UNIVERSIDADE FEDERAL DE PELOTAS

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Dissertação

Carcterização de compostos bioativos em *Tagetes minuta*, *Bixa orellana* e *Mentha piperita* como potenciais agentes antimicrobianos em cimentos endodônticos experimentais

Daniela Coelho dos Santos

Pelotas, 2017

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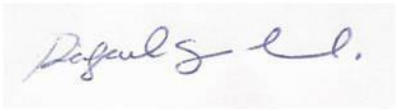

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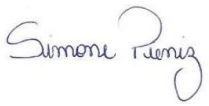

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Por isso, você é do tamanho dos seus sonhos.

Augusto Cury

PARTE I

RESUMO

DOS SANTOS, Daniela Coelho. **Caracterização de compostos bioativos em *Tagetes minuta*, *Bixa orellana* e *Mentha piperita* como potenciais agentes antimicrobianos em cimentos endodônticos experimentais.** 2017. 129f. Dissertação (Mestrado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas.

O objetivo deste estudo foi revisar sistematicamente na literatura e em bases de dados de patentes o que existe de evidência sobre o uso antimicrobiano de duas espécies vegetais (*Bixa orellana* e *Tagetes minuta*), bem como investigar um novo material endodôntico obturador experimental contendo óleos essenciais e extrato vegetais com potencial antimicrobiano dessas espécies, uma vez que infecções de origem bacteriana são apontadas como as principais causas de fracasso em endodontia. Três espécies foram utilizadas neste estudo: *B. orellana*, *Mentha piperita* e *T. minuta*. Inicialmente foi realizada duas revisões sistemáticas em bases de dados e bancos de patentes com o intituito de identificar na literatura o que já é conhecido sobre a atividade antimicrobiana de *T. minuta* e *B. orellana*. Após a revisão, as plantas foram coletadas e submetidas a extração dos óleos e extrato vegetais e, analisadas para identificação dos principais constituintes químicos. O próximo passo foi identificar a capacidade antimicrobiana dos mesmos. Para isso, foram realizados os ensaios de MIC e TCD contra três microrganismos da coleção ATCC associados a infecções endodônticas: *Candida albicans* (62342), *Enterococcus faecalis* (4083) *e Streptococcus mutans* (25175). Após a comprovação da capacidade antimicrobiana, o material vegetal foi incorporado em uma formulação experimental de um material selador do canal radicular na concentração de 0,5%. O material foi analisado por meio de ensaios físico-mecânicos (GC, TP, EP, AD, E, R) e o cimento endodôntico Real Seal®, foi utilizado como controle para fins de comparação de valores nos testes. Quanto aos resultados das reviões sistemáticas, conclui-se elevado potencial antimicrobiano das plantas em questão, porém, vale ressaltar que estudos citotóxicos e *in vivo* se fazem necessário. Os materias experimentais demonstraram bons resultados com valores dentro do recomendável pela ISO 6876, para todos os ensaios realizados, exceto R e EP. Quanto ao efeito antimicrobiano dos cimentos experimentais, a variável tempo teve influência na ação antimicrobiana, conforme maior o tempo de exposição ao microrganismo, maior o efeito antimicrobiano dos materiais, exceto para *C. albicans*, que não demonstrou efeito inibitório, quando comparado ao controle positivo. Por fim, conclui-se que os novos materiais obtidos possuem potencial antimicrobiano e mais estudos devem ser realizados para a comprovação desta propriedade.

Palavras-chave: produtos naturais, atividade antimicrobiana, cimento endodôntico, estudos *in vitro.*

ABSTRACT

DOS SANTOS, Daniela Coelho. **Characterization of bioactive compounds in *Tagetes minuta*, *Bixa orellana* and *Mentha piperita* as potential antimicrobial agents in experimental endodontic sealers.** 2017. 129f. Dissertação (Mestrado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas.

