *Streptococcus spp* (17). However, the antimicrobial effects of extracts from PC leaves have not yet been tested against endodontic pathogens. Thus, the aim of this study was to evaluate the antimicrobial activity of the aqueous (PCAE) and hydroethanolic (PCHE) extracts obtained from PC leaves against *Enterococcus faecalis*, *Pseudomonas aeruginosa, Actinomyces israelii*, and *Candida albicans*, under planktonic and biofilm growth conditions.

2. Materials and Methods

2.1. Plants extracts

The leaves were collected in the School of Dentistry of Araçatuba, State University-UNESP, Araçatuba, São Paulo, Brazil. The specimen, number HLF2006/7, was deposited at the Pharmacology and Research Herbarium, Phytotherapy Lab, São José do Rio Preto, São Paulo, Brazil. The leaves were washed and dried at 37°C for 1 week, for them become brittle. The leaves were then ground to a fine powder. Aqueous extract was obtained by decoction in deionized water (20g/250ml) for 5min at 100°C and at 55°C for an additional 1h. Hydroethanolic extract was obtained mixing 20g of powdered leaves with 250ml of 80% ethanol and shaking vigorously five times a day during 12 days (20). Both solutions were then filter sterilized with 0.22µm mixed cellulose ester membranes (Millipore; Billerica, USA). The hydroethanolic extract was concentrated under reduced pressures by using a rotary evaporator (MA120/TH - Marconi Laboratory Equipament, Piracicaba, SP, Brazil) linked vacuum pump (TE-058 - Tecnal Laboratoty Equipaments, Piracicaba, SP, Brazil). The aqueous extract was incubated at 37ºC for 72 hours. Both dried extracts were stored in dark bottles at –20° C until their use.

2.2. Antimicrobial properties of the plant extracts

2.2.1. Microbial conditions

The following microbial strains used in the present study were kindly provided by the Oswaldo Cruz Foundation (FIOCRUZ - Rio de Janeiro, São Paulo, Brazil): *Enterococcus faecalis* (ATCC 51299), *Actinomyces israelii* (ATCC 12102), *Pseudomonas aeruginosa* (ATCC 15442) and *Candida albicans* (ATCC 26790). The purity of the strains was confirmed by the Gram's method. Microbial suspensions were prepared from overnight grown culture in Brain Heart Infusion broth (Ref.M210/Lot.35340, Himedia, India) for bacteria strains or Sabouraud Dextrose broth (Ref.238230/Lot.2082199, Difco, France) for *C. albicans* and incubated at 37°C for 24 hours in a 5% CO_2 (Incubator Ultra Safe, HF212-

UV). We chose incubated all microorganisms in those atmospheric conditions to simulate low oxygen concentration inside root canals. Growth curve assays were performed for each microorganism in order to determine the optical density at the mid-log phase [approximately 0.5 (5-10x10^8 CFU/ml) for bacteria and 0.3 (5-10x10^6 CFU/ml) for *Candida albicans* at 550nm] to be used in the following experiments. The absorbance was measured using a microplate reader (Eon Microplate Spectrophotometer, BioTek Instruments, USA) to assess the cell growth.

2.2.2. Determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC).

MIC and MLC (minimal bactericidal/fungicidal concentration) were determined by the microdilution broth method, in 96-well microtiter plates, following the criteria previously described by NCCLS M7-A5 and M27-A2 (21, 22). The plants extracts were serially diluted in sterile distilled water in order to obtain the concentrations ranging from 30µg/ml to 4000µg/ml. Microbial cells suspension at the mid-log phase were harvested by centrifugation (Hanil Combi centrifuge, 514R) for 10 min, at 2.686xg, the supernatant was discarded and the pellet re-suspended in 2x concentrated Mueller-Hinton broth (Ref.275730/Lot.2319300, Difco, France) for bacteria or RPMI-1640 medium (R.8755/Lot.041M8303, Sigma-Aldrich, USA). The final concentration of bacterial suspension in the wells was 5-10x10^5CFU/ml and 2.5-5x10^3CFU/ml for *C. albicans*. Initially, the plant extracts were serially diluted in water and after microbial suspensions (correctly adjusted for the concentrations described above), were inoculated in each well. The plates were incubated at 37°C for 24 hours in a 5% CO_2 atmosphere. Afterwards, 15µl of 0.01% Resazurin (R7017 Sigma-Aldrich) was applied in each well and incubated for 4 hours to promote oxidation-reduction and determine the cell viability by color's change visually detected (Martin *et al*., 2003). After that, wells corresponding to MIC (the last blue well) and at least three previous wells were homogenized, serially diluted and plated on Mueller-Hinton agar or Sabouraud Dextrose agar to determine the MLC. The plates were incubated at 37°C for 24 hours in a 5% CO_2 atmosphere. The colonies were counted and the number of viable bacteria was determined in CFU/ml. The MLC was considered when the extracts/fractions killed than 50% to 100% of the tested microbial culture. Chlorhexidine digluconate (0.15µg/ml to 10mg/ml) and Amphotericin B (0.007µg/ml to 0.5mg/ml) were used as positive controls for the bacteria and *C. albicans*, respectively. The negative controls were cultures without antimicrobials agents in Mueller-Hinton broth or RPMI-1640 medium.

2.2.3. Biofilm formation in polystyrene microplates

Biofilm assays were conducted with the extracts that showed 100% growth inhibition on the MLC tests. These assays were conducting according to Budzyñska *et al*. (23) with some modifications. Sterile polystyrene 96-well microplates, U-shape bottom, were pretreated with 200µl of the stimulated saliva per well for 4 hours at 37°C in a 5% CO_2 atmosphere (coating phase). Saliva was collected during masticatory stimulation with a flexible film (Parafilm® M, Sigma-Aldrich), centrifuged at 2686xg for 10min, and the supernatant was filtered through a 0.22µm membrane filter (Corning Inc., Corning, USA). After the incubation time, the saliva was removed and 20µl of each microorganism suspension (approximately 5-10x10^6CFU/ml) was inoculated in each well containing 180 µl of BHI broth supplemented with 0.5% glucose for bacteria and Sabouraud Dextrose broth supplemented with 0.5% glucose for *C. albicans.* The plates were incubated at 37° in a 5% CO_2 atmosphere. After 48 hours, the culture medium was removed and the wells were washed with sterile saline (0.9% NaCl) for subsequent addition of 200µl of plant extract solution in each well. The concentrations used for these assays were 5 and 10 times higher than the MLC concentration. The microplates were incubated again in the same conditions for 24 hours. All wells were plated in Brain Heart Infusion agar or Sabouraud Dextrose agar and incubated for 24 hours. After this incubation period the reading of the plates were made by UFC count. Digluconate chlorhexidine (5/10xMLC) and Amphotericin B (5/10xMLC) were used as positive controls as well as biofilm in water without antimicrobial agents to assess viability of the tested microorganisms without supply nutrients or toxic agents for them.

2.3. Statistical analysis

Data from planktonic growth under MIC and MLC conditions were converted in logarithmic scale (log10 (CFU+1) and calculated the percentage (%) of microbial reduction compared to normal growth (control group). Box-whisker plots were performed to represent the distribution of non-parametric data obtained in the biofilm assays. Mann-Whitney tests were applied to compare one group with each other and these one with the positive control for biofilm assays.

Results

The MICs and MLCs values found in the present study ranged between 250µg/ml and 4000µg/ml (**Table 1**). Our results showed strong antibacterial activity of the *Psidium cattleianum* aqueous extract (PCAE) and *Psidium cattleianum* hydroethanolic extract (PCHE) against *E. faecalis* and *A. israelii* and weak activity for *P. aeruginosa* under planktonic conditions. Both extracts were not able to inhibit *C. albicans* growth by the

microdilution broth method. The percentages of microbial reduction under MIC and MLC for *Psidium cattleianum* extracts are demonstrated in **Figures 1** and **2**, respectively. The values of MICs completely inhibited the growth of *E. faecalis* and *P. aeruginosa* for PCAE and *P. aeruginosa* and *A. israelii* for PCHE. The *P. aeruginosa* was the most sensitive microorganism, and presented 100% of the growth reduction in the presence of the MICs obtained for both extracts. *C. albicans* was the most resistant strain for both tested substances and no extract had an effect on its growth (**Figure 1**). The MLC obtained for PCHE inhibited 100% growth of all the tested strains, except for *C. albicans*. The MLC obtained for PCAE had the same effect for *E. faecalis* and *P. aerugniosa.* The positive controls (Chlorhexidine and Amphotericin B) inhibited the growth of all tested strains (**Figure 2**). **Figure 3** shows box-whisker plots of the antibiofilm activity of the plant extracts. PCAE (10xMLC) was able to completely eliminate the *E. faecalis* biofilm. The same was observed for the PCHE (10xMLC) for the *P. aeruginosa* biofilm. Both concentrations of PCHE (5 or 10x MLC) also completely eliminated the *E. faecalis* biofilm after 24h of exposition.

Discussion

The complexity of root canals, the limitations of the chemomechanical preparation and the vulnerability of each species involved in the infection are well-recognized factors related to the failure of an endodontic treatment (24). *Enterococcus faecalis* is the microorganism most frequently isolated from the root canals of teeth with failed endodontic treatment (9, 10). There are controversies about the antimicrobial effect of calcium hydroxide (CH). Some studies demonstrated *E. faecalis* resistance to CH (4) while others showed the effectiveness of CH against this pathogen (25). *Actinomyces israelii* has been isolated from periapical tissues after endodontic treatment (9) due to its ability to colonize outside the root canal system. They could persist in refractory cases because of their ability to survive in periapical tissues when confronted by the host defenses and conventional antibiotic therapy (26, 27). *Pseudomonas aeruginosa* has been reported in persistent endodontic infections (11). *P. aeruginosa* biofilms are resistant to high concentrations of salts, dyes, antiseptics, antibiotics (8) and several studies also showed its resistance to commonly used endodontic disinfection protocols (28). Fungi are more frequently isolated after conventional endodontic treatment (29). *Candida albicans* is the most common fungus isolated from endodontic failure cases, perhaps because of its capacity to survive in a large range of pH values and tolerate the resultant alkalinity of the saturated CH solution (29).

The antimicrobial properties of PC have been recently studied (13, 14, 16-19). Because different methodologies and microorganisms were used in these investigations, the direct comparison of our results with the literature becomes difficult, but a parallel analysis was done. By the agar-diffusion method, the PC extracts showed antibacterial effect against *Staphylococcus epidermidis, Bacillus subtilis* and *Micrococcus luteus.* Similar to our results, the authors also did not observe an effect against *C. albicans* in any of the 18 tested extracts, including the PC extract (13). PCAE showed antibacterial effect in high concentration in a reduced exposure time (5 minutes) or in a low concentration in 60 minutes against the *Streptococcus mutans* biofilm (16). After treatment with PCAE, in an *in situ* biofilm assay, total anaerobic microorganism, total streptococci and *S. mutans* counts decreased when compared with water or antiseptic treatments (17). In the present study, although the methodology and microorganisms were completely different, the PCAE also presented antibacterial effect against the *E. faecalis* and *P. aeruginosa* biofilms and eliminated the *E. faecalis* biofilm at higher concentrations (10xMLC). In order to reduce the time for calcium hydroxide activity as an endodontic dressing, PCHE was used as a vehicle for calcium hydroxide in a biofilm assay. The tested formulation eliminated *E. faecalis* in 24 hours (30). Our results also showed *E. faecalis* elimination when exposed to PCHE (5x and 10x MLC), suggesting that only the presence of the PC extract produces bactericidal effect.

PCAE and PCHE exhibited antibacterial activity against the periodontal pathogens such as *A. Actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis* and *P. intermedia* with MIC values between 500 and 2000µg/ml and MLC values between 4000 and 8000µg/ml (18). The large range between the MIC and MLC values were also observed in our results for the PCAE extract MIC (250µg/ml) and MLC (>4000µg/ml) against *A. israelii*. The same was also reported in studies using antibiotics and propolis against the same bacterium (26, 27). It is suggested that the slow rate of *A. israelii* elimination is due to the genotypic tolerance of the species. (26)

P. aeruginosa was resistant to PC hexane, ethyl acetate and methanol fruit extracts by disc diffusion (19). In our study, although others solvents (water and 80% ethanol) and other tree parts (leaves) were employed to obtain the PC extracts, it can observed the effect of PC extracts for both solvents against *P.aeruginosa*. Different results can be observed in other studies with plant material. This can occur due to the different methods of extraction employed and the different solvents used for obtaining the crude extracts (31). Furthermore, the presence and concentration of the compounds may be different in each part of the tree (32).

The presence of the phenolic compounds - three flavonoids (kaempferol, quercetin and cyaniding) and tannin (ellagic acid) - have been related as the factor responsible for the antibacterial activity of the PC (16). Flavonoids are secondary metabolites naturally synthesized by plants, capable of penetrating into bacterial cells due to their lipophilic characteristic (33). Quercetin-5.3-dimethylether from *Combretum erythrophyllum* displayed MICs values of 50 and 100µg/ml for *Enterococcus faecalis* and *Pseudomonas aeruginosa,* respectively (34). Tannins can be toxic to filamentous fungi, yeasts and bacteria due their ability to inactivate microbial adhesins, enzymes, and cell envelopes that transport proteins (35).

Our results showed that PCAE and PCHE are potential antibacterial agents and could be employed for endodontic purposes due to their effectiveness against important pathogens associated with persistent endodontic infections.

Acknowledgements

The microbial strains were gently assigned by Oswaldo Cruz Foundation (FIOCRUZ). This study was funded by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) from Brazil.

References

1. Siqueira JF Jr, Rôças IN. Clinical implications and microbiology of bacterial persistence after treatment procedures. Journal of Endodontics. 2008; Nov;34(11):1291-1301.e3.
2. Vaghela DJ, Kandaswamy D, Venkateshbabu N, Jamini N, Ganesh A. Disinfection of dentinal tubules with two different formulations of calcium hydroxide as compared to 2% chlorhexidine: As intracanal medicaments against *Enterococcus faecalis* and *Candida albicans*: An in vitro study. Journal of Conservative Dentistry. 2011; Apr-Jun; 14(2): 182–186.
3. Turk BT, Sen BH, Ozturk T. *In vitro* antimicrobial activity of calcium hydroxide mixed with different vehicles against *Enterococcus faecalis* and *Candida albicans*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108 2:297–301
4. Gomes BP, Souza SF, Ferraz CC, Teixeira FB, Zaia AA, Valdrighi L, Souza-Filho FJ. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against

Enterococcus faecalis in bovine root dentine in vitro. Int Endod J. 2003; Apr;36(4):267-75.

5. Gomes BP, Vianna ME, Sena NT, Zaia AA, Ferraz CC, de Souza Filho FJ. In vitro evaluation of the antimicrobial activity of calcium hydroxide combined with chlorhexidine gel used as intracanal medicament. Oral Surg Oral Med Oral Pathol Oral Radiol Endod; 2006;102:544-550.
6. de Souza-Filho FJ, Soares A de J, Vianna ME, Zaia AA, Ferraz CC, Gomes BP. Antimicrobial effect and pH of chlorhexidine gel and calcium hydroxide alone and associated with other materials. Brazilian Dental Journal. 2008; 19:28-33.
7. Cintra LT, Watanabe S, Samuel RO, da Silva Facundo AC, de Azevedo Queiroz IO, Dezan-Júnior E, Gomes-Filho JE. The use of NaOCl in combination with CHX produces cytotoxic product. Clin Oral Investig. 2014; Apr;18(3):935-40.
8. Phee A, Bondy-Denomy J, Kishen A, Basrani B, Azarpazhooh A, Maxwell K. Efficacy of bacteriophage treatment on *Pseudomonas aeruginosa* biofilms. Journal of Endodontics. 2013; Mar;39(3):364-9.
9. Molander A, Reit C, Dahlen G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. International Endodontic Journal. 1998; 31:1–7.
10. Cogulu D, Uzel A, Oncag O, Eronat C. PCR-based identification of selected pathogens associated with endodontic infections in deciduous and permanent teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008; Sep;106(3):443-9.
11. Cheung GS, Ho MW. Microbial flora of root canal-treated teeth associated with asymptomatic periapical radiolucent lesions. Oral Microbiol Immunol. 2001; Dec;16(6):332-7.
12. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999 May 21;284(5418):1318-22.
13. de Souza GC, Haas AP, von Poser GL, Schapoval EE, Elisabetsky E. Ethnopharmacological studies of antimicrobial remedies in the south of Brazil. J Ethnopharmacol. 2004 Jan;90(1):135-43.
14. Medina AL, Haas LIR, Chaves FC, Salvador M, Zambiazi RC, da Silva WP, Nora L, Rombaldi CV. Araçá (*Psidium cattleianum* Sabine) fruit extracts with antioxidant and antimicrobial activities and antiproliferative effect on human cancer cells. Food Chemistry 128; 2011- 916–922
15. Biegelmeyer R, Andrade JM, Aboy AL, Apel MA, Dresch RR, Marin R, Raseira Mdo C, Henriques AT. Comparative analysis of the chemical composition and

antioxidant activity of red (*Psidium cattleianum*) and yellow (*Psidium cattleianum* var. lucidum) strawberry guava fruit. J Food Sci. 2011 Sep;76(7):C991-6.