The objective of this study was to systematically review in literature and in patent databases the existence of evidence on the antimicrobial use of two plant species (*Bixa orellana* and *Tagetes minuta*), as well as investigating a new experimental endodontic obturator material containing essential oils and plant extract with antimicrobial potential of these species, since bacterial infections are indicated as the main causes of endodontic failure. Three species were used in this study: *B. orellana*, *Mentha piperita* and *T. minuta*. Initially, two systematic reviews were carried out on databases and patent databases with the aim of identifying in the literature what is already known about the antimicrobial activity of *T. minuta* and *B. orellana*. After the review, the plants were collected and submitted to extraction of the oils and vegetable extract and analyzed for identification of the main chemical constituents. The next step was to identify their antimicrobial capacity. For this, MIC and TCD assays were performed against three microorganisms from the ATCC collection associated with endodontic infections: *Candida albicans* (62342), *Enterococcus faecalis* (4083) and *Streptococcus mutans* (25175). After proving the antimicrobial capacity, the plant material was incorporated into an experimental formulation of a 0.5% root canal sealer. The material was analyzed by physico-mechanical tests (GC, TP, EP, AD, E, R) and the Royal Seal® endodontic cement was used as control for comparison purposes. Regarding the results of the systematic reviews, the high antimicrobial potential of the plants in question is concluded, but it is worth mentioning that cytotoxic and in vivo studies are necessary. The experimental materials showed good results with values within the recommended by ISO 6876, for all tests performed except R and EP. As for the antimicrobial effect of the experimental cements, the time variable had an influence on the antimicrobial action, the longer the exposure time to the microorganism, the greater the antimicrobial effect of the materials, except for *C. albicans*, which showed no inhibitory effect when compared to the positive control. Por fim, conclui-se que os novos materiais obtidos possuem potencial antimicrobiano e mais estudos devem ser realizados para a comprovação desta propriedade

Key words: natural products, antimicrobial activity, endodontic cement, *in vitro* studies.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AD – Alteração Dimensional

ATCC – *American Type Culture Collection*

CIM – Concentração Inibitória Mínima

E – Escoamento

EP – Espessura de Película

GC – Grau de Conversão

IBGE – Instituto Brasileiro de Geografia e Estatística

ISO – *International Organization for Standardization*

OMC – Organização Mundial do Comércio

OMPI – Organização Mundial de Propriedade Intelectual

R – Radiopacidade

SUS – Sistema Único de Saúde

TCD – Teste de Contato Direto

TP – Tempo de Presa

Sumário

PARTE II

1 Introdução

Os óleos essenciais e extratos de plantas aromáticas e medicinais são potencialmente úteis como agentes antimicrobianos e seu uso como medicamentos tem sido reconhecido (HOLLEY & PATEL, 2005). O uso de óleos essenciais e extratos como agentes antimicrobianos apresenta duas características principais: I) a sua origem natural o que significa mais segurança para os usuários e meio ambiente; II) menor risco de aumento da resistência microbiana a sua ação, porque estes são misturas de vários compostos que, aparentemente, apresentam diferentes ações antimicrobianas tornando mais difícil a adaptabilidade de microrganismos (MELO E SILVA, DE PAULA, ESPINDOLA, 2009; BUENO-SILVA *et al*., 2013).

Na área odontológica, há uma grande procura por novos produtos com potencial antimicrobiano e de baixa toxidade, já que a maioria das patologias orais é causada por microrganismos. Um dos desafios clínicos de interesse odontológico e de desenvolvimento são materiais capazes de obturar o canal radicular conferindo ao mesmo vedamento adequado (HAMMAD, QUALTROUGH, SILIKAS, 2009). A importância da presença de características antimicrobianas nos materiais seladores do canal radicular se dá principalmente pela incidência de infecções endodônticas recorrentes e resistentes, os efeitos adversos de alguns agentes químicos antibacterianos atualmente utilizados em odontologia, considerações financeiras dos países em desenvolvimento, além da necessidade de prevenção e opções de tratamento alternativos que sejam seguros, eficazes e econômicos (KESLER SHVERO *et al*., 2012; HEYDER *et al*., 2013).