16. Brighenti FL, Luppens SB, Delbem AC, Deng DM, Hoogenkamp MA, Gaetti-Jardim EJr, Dekker HL, Crielaard W, ten Cate JM. Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* viability, protein expression and acid production. Caries Res. 2008;42(2):148-54.
17. Brighenti FL, Gaetti-Jardim E Jr, Danelon M, Evangelista GV, Delbem AC. Effect of *Psidium cattleianum* leaf extract on enamel demineralisation and dental biofilm composition in situ. Arch Oral Biol. 2012 Aug;57(8):1034-40.
18. Gaetti-Jardim Júnior E, Landucci LF, Arafat OKK, Ranieri RV, Ramos MMB, Ciesielski FIN, Schweitzer CM, Okamoto AC. Antimicrobial activity of six plant extracts from the Brazilian savanna on periodontal pathogens. Int. J. Odontostomat., 5(3):249-256, 2011.
19. McCook-Russell KP, Nair MG, Facey PC, Bowen-Forbes CS. Nutritional and nutraceutical comparison of Jamaican *Psidium cattleianum* (strawberry guava) and *Psidium guajava* (common guava) fruits. Food Chem. 2012 Sep 15;134(2):1069-73.
20. Machado AC, Dezan Junior E, Gomes-Filho JE, Cintra LT, Ruviére DB, Zoccal R, Damante CA, Jardim Junior EG. Evaluation of tissue reaction to Aroeira (*Myracrodruon urundeuva*) extracts: a histologic and edemogenic study. J Appl Oral Sci. 2012; Jul-Aug;20(4):414-8.
21. NCCLS: National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeast: approved standard. National Committee for Clinical Laboratory Standards, Wayne, PA, CLSI document ,2002, M27-A2.
22. NCCLS: National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 5th ed., Wayne, PA, National Committee for Clinical Laboratory Standards, CLSI document, 2002, M7-A5.
23. Budzyńska A1, Wieckowska-Szakiel M, Sadowska B, Kalemba D, Rózalska B. Antibiofilm activity of selected plant essential oils and their major components. Pol J Microbiol. 2011;60(1):35-41.
24. Gomes BP, Pinheiro ET, Gadê-Neto CR, Sousa EL, Ferraz CC, Zaia AA, Teixeira FB, Souza-Filho FJ. Microbiological examination of infected dental root canals. Oral Microbiol Immunol 2004;19:71-6

25. Han GY, Park SH, Yoon TC. Antimicrobial activity of $Ca(OH)_2$ containing pastes with *Enterococcus faecalis* in vitro. Journal of Endodontics. 2001; May; 27(5):328-32.
26. Barnard D, Davies J, Figdor D. Susceptibility of *Actinomyces israelii* to antibiotics, sodium hypochlorite and calcium hydroxide. International Endodontic Journal. 1996; 29:320-6.
27. Ferreira FB, Torres SA, Rosa OP, Ferreira CM, Garcia RB, Marcucci MC, Gomes BP. Antimicrobial effect of propolis and other substances against selected endodontic pathogens. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007; Nov;104(5):709-16.
28. Estrela C, Pimenta FC, Ito IY, Bammann LL. In vitro determination of direct antimicrobial effect of calcium hydroxide. Journal of Endodontics. 1998; 24:15–7.
29. Siqueira JF Jr, Sen BH. Fungi in endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004; May;97(5):632-41.
30. Dezan-Jr E, Sangalli J, Gomes-Filho JE, Gaetti-Jardim JrE. *Psidium cattleianum* plus Ca(OH)2 antimicrobial efficacy against *Enterococcus faecalis*. IADR; Jul; 15-16, 2010, J Dent Res; 2010; 89,Special Issue B.
31. Passari LM, Scarminio IS, Bruns RE. Experimental designs characterizing seasonal variations and solvent effects on the quantities of coumarin and related metabolites from *Mikania laevigata*. Anal Chim Acta. 2014Apr 22;821:89-96.
32. Tortoriello J, Meckes-Fischer M, Villarreal ML, Berlin B, Berlin E. Spasmolytic activity of medicinal plants used to treat gastrointestinal and respiratory diseases in the Highland of Chiapas. Phytomedicine. 1995 Jul;2(1):57-66.
33. Williamson G, Day AJ, Plumb GW, Couteau D. Human metabolic pathways of dietary flavonoids and cinnamates. Biochem Soc Trans. 2000 Feb;28(2):16-22.
34. Martini ND, Katerere DR, Eloff JN. Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). J Ethnopharmacol. 2004 Aug;93(2-3):207-12.
35. Cowan MM: Plant products as antimicrobial agents. Clin Microbiol Rev 1999; 12: 564–582.

Table 1. Minimum inhibitory concentration (MIC) and minimal lethal concentration (MLC) of *Psidium cattleianum* extracts.

Antimicrobial Tested	Microorganism	MIC (µg/ml)	MLC 50%-99,99% (µg/ml)	MLC 100% (µg/ml)
Psidium cattleianum aqueous extract	*E.faecalis*	500	-	500
	P.aeruginosa	4000	-	4000
	C.albicans	>4000	>4000	>4000
	A.israelii	250	>4000	>4000
Psidium cattleianum hidroethanolic extract	*E.faecalis*	250	-	500
	P.aeruginosa	4000	-	4000
	C.albicans	>4000	>4000	>4000
	A.israelii	250	-	250
Chlorhexidine digluconate (CHX)	*E.faecalis*	6,5	-	9,8
	P.aeruginosa	39	-	78
	A.israelii	1,2	-	1,2
Amphotericin B (AB)	*C.albicans*	0,16	-	0,49

Figure 1. Percentage (%) of microbial reduction compared to normal growth under minimum inhibitory concentration **(MIC)** of *Psidium cattleianum* extracts. Data were expressed in mean of log (CFU+1).

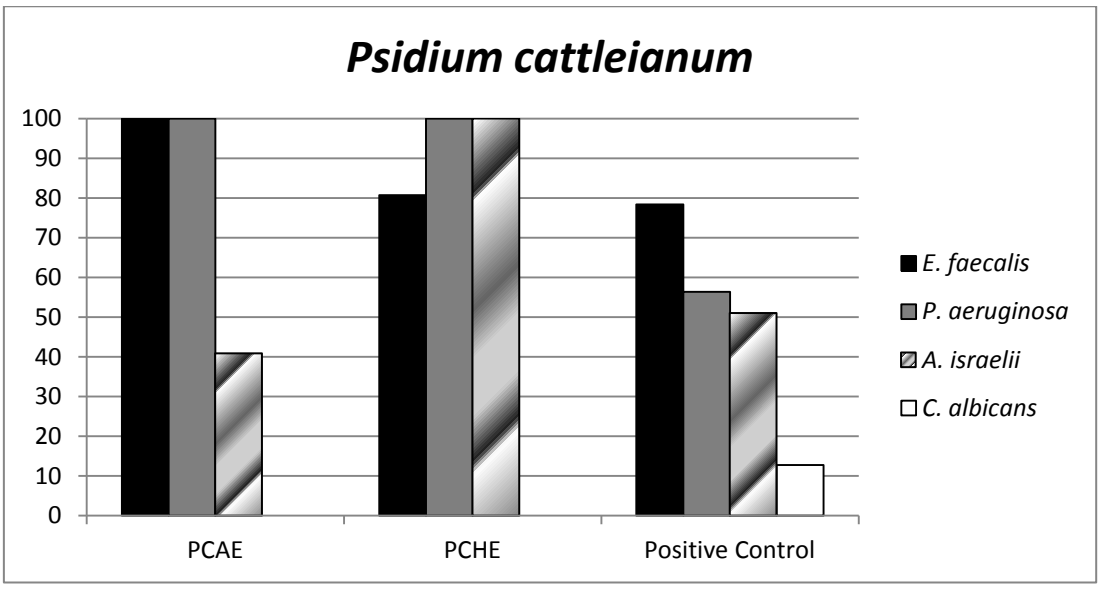


Figure 2. Percentage (%) of microbial reduction compared to normal growth under minimum lethal concentration **(MLC)** of *Psidium cattleianum* extracts. Data were expressed in mean of log (CFU+1).

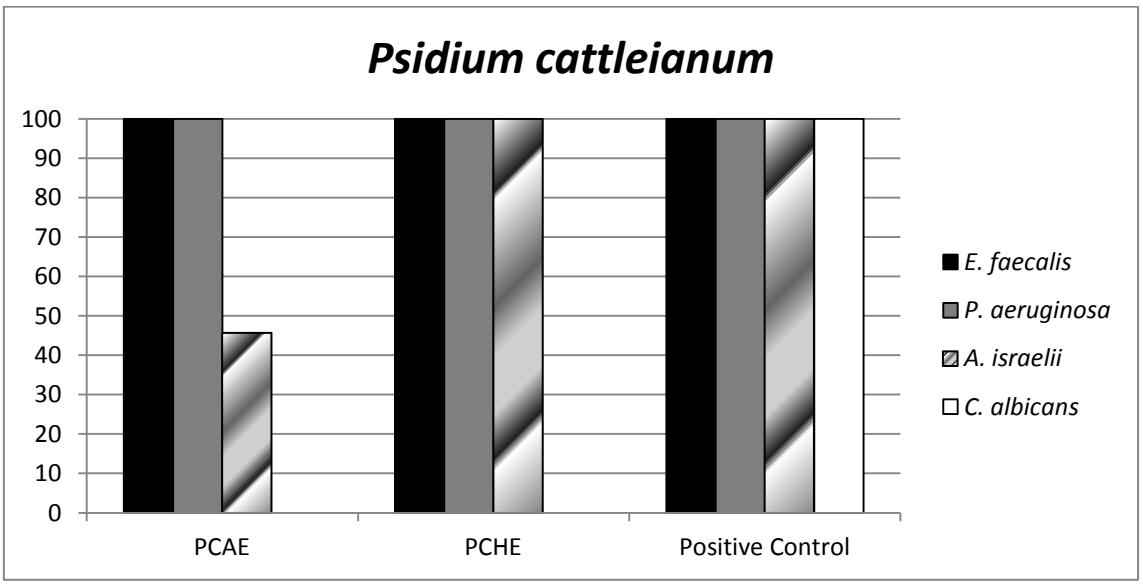
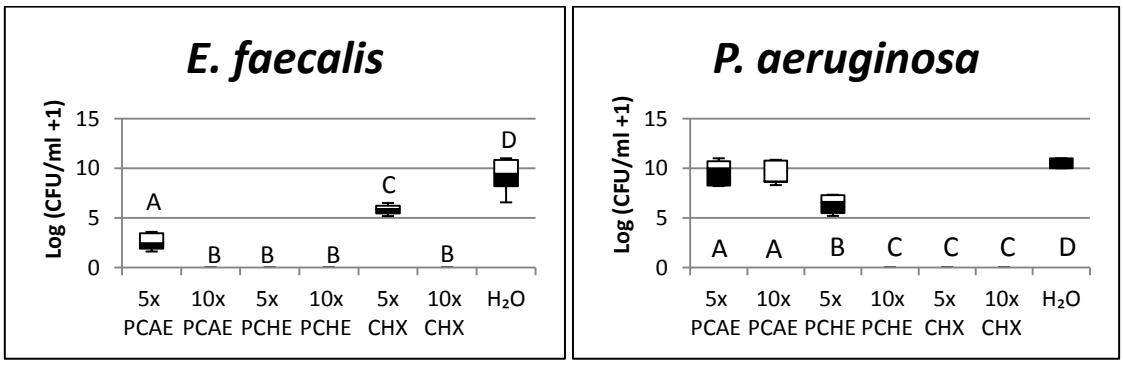
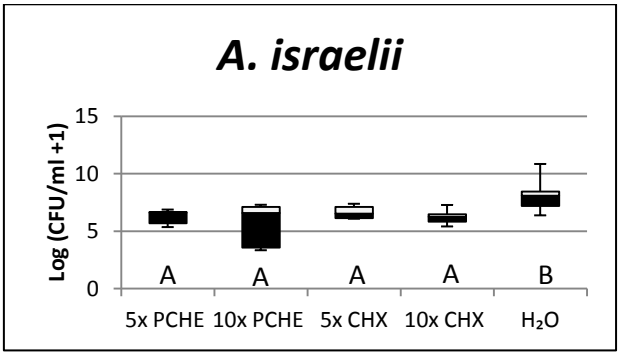


Figure 3. Box-whisker plots of the antibiofilm activity of the *Psidium cattleianum* extracts against pathogenic microorganisms. Different capital letters show statistical difference among the groups and controls (H_2O and Chlorhexidine), according to Mann-Whitney tests. Bars indicate minimum and maximum values. Black and white boxes indicate lower and upper quartiles, respectively. Line in the middle of boxes is median.





Referências Bibliográficas Gerais

1. Badr AE, Omar N, Badria FA. A laboratory evaluation of the antibacterial and cytotoxic effect of Liquorice when used as root canal medicament. Int Endod J. 2011 Jan;44(1):51-8.
2. Barnard D, Davies J, Figdor D. Susceptibility of *Actinomyces israelii* to antibiotics, sodium hypochlorite and calcium hydroxide. International Endodontic Journal. 1996; 29:320-6.
3. Bodet C, La VD, Gafner S, Bergeron C, Grenier D. A licorice extract reduces lipopolysaccharide-induced proinflammatory cytokine secretion by macrophages and whole blood. J Periodontol. 2008 Sep;79(9):1752-61.
4. Borssén E, Sundqvist G. Actinomyces of infected dental root canals Oral Surgery, Oral Medicine, Oral Pathology, Volume 51, Issue 6, June 1981, Pages 643–648.
5. Brighenti FL, Gaetti-Jardim E Jr, Danelon M, Evangelista GV, Delbem AC. Effect of *Psidium cattleianum* leaf extract on enamel demineralisation and dental biofilm composition in situ. Arch Oral Biol. 2012 Aug;57(8):1034-40.
6. Brighenti FL, Luppens SB, Delbem AC, Deng DM, Hoogenkamp MA, Gaetti-Jardim EJr, Dekker HL, Crielaard W, ten Cate JM. Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* viability, protein expression and acid production. Caries Res. 2008;42(2):148-54.
7. Cechinel Filho V, Yunes RA. Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais. Conceitos sobre modificação estrutural para otimização da atividade. Química Nova, 1998; 21(1):99-105.
8. Cheung GS, Ho MW. Microbial flora of root canal-treated teeth associated with asymptomatic periapical radiolucent lesions. Oral Microbiol Immunol. 2001; Dec;16(6):332-7.
9. Cogulu D, Uzel A, Oncag O, Eronat C. PCR-based identification of selected pathogens associated with endodontic infections in deciduous and permanent teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008; Sep;106(3):443-9.
10. Cowan MM: Plant products as antimicrobial agents. Clin Microbiol Rev 1999; 12: 564–582.

11. da Silva LAB, Perassi FT, Ito IY, Yamashita JC, Bonifácio KC, Tanomaru-Filho M. A presença de fungos nas infecções endodônticas. Revista da Faculdade de Odontologia de Lins. V.12; n.1 e 2; jan/dez 2000.
12. Delgado RJ, Gasparoto TH, Sipert CR, Pinheiro CR, de Moraes IG, Garcia RB, Duarte MA, Bramante CM, Torres SA, Garlet GP, Campanelli AP, Bernardineli N. Antimicrobial activity of calcium hydroxide and chlorhexidine on intratubular *Candida albicans*. International Journal of Oral Science. 2013; Apr; 5(1): 32–36.
13. de Menezes TE, Botazzo Delbem AC, Lourenção Brighenti F, Cláudia Okamoto A, Gaetti-Jardim E Jr. Protective efficacy of *Psidium cattleianum* and *Myracrodruon urundeuva* aqueous extracts against caries development in rats. Pharm Biol. 2010 Mar;48(3):300-5
14. de Souza-Filho FJ, Soares A de J, Vianna ME, Zaia AA, Ferraz CC, Gomes BP. Antimicrobial effect and pH of chlorhexidine gel and calcium hydroxide alone and associated with other materials. Brazilian Dental Journal. 2008; 19:28-33.
15. Dezan-Jr E, Sangalli J, Gomes-Filho JE, Gaetti-Jardim JrE. *Psidium cattleianum* plus Ca(OH)2 antimicrobial efficacy against *Enterococcus faecalis*. IADR; Jul; 15-16, 2010, J Dent Res; 2010; 89,Special Issue B.
16. Dumani A, Yoldas O, Yilmaz S, Koksal F, Kayar B, Akcimen B, Seydaoglu G. Polymerase chain reaction of *Enterococcus faecalis* and *Candida albicans* in apical periodontitis from Turkish patients. J Clin Exp Dent. 2012 Feb 1;4(1):e34-9.
17. Estrela C, Holland R, Estrela CR, Alencar AH, Sousa-Neto MD, Pécora JD. Characterization of successful root canal treatment. Braz Dent J. 2014 Jan-Feb;25(1):3-11.
18. Ferreira FB, Torres SA, Rosa OP, Ferreira CM, Garcia RB, Marcucci MC, Gomes BP. Antimicrobial effect of propolis and other substances against selected endodontic pathogens. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007; Nov;104(5):709-16.
19. Fujii R, Saito Y, Tokura Y, Nakagawa KI, Okuda K, Ishihara K. Characterization of bacterial flora in persistent apical periodontitis lesions. Oral Microbiol Immunol. 2009; Dec;24(6):502-5.
20. Gaetti-Jardim Júnior E, Landucci LF, Arafat OKK, Ranieri RV, Ramos MMB, Ciesielski FIN, Schweitzer CM, Okamoto AC. Antimicrobial activity of six

plant extracts from the Brazilian savanna on periodontal pathogens. Int. J. Odontostomat., 5(3):249-256, 2011.