Embora haja diferentes agentes endodônticos com características antimicrobianas disponíveis comercialmente, há a preocupação em relação à biocompatibilidade destas substâncias e os seus efeitos tóxicos, alérgicos e mutagênicos (MOZAYENI *et al*., 2012). Assim, a busca pelo desenvolvimento de produtos alternativos seguros e eficazes continuam sendo os fitoquímicos naturais isolados de plantas considerados como potenciais alternativas terapêuticas (PALOMBO, 2011). Visto isso, na tentativa de proporcionar um maior conjunto de características positivas e favoráveis para um tratamento endodôntico de sucesso, pesquisas vêm sendo realizadas visando o

desenvolvimento de seladores e obturadores para aplicações endodônticas (PAMEIJER AND ZMENER, 2010; CHADA *et al*., 2012).

Sendo assim, este estudo visa desenvolver um cimento selador endodôntico polimerizável, que possa ser aplicado tanto no selamento dos canais radiculares e/ou do preparo coronário de acesso endodôntico, quanto como carreador de substâncias bioativas de medicação intracanal. Ainda, o material deve apresentar baixo módulo de elasticidade, compatibilidade com a dentina úmida (proporcionada pela utilização de monômeros surfactantes), radiopacidade, capacidade de expansão para auxiliar no selamento, profundidade de polimerização adequada, biocompatibilidade e presença de substâncias naturais antimicrobianas.

Há um desafio clinico permanente na Odontologia em diminuir a incidência de infecções endodônticas recorrentes e resistentes e um questionamento tecnológico a respeito dos efeitos adversos (toxicidade, alergias e mutagênese) de alguns agentes químicos antibacterianos atualmente utilizados. Materiais temporários com versatilidade de aplicação (podendo ser aplicado tanto no preparo coronário quanto no sistema de canais radiculares), com potencial antimicrobiano e utilizando alta tecnologia à base de resina e extratos naturais com potencial terapêutico são escassos no mercado odontológico voltado para a Endodontia, tanto estrangeiro quanto nacional. Sendo assim, este estudo torna-se altamente promissor para o mercado odontológico brasileiro, uma vez que possuímos matéria prima promissora em nosso país.

2. Objetivos

2.2.1 Geral

Revisar sistematicamente a literatura científica e as bases de dados de patentes sobre o potencial antimicrobiano de duas espécies vegetais (*T. minuta* e *B. orellana*), bem como obter e caracterizar quimicamente os extratos e óleos essenciais vegetais de *T. minuta*, *B. orellana* e *M. piperita* para a preparação de novos materiais odontológicos de uso endodôntico.

2.2.2 Específicos

1) Realizar duas revisões sistemáticas, baseadas na literatura científica e bancos de dados de patentes, sobre o potencial antimicrobiano de *B. orellana* e *T. minuta.*

2) Cultivar as espécies vegetais, obter e caracterizar os extratos e óleos essenciais vegetais;

3) Testar as atividade antimicrobiana dos óleos essenciais e extratos brutos das plantas em estudo;

4) Caracterizar, avaliar e comparar as propriedades físico-mecânicas desse novo material contendo óleo ou extrato vegetal;

5) Desenvolver um material odontológico com propriedades antimicrobianas promovidas por plantas medicinais testadas e selecionadas de acordo com seu potencial efeito.

3. Revisão de Literatura

3.1 Revisão sistemática

A revisão sistemática da literatura é um estudo que tem por objetivo reunir estudos semelhantes, publicados ou não, avaliando-os criticamente em sua metodologia e reunindo-os numa análise estatística, a metanálise, quando possível. Por sintetizar estudos primários semelhantes e de boa qualidade é considerada o melhor nível de evidência para tomadas de decisões sobre algum tema (ATALLAH & CASTRO, 1998).

Para evitar viés de análise na revisão sistemática, os métodos de seleção e análise dos dados são estabelecidos antes de a revisão ser conduzida, num processo rigoroso e bem definido. A revisão sistemática inicia-se com a elaboração da questão e de um projeto de revisão. A seguir é realizada a busca da literatura com o objetivo de identificar o maior número possível de estudos relacionados à questão. Uma vez selecionados, aplicam-se critérios para avaliação da qualidade metodológica conforme o delineamento do estudo original. Como as revisões realizadas pela Colaboração Cochrane avaliam efetividade de intervenções, apenas ensaios clínicos controlados, em sua maioria randomizados, são incluídos. Quando os estudos forem semelhantes, os resultados podem ser finalmente sintetizados numa metanálise (SAMPAIO & MANCINI, 2007).