21. Gomes BP, Pinheiro ET, Jacinto RC, Zaia AA, Ferraz CC, Souza-Filho FJ. Microbial analysis of canals of root-filled teeth with periapical lesions using polymerase chain reaction. J Endod. 2008 May;34(5):537-40.
22. Gomes BP, Souza SF, Ferraz CC, Teixeira FB, Zaia AA, Valdrighi L, Souza-Filho FJ. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against *Enterococcus faecalis* in bovine root dentine in vitro. Int Endod J. 2003; Apr;36(4):267-75.
23. Gomes BP, Vianna ME, Sena NT, Zaia AA, Ferraz CC, de Souza Filho FJ. In vitro evaluation of the antimicrobial activity of calcium hydroxide combined with chlorhexidine gel used as intracanal medicament. Oral Surg Oral Med Oral Pathol Oral Radiol Endod; 2006;102:544-550.
24. Haapasalo M, Orstavik D. In vitro infection and disinfection of dentinal tubules. J Dent Res. 1987; 66:1375-9.
25. Hancock HH, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001 May;91(5):579-86.
26. Martin A, Camacho M, Portaels F, Palomino JC. Resazurin Microtiter Assay Plate Testing of Mycobacterium tuberculosis Susceptibilities to Second-Line Drugs: Rapid, Simple, and Inexpensive Method. Antimicrobial agents and chemotherapy, Nov. 2003, p. 3616–3619.
27. Matias EF, Alves EF, Santos BS, Sobral de Souza CE, de Alencar Ferreira JV, Santos de Lavor AK, Figueredo FG, Ferreira de Lima L, Vieira Dos Santos FA, Neves Peixoto FS, Viana Colares A, Augusti Boligon A, Saraiva Rde A, Athayde ML, da Rocha JB, Alencar Menezes IR, Douglas Melo Coutinho H, da Costa JG. 2013. Biological Activities and Chemical Characterization of *Cordia verbenacea* DC. as Tool to Validate the Ethnobiological Usage. Evidence-Based Complementary and Alternative Medicine 2013a:164215.
28. Matias EF, Santos KK, Falcão-Silva VS, Siqueira-Junior JP, Costa JG, Coutinho HD. Modulation of the norfloxacin resistance in *Staphylococcus aureus* by *Cordia verbenacea* DC. Indian J Med Res. 2013b; Jan;137(1):178-82.
29. Meccia G, Rojas LB, Velasco J, Díaz T, Usubillaga A, Arzola JC, Ramos S. Chemical composition and antibacterial activity of the essential oil of *Cordia*

verbenacea from the Venezuelan Andes. Nat Prod Commun. 2009. Aug;4(8):1119-22.

30. Michielin EM, Salvador AA, Riehl CA, Smânia A Jr, Smânia EF, Ferreira SR. Chemical composition and antibacterial activity of *Cordia verbenacea* extracts obtained by different methods. Bioresource Technology. 2009; 100: 6615–6623.
31. Nair PN, Sjögren U, Figdor D, Sundqvist G. Persistent periapical radiolucencies of root-filled human teeth, failed endodontic treatments, and periapical scars. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1999 May;87(5):617-27.
32. Oh JH, Jeong YJ, Koo HJ, Park DW, Kang SC, Khoa HV, Le le B, Cho JH, Lee JY. Antimicrobial activities against periodontopathic bacteria of Pittosporum tobira and its active compound. Molecules. 2014 Mar 24;19(3):3607-16.
33. Ozbek SM, Ozbek A, Erdorgan AS. Analysis of *Enterococcus faecalis* in samples from Turkish patients with primary endodontic infections and failed endodontic treatment by real-time PCR SYBR green method. J Appl Oral Sci. 2009 Sep-Oct;17(5):370-4.
34. Paradella TC, Koga-Ito CY, Jorge AOC. *Enterococcus faecalis*: considerações clínicas e microbiológicas. Rev Odontol UNESP. 2007; 36(2): 163-68.
35. Pimentel SP, Barrella GE, Casarin RC, Cirano FR, Casati MZ, Foglio MA, Figueira GM, Ribeiro FV. Protective effect of topical *Cordia verbenacea* in a rat periodontitis model: immune-inflammatory, antibacterial and morphometric assays. BMC Complementary and Alternative Medicine Nov 2012; 21;12:224.
36. Raphael D, Wong TA, Moodnik R, Borden BG. The effect of temperature on the bactericidal efficiency of sodium hypochlorite. J Endod. 1981 Jul;7(7):330-4.
37. Rodrigues FF, Oliveira LG, Rodrigues FF, Saraiva ME, Almeida SC, Cabral ME, Campos AR, Costa JG. Chemical composition, antibacterial and antifungal activities of essential oil from *Cordia verbenacea* DC leaves. Pharmacognosy Res. 2012, Jul;4(3):161-5.
38. Sakamoto M, Siqueira JF Jr, Rôças IN, Benno Y. Molecular analysis of the root canal microbiota associated with endodontic treatment failures. Oral Microbiol Immunol. 2008 Aug;23(4):275-81.
39. Scannapieco FA, Yu J, Raghavendran K, Vacanti A, Owens SI, Wood K, Mylotte JM. A randomized trial of chlorhexidine gluconate on oral bacterial pathogens in mechanically ventilated patients. Critical Care. 2009;13(4):R117.

40. Siqueira JF Jr, Guimarães-Pinto T, Rôças IN. Effects of chemomechanical preparation with 2.5% sodium hypochlorite and intracanal medication with calcium hydroxide on cultivable bacteria in infected root canals. J Endod. 2007 Jul;33(7):800-5.
41. Siqueira JF Jr, Rôças IN. Diversity of endodontic microbiota revisited. J Dent Res. 2009 Nov;88(11):969-81.
42. Siqueira JF Jr, Rôças IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004 Jan;97(1):85-94.
43. Siren EK, Haapasalo MP, Ranta K, Salmi P, Kerosuo EN. Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. Int Endod J. 1997 Mar;30(2):91-5.
44. Smith K, Hunter IS. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. Journal of Medical Microbiology. 2008; Aug;57:966-73.
45. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. J Endod. 2006 Feb;32(2):93-8.
46. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998 Jan;85(1):86-93.
47. Turk BT, Sen BH, Ozturk T. *In vitro* antimicrobial activity of calcium hydroxide mixed with different vehicles against *Enterococcus faecalis* and *Candida albicans*. . Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108 2:297–301
48. Vaghela DJ, Kandaswamy D, Venkateshbabu N, Jamini N, Ganesh A. Disinfection of dentinal tubules with two different formulations of calcium hydroxide as compared to 2% chlorhexidine: As intracanal medicaments against *Enterococcus faecalis* and *Candida albicans*: An in vitro study. Journal of Conservative Dentistry. 2011; Apr-Jun; 14(2): 182–186.
49. Vojtová V, Kolár M, Hricová K, Uvízl R, Neiser J, Blahut L, Urbánek K. Antibiotic utilization and *Pseudomonas aeruginosa* resistance in intensive care units. New Microbiologica. 2011; Jul;34(3):291-8.

50. Waltimo TM, Sirén EK, Torkko HL, Olsen I, Haapasalo MP. Fungi in therapy-resistant apical periodontitis. Int Endod J. 1997 Mar;30(2):96-101.
51. Xia T, Baumgartner JC. Occurrence of Actinomyces in infections of endodontic origin. J Endod. 2003 Sep;29(9):549-52.
52. Yatsuda R, Rosalen PL, Cury JA, Murata RM, Rehder VL, Melo LV, Koo H. Effects of Mikania genus plants on growth and cell adherence of *mutans streptococci*. Journal of Ethnopharmacology. 2005; Feb 28;97(2):183-9.

Anexos

Anexo A

Protocolo da Coloração de GRAM

1. Fazer um esfregaço bem homogênio, a partir de uma cultura em caldo, fixar pelo calor e esperar esfriar;
2. Cobrir a lâmina com cristal violeta e deixar agir por 1 minuto;
3. Lavar rapidamente em água
4. Cobrir com solução de lugol por 1 minuto;
5. Lavar em água corrente;
6. Descorar com álcool acetona (aproximadamente 15 segundos)
7. Corar com fucsina por 30 segundos;
8. Lavar em água, deixar secar e observar na objetiva desejada.

Anexo B

Protocolo da Curva de Crescimento

Dia 1: Reativar o microrganismo em BHI agar para as bactérias e SD agar para *C. albicans* (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar de acordo com as exigências de oxigênio requeridas pelo microrganismo/experimento.

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

Dia 3 (dia do experimento):

- Realizar a leitura da cultura overnight em espectrofotômetro (550nm);

- Pipetar o volume do inoculo (cultura overnight) pré-determinado de acordo com cada microrganismo, em cada tubo contendo 5ml de meio de cultura em caldo em temperatura ambiente e incubar (0h).