A posição ocupada pela revisão sistemática na hierarquia da evidência ilustra a sua importância para a pesquisa. Nessa hierarquia, quando procuramos por evidência sobre a eficácia de intervenção ou tratamento, estudos de revisão sistemática, tendem geralmente a disponibilizar evidência mais forte, ou seja, são estudos mais adequados para responder a perguntas sobre a eficácia de uma intervenção.

3.2 Propriedade Intelectual

Propriedade Intelectual é uma área que, por meio de leis, garante a inventores ou responsáveis por qualquer produção do intelecto: seja nos domínios industriais, científicos, literários ou artísticos (BASSO, 2007). Segundo definição da OMPI - Organização Mundial de Propriedade Intelecutal, a Propriedade Intelectual está dividida em duas categorias: Propriedade Industrial, que inclui as patentes (invenções), marcas, desenho industrial, indicação

geográfica e proteção de cultivares, e direitos autorais abrangendo trabalhos literário e artísticos.

A Organização Mundial do Comércio - OMC criou o Trips – *Trade Related Aspects of Intellectual Property Rights* (Acordo sobre Aspectos do Direito de Propriedade Intelectual Relacionados ao Comércio), do qual o Brasil tornou-se signatário desde 1994. O Trips estabelece um padrão de proteção mínima à propriedade intelectual, e os países que o assinaram obrigaram-se a revisar suas leis nacionais de modo a adaptá-las a esse padrão.

Patente é um direito exclusivo concedido a uma invenção, que consista em um produto ou um processo que prevê uma nova maneira de fazer algo, ou oferece uma nova solução técnica para um problema. O inventor precisa atender aos requisitos de novidade, atividade inventiva e aplicação industrial. Pode-se afirmar que a patente é um documento expedido pelo órgão competente do Estado que reconhece o direito de propriedade industrial reivindicado pelo titular. (DE CARVALHO, 2009)

3.3 Tratamento Endodôntico

Injúrias pulpares irreversíveis causadas por microrganismos provenientes de lesões cariosas profundas são um dos principais fatores que conduzem a necessidade de terapia endodôntica. O desejado “sucesso clínico” no tratamento destes casos depende, principalmente de adequada limpeza, desinfecção e completa obturação do sistema de canais radiculares. Este tratamento pode ser dividido em três etapas básicas que compõe o procedimento clínico: a remoção do tecido pulpar, o preparo biomecânico e a obturação dos canais radiculares. O objetivo desta última etapa é promover o selamento de todo o sistema de canais, incluindo a embocadura dos túbulos dentinários periféricos e canais acessórios, visando propiciar um ambiente favorável ao reparo dos tecidos periapicas e impedir a reinfecção pela passagem de microrganismos e seus subprodutos (EUROPEAN SOCIETY OF ENDODONTOLOGY, 2006). Uma obturação adequada é aquela que preenche tridimensionalmente o canal até o comprimento de trabalho, atuando como uma barreira física e efetiva na prevenção de uma reinfecção. Dessa forma, a qualidade da obturação é fator determinante no prognóstico do tratamento endodôntico (GUNDUZ *et al*., 2011).

Além da correta técnica operatória, o emprego de materiais obturadores com adequadas propriedades físicas, químicas e biológicas são fundamentais a este processo, e devem ser criteriosamente selecionados pelo clínico.

O tratamento endodôntico tem por objetivo final restabelecer a integridade dos tecidos perirradiculares e preservar o elemento dental após a descontaminação e limpeza do sistema de canais infectados através do preparo químico-mecânico (HAMMAD, QUALTROUGH, SILIKAS, 2009). A cavidade oral apresenta mais de 500 espécies, já isoladas e estudadas, de microrganismos. Estes são formadores de biofilme nos tecidos duros dentais e moles sendo os principais causadores da cárie e doença periodontal (ZAURA *et al*., 2009). A bactéria *E. faecalis* é o microrganismo mais prevalente em casos de infecção persistente (PINHEIRO, 2003) é a principal razão para o fracasso do tratamento endodôntico (SJOGREN, 1997). Em muitos tratamentos, é necessário substituir algum tecido para restabelecer função ou estética, porém, nenhum desses garante sucesso na eliminação total de biofilme ou contra infecção secundária (ZAURA *et al*., 2009).