*Volumes: 50µl para cultura de *E. faecalis*; 200µl para *C. albicans*; 500µl para *P. aeruginosa* e *A.israelii.*

*10 tubos

*Enumerar os tubos em duplicata: 1 e 1', 2 e 2'...

- Realizar a leitura da D.O. em espectrofotômetro (550nm) de uma alíquota de dois tubos (duplicata); e o plaqueamento de uma dessas, de hora em hora;

*Diluição: 50µl do tubo de leitura em 450µl de solução salina 0,9% = 1ºeppendorf (deverá ser plaqueado no quadrante 1), e assim sucessivamente; o quadrante 0 corresponde à cultura não diluída, direto do tubo.

* Plaqueamento: dividir a placa de petri em 8 quadrantes (0 à 7; cada quadrante irá corresponder à diluição realizada). Pipetar no mínimo 2 gotas, cada uma com 25µl, por quadrante.

- A leitura/plaqueamento deverão ser realizadas até que a D.O. comece a se repetir (fase estacionária);

Dia 4: Após 24h de incubação, realiza-se a contagem das UFCs.

-Ler um quadrante (que tenha de 30 à 300 UFC) por placa e anotar tanto a leitura quanto a diluição selecionada para a contagem.

-Determinar em UFC/ml

M dia da Contagem x Fator de Correção x 10

Onde: Fator de Correção = 40 (quando se utilizar 25µl/gota)

X= número do quadrante correspondente à diluição

Anexo C

Protocolo de Determinação da CIM e CLM

Dia 1: Reativar o microrganismo em BHI agar para as bactérias e SD agar para *C. albicans* (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar de acordo com as exigências de oxigênio requeridas pelo microrganismo/experimento.

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

Dia 3: Diluição dos antimicrobianos teste:

*Pipetar 50µl do extrato ressuspendido na concentração de 8mg/ml no primeiro e segundo poço de uma placa de 96 poços;

*Pipetar 50µl de água em cada poço, exceto o primeiro;

*Ressuspender o segundo poço e transferir 50µl para o terceiro poço, e assim sucessivamente (diluição seriada);

*Tampar a placa, cobrir com papel alumínio e manter na geladeira.

- Preparar o inoculo de acordo com a curva de crescimento: D.O. 0,5 para as bactérias e 0,3 para *C. albicans*.

*Mueller-Hinton caldo para as bactérias e RPMI-1640 para *C. albicans*

*Diluir 1000x no meio de cultura 2x concentrado (500µl do inoculo em 4,5ml de meio – 3 tubos)

- Ressuspender 50µl do inoculo em cada poço; tampar a placa e incubar por 24h.

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

- Plaquear o último poço com coloração azulada (CIM) e, no mínimo, 2 poços anteriores para se determinar a CLM.

Dia 5: Leitura das placas

Anexo D

Protocolo do Ensaio de Biofilme

Dia 1: Reativar o microrganismo em BHI agar para as bactérias e SD agar para *C. albicans* (estriar 15µl da cultura estoque – Técnica do esgotamento) e incubar de acordo com as exigências de oxigênio requeridas pelo microrganismo/experimento.

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

Dia 3: Preparar a placa de 96 poços com fundo em U (não tratada): pipetar 200µl de saliva estimulada (esterilizada por uma membrana de 0,22µm) em cada poço e incubar por um período de 4h;

- Preparar o inoculo de acordo com a curva de crescimento: D.O. 0,5 para todos os microrganismos teste;

- Remover a saliva com ponteira estéril e acrescentar 20µl do inoculo e 180µl de BHI ou SDB suplementados com 0,5% de glicose; incubar por 48h.

Dia 5: Remover o sobrenadante com ponteira estéril, e lavar os poços com 200µl de solução salina 0,9% durante 3 minutos, remover toda a solução com ponteira estéril;

- Acrescentar 200µl do antimicrobiano na concentração 5 e 10 vezes maiores que a CLM pré-determinada;

*Incluir as mesmas concentrações para os controles positivos (Clorexidina e Anfotericina B);

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

*Deixar três poços com 200µl de água destilada estéril (controle negativo);

- Incubar por 24h.

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

-Diluição e plaqueamento de todos os poços.

Dia 7: Leitura das placas.

Anexo E

Guide for Authors (Journal of Ethnopharmacology)

Introduction

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

Please note that figures and tables should be embedded in the text as close as possible to where they are initially cited. It is also mandatory to upload separate graphic and table files as these will be required if your manuscript is accepted for publication.

Classification of your paper

Please note that upon submitting your article you will have to select **at least one classification** and **at least three of the given keywords**. You can preview the list of classifications and keywords (here). This information is needed by the Editors to more quickly process your article. In addition to this, you can submit free keywords as described below under "Keywords".

The "rules of 5"

The Editors and Editorial Board have developed the "Rules of 5" for publishing in JEP. We have produced five clear criteria that each author needs to think about before submitting a manuscript and setting the whole process of editing and reviewing at work. Click here.

For more details on how to write a world class paper, please visit our Pharmacology Author Resources page.

Authors are encouraged to submit video material or animation sequences to support and enhance your scientific research. For more information please see the paragraph on video data below.

Types of paper

The *Journal of Ethnopharmacology* will accept the following contributions:

Original research articles - whose length is not limited and should include Title, Abstract, Methods and Materials, Results, Discussion, Conclusions, Acknowledgements and References. As a guideline, a full length paper normally occupies no more than 10 printed pages of the journal, including tables and illustrations.
2. Ethnopharmacological communications (formerly Short Communications) - whose average length is not more than 4 pages in print (approx. 2000-2300 words, including abstract and references). A maximum of 2 illustrations (figures or tables) is allowed. See paragraph below for description and format.
3. Letters to the Editors.
4. Reviews - Authors intending to write review articles should consult and send an outline to the Reviews Editor (see inside front cover for contact information) before preparing their manuscripts. The organization and subdivision of review articles can be arranged at the author's discretion. Authors should keep in mind that a good review sets the trend and direction of future research on the subject matter being reviewed. Tables, figures and references are to be arranged in the same way as research articles in the journal. Reviews on topics that address cutting-edge problems are particularly welcome.
Outlines for potential reviews need to include:

- A detailed abstract using the structure provided in the guidelines
- An annotated table of contents
- A short CV of the lead author

5. Book reviews - Books for review should be sent to the Reviews Editor. 6. Commentaries - *invited*, peer-reviewed, critical discussion about crucial aspects of the field but most importantly methodological and conceptual-theoretical developments in the field and should also provide a standard, for example, for pharmacological methods to be used in papers in the *Journal of Ethnopharmacology*. The scientific dialogue differs greatly in the social / cultural and natural sciences, the discussions about the common foundations of the field are ongoing and the papers published should contribute to a transdisciplinary and multidisciplinary discussion. The length should be a maximum of 2-3 printed pages or 2500 words. Please contact the Reviews Editor j.ethnopharmacol@pharmacy.ac.uk with an outline.

7. Conference announcements and news.



Before You Begin

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/journal-authors/ethics.

Policy and ethics - In the covering letter, the author must also declare that the study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights. See below for further information. The ethnopharmacological importance of the study must also be explained in the cover letter.

Animal and clinical studies - Investigations using experimental animals must state in the Methods section that the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in for example the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or the US guidelines (NIH publication #85-23, revised in 1985). Investigations with human subjects must state in the Methods section that the research followed guidelines of the Declaration of Helsinki and Tokyo for humans, and was approved by the institutional human experimentation committee or equivalent, and that informed consent was obtained. The Editors will reject papers if there is any doubt about the suitability of the animal or human procedures used.

Biodiversity rights - Each country has its own rights on its biodiversity. Consequently for studying plants one needs to follow the international, national and institutional rules concerning the biodiversity rights.

Author contributions

For each author the contribution to the publication should be mentioned.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://help.elsevier.com/app/answers/detail/a_id/286/p/7923.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/postingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the

work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Article transfer service

This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. More information about this can be found here: http://www.elsevier.com/authors/article-transfer-service.

Copyright
This journal offers authors a choice in publishing their research: Open access and Subscription.

For subscription articles

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see http://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement. Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

For open access articles

Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see http://www.elsevier.com/OAauthoragreement). Permitted reuse of open access articles is determined by the author's choice of user license (see http://www.elsevier.com/openaccesslicenses).

Retained author rights

As an author you (or your employer or institution) retain certain rights. For more information on author rights for:

Subscription articles please see http://www.elsevier.com/journal-authors/author-rights-and-responsibilities.
Open access articles please see http://www.elsevier.com/OAauthoragreement.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Open access

This journal offers authors a choice in publishing their research:

Open access

• Articles are freely available to both subscribers and the wider public with permitted reuse
• An open access publication fee is payable by authors or their research funder
Subscription
• Articles are made available to subscribers as well as developing countries and patient groups through our access programs (http://www.elsevier.com/access)

• No open access publication fee

All articles published open access will be immediately and permanently free for everyone to read and download. Permitted reuse is defined by your choice of one of the following Creative Commons user licenses:
Creative Commons Attribution (CC BY): lets others distribute and copy the article, to create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-ShareAlike (CC BY-NC-SA): for non-commercial purposes, lets others distribute and copy the article, to create extracts, abstracts and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text and data mine the article, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, do not modify the article in such a way as to damage the author's honor or reputation, and license their new adaptations or creations under identical terms (CC BY-NC-SA).