Portanto, esse sucesso depende da atividade antimicrobiana dos materiais utilizados nesses tratamentos, desde o preparo químico-mecânico, medicação intracanal nas interseções clínicas, e também, nos cimentos endodônticos utilizados na obturação endodôntica. Após a comercialização de "auto-priming", auto-condicionantes, e tecnologias de cimentos resinosos auto-adesivos em odontologia restauradora, foram lançados no mercado os cimentos endodônticos de baixa viscosidade à base de resina de metacrilato com a proposta de comercialização de um material obturador intrarradicular capaz de se aderir a resinas de metacrilato. Este gênero de cimentos endodônticos adesivos foi promovido no mercado odontológico com a propriedade de criar monoblocos dentro do espaço do canal radicular. O termo "monobloco" sugere que o interior do conduto radicular obturado torna-se perfeitamente preenchido com uma massa sólida isenta de espaços, que consiste em diferentes materiais e interfaces obturadoras, com as vantagens pretendidas de melhorar simultaneamente a vedação e a resistência à fratura dos canais obturados. Os cimentos endodônticos auto-adesivos mais recentemente introduzidos (3ª e 4ª geração) também sugerem benefícios adicionais de etapas de aplicação clínica reduzidas e melhorias gerais na sua facilidade de utilização.

Adicionalmente a isso, embora haja diferentes agentes endodônticos com características antimicrobianas disponíveis comercialmente, há a preocupação em relação à biocompatibilidade destas substâncias e os seus efeitos tóxicos, alérgicos e mutagênicos (MOZAYENI *et al*., 2012). A importância da presença de características antimicrobianas nos materiais seladores do canal radicular se dá principalmente pela incidência de infecções endodônticas recorrentes e resistentes, e os efeitos adversos de alguns agentes químicos antibacterianos atualmente utilizados em odontologia (KESLER SHVERO *et al*., 2012; HEYDER *et al*., 2013), pois há casos em que o tratamento endodôntico necessita ser realizado em mais de uma sessão e o passo clínico da obturação (vedamento) dos canais radiculares precisa ser adiado. Nesses intervalos entre sessões clinicas, faz-se o uso de substâncias seladoras ou intermediárias, como a medicação intracanal.

Assim, a busca pelo desenvolvimento de materiais odontológicos seguros e eficazes continua sendo os fitoquímicos naturais, isolados de plantas de potencial terapêutico e alvos promissores de novos produtos com propriedades farmacológicas.

3.3.1 Materiais seladores do canal radicular disponíveis no mercado

As seguintes informações foram cedidas pelo fabricante.

3.3.1.1 AH Plus© (Dentsply)

AH Plus é o cimento endodôntico para obturação definitiva do sistema de canais radiculares em conjunto com cones de guta percha. É um cimento resinoso à base de resina epóxica e sua apresentação é pasta+pasta. Oferece excelente biocompatibilidade, radiopacidade, estabilidade de cor, ótima viscosidade, rapidez e segurança. Sua formulação reduz o risco de inflamação periapical pós-operatória. Esta marca permite sua remoção, caso seja necessário.

Figura 1. AH plus

3.3.1.2 EndoREZ© (Ultradent)

O EndoREZ é um cimento para canais radiculares à base de resina UDMA com propriedades hidrofílicas para uma selagem consideravelmente superior. A sua fórmula especial evita o uso de agentes adesivos problemáticos no canal. As pontas EndoREZ Points são revestidas com resina e formam uma união química completa. É um material de dupla polimerização, biocompatível e radiopaco, permitindo uma rápida identificação e avaliação nas radiografias. Este cimento pode ser removido em caso de preparos para núcleos e re-tratamento.