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND): for non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

To provide open access, this journal has a publication fee which needs to be met by the authors or their research funders for each article published open access.

Your publication choice will have no effect on the peer review process or acceptance of submitted articles.
The open access publication fee for this journal is **$3000**, excluding taxes. Learn more about Elsevier's pricing policy: http://www.elsevier.com/openaccesspricing.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.

Additional information

Authors who want to submit a manuscript should consult and peruse carefully recent issues of the journal for format and style. Authors must include the following contact details on the title page of their submitted manuscript: full postal address; fax; e-mail. All manuscripts submitted are subject to peer review. The minimum requirements for a manuscript to qualify for peer review are that it has been prepared by strictly following the format and style of the journal as mentioned, that it is written in good English, and that it is complete. Manuscripts that have not fulfilled these requirements will be returned to the author(s).

In addition, you are recommended to adhere to the research standards described in the following articles:

Cos P., Vlietinck A.J., Berghe D.V., et al. (2006) Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. Journal of Ethnopharmacology, 106: 290-302.

Matteucci, E., Giampietro, O. (2008) Proposal open for discussion: defining agreed diagnostic procedures in experimental diabetes research. Journal of Ethnopharmacology,115: 163-172.

Froede, T.SA. and Y.S. Medeiros, Y.S. (2008) Animal models to test drugs with potential antidiabetic activity. Journal of Ethnopharmacology 115: 173-183. Gertsch J. (2009) How scientific is the science in ethnopharmacology? Historical perspectives and epistemological problems. Journal of Ethnopharmacology, 122: 177-183.

Chan K., et al. (2012) Good practice in reviewing and publishing studies on herbal medicine, with special emphasis on traditional Chinese medicine and Chinese Materia Medica. Journal of Ethnopharmacology 140: 469-475.

Heinrich, M., Edwards. S., Moerman. D.E.. and Leonti. M. (2009), Ethnopharmacological field studies: a critical assessment of their conceptual basis and methods. J. Ethnopharmacol, 124: 1-17.



Preparation

Reference formatting

References in the manuscript should be given with the name of the first author (in case of 2 authors, both names, and in case of more first author et al.), and the year of publication, in the reference list any style or format can be used as long as the style is consistent. In the list all author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Use of word processing software

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

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

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

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

Glossary
Please supply, as a separate list, the definitions of field-specific terms used in your article.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The author should divide the abstract with the **headings *Ethnopharmacological relevance, Materials and Methods, Results,* and *Conclusions***.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples. Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Keywords
After having selected a classification in the submission system, authors must in the same step select 5 keywords. These keywords will help the Editors to categorize your article accurately and process it more quickly. A list of the classifications and set keywords can be found here.

In addition, you can provide a maximum of 6 specific keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Chemical compounds

You can enrich your article by providing a list of chemical compounds studied in the article. The list of compounds will be used to extract relevant information from the NCBI PubChem Compound database and display it next to the online version of the article on ScienceDirect. You can include up to 10 names of chemical compounds in the article. For each compound, please provide the PubChem CID of the most relevant record as in the following example: Glutamic acid (PubChem CID:611). The PubChem CIDs can be found via http://www.ncbi.nlm.nih.gov/pccompound. Please position the list of compounds immediately below the 'Keywords' section. It is strongly recommended to follow the exact text formatting as in the example below:

Chemical compounds studied in this article Ethylene glycol (PubChem CID: 174); Plitidepsin (PubChem CID: 44152164); Benzalkonium chloride (PubChem CID: 15865)

More information is available at: http://www.elsevier.com/PubChem.

Plant names

In the Materials and Methods section there must be a separate heading for describing the material used. That includes official name, local name, English name (if known), GPS position in case of collection in the wild or cultivation, a voucher specimen must be deposited in an official herbarium for possible future comparison. In the text it should be stated that the plant name has been checked with www.theplantlist.org mentioning the data of accessing that website.

In case of commercially procured material should mention the source, batch number, quality control data. Data on chemical characterization (metabolomics, chromatographic methods) should also be presented, in case of known active compounds their quantitative analysis should be presented.

Acknowledgements

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

Database linking

Elsevier encourages authors to connect articles with external databases, giving their readers one-click access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.
Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Embed the used fonts if the application provides that option.

• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Size the illustrations close to the desired dimensions of the printed version.

• Submit each illustration as a separate file.

A detailed guide on electronic artwork is available on our website:

http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below): EPS (or PDF): Vector drawings, embed all used fonts. TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi. TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi. TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Please note that figures and tables should be embedded in the text as close as possible to where they are initially cited. It is also mandatory to upload separate graphic and table files as these will be required if your manuscript is accepted for publication.
Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with "Unpublished results". "*Personal communication*" will not be accepted as a reference. Citation of a reference as "in press" implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: All citations in the text should refer to:

Single author: the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by "et al." and the year of publication. Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: "as demonstrated (Allan, 1996a, 1996b, 1999; Allan and Jones, 1995). Kramer et al. (2000) have recently shown ...."

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication. Please use full journal names.
Examples:
Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2000. The art of writing a scientific article. Journal of Scientific Communication. 163, 51-59.

Reference to a book:

Strunk Jr., W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York.
Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281-304.

When a web citation with a link is being used as a reference, the link should be checked, and that date reported with the link as "accessed on March 29, 2012":

American Chemical Society. 2012. Ethical guidelines to publication of chemical research. http://pubs.acs.org/userimages/ContentEditor/1218054468605/ethics.pdf. Accessed on March 31, 2012.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

• Phone numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

•All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print

• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes.

Anexo F

Guide for Authors (Journal of Endodontics)

Introduction

The *Journal of Endodontics* is owned by the American Association of Endodontists. Submitted manuscripts must pertain to endodontics and may be original research (eg, clinical trails, basic science related to the biological aspects of endodontics, basic science related to endodontic techniques, case reports, or review articles related to the scientific or applied aspects of endodontics). Clinical studies using CONSORT methods (http://www.consort-statement.org/consort-statement/) or systematic reviews using meta-analyses are particularly encouraged. Authors of potential review articles are encouraged to first contact the Editor during their preliminary development via e-mail at *JEndodontics@UTHSCSA.edu*. Manuscripts submitted for publication must be submitted solely to *JOE*. They must not be submitted for consideration elsewhere or be published elsewhere.

Disclaimer

The statements, opinions, and advertisements in the *Journal of Endodontics* are solely those of the individual authors, contributors, editors, or advertisers, as indicated. Those statements, opinions, and advertisements do not affect any endorsement by the American Association of Endodontists or its agents, authors, contributors, editors, or advertisers, or the publisher. Unless otherwise specified, the American Association of Endodontists and the publisher disclaim any and all responsibility or liability for such material.

Before You Begin

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/journal-authors/ethics.

Human and animal rights

If the work involves the use of animal or human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans http://www.wma.net/en/30publications/10policies/b3/index.html; EU Directive 2010/63/EU for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; Uniform Requirements for manuscripts submitted to Biomedical journals http://www.icmje.org. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

Conflict of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. See also http://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://help.elsevier.com/app/answers/detail/a_id/286/p/7923.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/postingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in

the same form, in English or in any other language, without the written consent of the copyright-holder.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:*Before the accepted manuscript is published in an online issue*: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Reporting clinical trials

Randomized controlled trials should be presented according to the CONSORT guidelines. At manuscript submission, authors must provide the CONSORT checklist accompanied by a flow diagram that illustrates the progress of patients through the trial, including recruitment, enrollment, randomization, withdrawal and completion, and a detailed description of the randomization procedure. The CONSORT checklist and template flow diagram can be found on http://www.consort-statement.org.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright see http://www.elsevier.com/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

Retained author rights

As an author you (or your employer or institution) retain certain rights; for details you are referred to: http://www.elsevier.com/authorsrights.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible

grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (

http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission
Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.
Submit your article

Please submit your article via http://ees.elsevier.com/joe/.



Preparation

General Points on Composition

Authors are strongly encouraged to analyze their final draft with both software (eg, spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery because their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision or that have unacceptable grammar or syntax. The following list represents common errors in manuscripts submitted to the Journal of Endodontics:
a. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section "c," below), or sentences with little to no transition within a paragraph.

b. Keep to the point. The subject of the sentence should support the subject of the paragraph For example, the introduction of authors' names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, "In 1983, Langeland et al, reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals" can be edited to: "Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al, 1983)." In this example, the paragraph's subject is sodium hypochlorite and sentences should focus on this subject.

c. Sentences are stronger when written in the active voice, that is, the subject performs the action. Passive sentences are identified by the use of passive verbs such as "was," "were," "could," etc. For example: "Dexamethasone was found in this study to be a factor that was associated with reduced inflammation," can be edited to: "Our results demonstrated that dexamethasone reduced inflammation." Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

d. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus, or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph. (This section was written deliberately with sentences of varying length to illustrate this point.)
e. Use parallel construction to express related ideas. For example, the sentence, "Formerly, endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method," can be edited to "Formerly, endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation." The use of parallel construction in sentences simply means that similar

ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related. f. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, "Accordingly, when conclusions are drawn from the results of this study, caution must be used," can be edited to "Caution must be used when conclusions are drawn from the results of this study."

g. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.

h. Authors should be aware that the JOE uses iThenticate, plagiarism detection software, to ensure originality and integrity of material published in the journal. The use of copied sentences, even when present within quotation marks, is highly discouraged. Instead, the information of the original research should be expressed by the new manuscript author's own words, and a proper citation given at the end of the sentence. Plagiarism will not be tolerated and manuscripts will be rejected or papers withdrawn after publication based on unethical actions by the authors. In addition, authors may be sanctioned for future publication.

Use of word processing software

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

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

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Structured abstract

A structured abstract, by means of appropriate headings, should provide the context or background for the research and should state its purpose, basic procedures (selection of study subjects or laboratory animals, observational and analytical methods), main findings (giving specific effect sizes and their statistical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations.

Abstract Headings

Introduction, Methods, Results, Conclusions

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

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

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

Original Research Article Guidelines

Title Page

The title describes the major emphasis of the paper. It must be as short as possible without loss of clarity. Avoid abbreviations in the title because this may lead to imprecise coding by electronic citation programs such as PubMed (eg, use sodium hypochlorite rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at www.icmje.org). Include the manuscript title; the names and affiliations of all authors; and the name, affiliation, and full mailing address (including e-mail) of the corresponding author. This author will be responsible for proofreading page proofs and ordering reprints when applicable. Also highlight the contribution of each author in the cover letter.
Abstract
The Abstract concisely describes the purpose of the study in 250 or fewer words. It must be organized into sections: Introduction, Methods, Results, and Conclusions. The hypothesis is described in the Abstract Introduction. The Abstract describes the new contributions made by this study. The Abstract word limitation and its wide distribution (eg, PubMed) make it challenging to write clearly. This section is written last by many authors. Write the abstract in past tense because the study has been completed. Provide 3-5 keywords.

Introduction
The introduction briefly reviews the pertinent literature in order to identify the gap in knowledge that the study is intended to address and the limitations of previous studies in the area. Clearly describe the purpose of the study, the tested hypothesis, and its scope. Many successful manuscripts require no more than a few paragraphs to accomplish these goals; therefore, do not perform extensive literature review or discuss the results of the study in this section.

Materials and Methods

The Materials and Methods section is intended to permit other investigators to repeat your experiments. There are 4 components to this section: (1) detailed description of the materials used and their components, (2) experimental design, (3) procedures employed, and (4) statistical tests used to analyze the results. Most manuscripts should cite prior studies that used similar methods and succinctly describe the essential aspects used in the present study. A "methods figure" will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then you must carefully describe the method and include validation experiments. If the study used a commercial product, the manuscript must either state that you followed manufacturer's protocol or specify any changes made to the protocol. If the study used an *in vitro* model to simulate a clinical outcome, describe either experiments made to validate the model or previous literature that proved the clinical relevance of the model. The statistical analysis section must describe which tests were used to analyze which dependent measures; *P* values must be specified. Additional details may include randomization scheme, stratification (if any), power analysis as a basis for sample size computation, dropouts from clinical trials, the effects of important confounding variables, and bivariate versus multivariate analysis.

Results
Only experimental results are appropriate in this section; do not include methods, discussion, or conclusions. Include only those data that are critical for the study, as defined by the aim(s). Do not

include all available data without justification; any repetitive findings will be rejected from publication. All Figures, Charts, and Tables must be cited in the text in numerical order and include a brief description of the major findings. Consider using Supplemental Figures, Tables, or Video clips that will be published online. Supplemental material often is used to provide additional information or control experiments that support the results section (eg, microarray data).

Figures
There are 2 general types of figures: type 1 includes photographs, radiographs, or micrographs; type 2 includes graphs. *Type 1:* Include only essential figures and use composite figures containing several panels of photographs, if possible. Each panel must be clearly identified with a letter (eg, A, B, C), and the parts must be defined in the figure legend. A figure that contains many panels counts as 1 figure. *Type 2:* Graphs (ie, line drawings including bar graphs) that plot a dependent measure (on the Y axis) as a function of an independent measure (usually plotted on the X axis). One example is a graph depicting pain scores over time. Use graphs when the overall trend of the results is more important than the exact numeric values of the results. A graph is a convenient way to report that an ibuprofen-treated group reported less pain than a placebo-treated group over the first 24 hours, but pain reported was the same for both groups over the next 96 hours. In this case, the trend of the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

Tables
Tables are appropriate when it is critical to present exact numeric values; however, not all results need be placed in either a table or figure. Instead of a simple table, the results could state that there was no inhibition of growth from 0.001%-0.03% NaOCl, and a 100% inhibition of growth from 0.03%-3% NaOCl (N=5/group). If the results are not significant, then it is probably not necessary to include the results in either a table or as a figure.

Acknowledgments
All authors must affirm that they have no financial affiliation (eg, employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements, or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past 3 years. Disclose any potential conflict of interest. Append a paragraph to the manuscript that fully discloses any financial or other interest that poses a conflict. Disclose all sources and attribute all grants, contracts, or donations that funded the study. Specific wording: "The authors deny any conflicts of interest related to this study."

References
The reference style can be learned from reading past issues of *JOE*. References are numbered in order of citation. Place text citation of the reference Arabic number in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

Other Article Types and Guidelines

Manuscripts submitted to *JOE* that are not Original Articles must fall into one of the following categories. Abstract limit: 250 words. Note that word limits, listed by type, do not include figure legends or References. If you are not sure whether your manuscript falls within one of the categories listed or if you would like to request pre-approval to submit additional figures, contact the Editor at *JEndodontics@uthscsa.edu*.

CONSORT Randomized Clinical Trial

Must strictly adhere to the Consolidated Standards of Reporting Trials—CONSORT—minimum guidelines for publication of randomized clinical trials (http://www.consort-statement.org). Word limit: 3500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables: 4.

Review Article

Either narrative articles or systemic reviews/meta-analyses. Case Report/Clinical Techniques articles, even when they include an extensive review of the literature, are categorized as Case Report/Clinical Techniques. Word limit: 3500. Headings: Abstract, Introduction, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables: 4.

Clinical Research

Prospective or retrospective studies of patients or patient records, research on biopsies excluding the use of human teeth for technique studies. Word limit: 3500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables: 4.

Basic Research—Biology

Animal or culture studies of biological research on physiology, development, stem cell differentiation, inflammation, or pathology. Primary focus is on biology. Word limit: 2500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables: 4.

Basic Research—Technology

Focus primarily on research related to techniques and materials used, or on potential clinical use, in endodontics. Word limit: 2500. Headings: Abstract, Introduction, Material and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 3. Maximum number of tables: 3.

Case Report/Clinical Techniques

Reports of an unusual clinical case or use of a cutting edge technology in a clinical case. Word limit: 2500. Headings: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments. Maximum number of figures: 4. Maximum number of tables: 4.

Units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Database linking

Elsevier encourages authors to connect articles with external databases, giving their readers one-click access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Artwork
Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Embed the used fonts if the application provides that option.

• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately

• Size the illustrations close to the desired dimensions of the printed version.

• Submit each illustration as a separate file.

A detailed guide on electronic artwork is available on our website: http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.

Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.

Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.

TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.

TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF) or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) in addition to color reproduction in print. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.
Tables
Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not allowed in the reference list, but they may be mentioned in the text. Citation of a reference as "in press" implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Web References

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references are included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: Indicate references by Arabic numerals in parentheses, numbered in the order in which they appear in the text. *List:* Number the references in the list in the order in which they appear in the text. List 3 authors then et al.

Examples:
Journal article:

1. Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun. 2010;163:51–59.
Book:

2. Strunk W Jr, White EB. *The Elements of Style,* 4th ed. New York: Longman; 2000. Chapter in an edited book:

3. Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, eds. *Introduction to the Electronic Age.* New York: E-Publishing; 2009:281–304. *Journal abbreviations source*

Journal names are abbreviated according to Index medicus.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides
The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including

ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.
Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
• E-mail address

• Full postal address

• Phone numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print

• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes

For any further information please visit our customer support site at http://support.elsevier.com.