Figura 2. EndoREZ

3.3.1.3 Sistema Real Seal©

O Sistema Real Seal é composto por um cimento resinoso (a base de resina plástica uretano dimetacrilato), cones principais e acessórios de Resilon, primer (solução monomérica acidificada aquosa, que além de preparar a dentina

para receber o material, também é um ativador) e resina diluente (para mudar a consistência do cimento). Esse cimento apresenta dupla polimerização, boa radiopacidade, é considerado biocompatível e apresenta uma boa capacidade seladora. Quanto a formação do monobloco, como afirma o fabricante, surgiu com o intuito de formar um sistema de material obturador com mesma natureza química de forma com que eles possam se aderir e formar um compacto de natureza única, assim os cones de Resilon, estariam aderidos ao cimento, e estes à parede do canal, formando um compacto inacessível a infiltração, na tentativa de diminuir a infiltração bacteriana e o risco de fratura radicular por aumentar a adesão às paredes de dentina.

Figura 3. Sistema Real Seal

3.4 Plantas Medicinais

As plantas medicinais são utilizadas em países em desenvolvimento como tratamento alternativo para problemas de saúde. Muitos extratos de plantas e óleos essenciais isolados de plantas medicinais têm demonstrado exercer atividade biológica *in vivo* e *in vitro*, pelo qual se justifica a avaliação destas plantas tradicionais centrado na caracterização da atividade antimicrobiana das mesmas (BETTEGA *et al.*, 2011). Produtos naturais usados como fitoterápicos como o “alho” (*Allium sativum*), a “hortelã” (*Mentha piperita*), a “guaçatonga“ (*Casearia sylvestris*), o “confrei” (*Symphytum officinale*), o “crajirú” (*Arrebidaea chica*), o “alecrim” (*Rosmarinus officinalis*) e a “bardana” (*Arctium minus*), dentre outros, têm demonstrando propriedades medicinais no combate a doenças que acometem o ser humano.

Os chamados medicamentos “fitoterápicos” são preparações vegetais padronizadas que consistem de uma mistura complexa de uma ou mais substâncias presentes na planta que são preparados adequadamente e, posteriormente prescritos em obediência à legislação vigente (DI STASI, 2007). De modo geral, os compostos fitoterápicos podem ser utilizados nas mais variadas fórmulas, como cápsulas, comprimidos, géis, pomadas, soluções aquosas, soluções hidroalcoólicas e infusões, que são conhecidas como chás (FRANCISCO, 2010).

A produção de medicamentos e o tratamento farmacológico de inúmeras patologias tiveram seu início com a utilização de plantas medicinais. Um dos fatores que mais contribui para a pesquisa do potencial bioativo de plantas é a resistência a antibióticos convencionais adquirida por diversos microrganismos pois apesar da grande variedade de fármacos antimicrobianos amplamente distribuídos no mercado, cepas resistentes aparecem continuamente. Esta resistência, associada à toxicidade, interações medicamentosas, e biodisponibilidade insuficiente dos antimicrobianos disponíveis tornam o tratamento mais difícil e estimulam a busca por novas alternativas terapêuticas. Dentre estes, plantas medicinais e seus compostos bioativos tem sido estudados devido sua ampla utilização na medicina popular.

Os óleos essenciais e extratos derivados de plantas aromáticas e medicinais são potencialmente úteis como agentes antimicrobianos e a sua utilização como medicamentos tem sido amplamente reconhecida (HOLLEY e PATEL, 2005). As razões para o uso dessas plantas com propósito medicinal é objetivo de vários estudos (FRANCISCO, 2010) e alguns fatores contribuem para a propagação desta terapia como: a origem natural das drogas, a crença na sua utilização segura e a alegada diminuição de efeitos colaterais quando comparado a fármacos convencionais (BAHMANI & EFTEKHARI, 2012). Visto isso, a Organização Mundial da Saúde (OMS) constatou a importância de se incorporar a medicina moderna e a medicina tradicional na implementação de sistemas de saúde a fim de se melhorar a saúde da população.

Seguindo essa proposição, no Brasil, de acordo com dados oficiais do Sistema Único de Saúde (SUS), dezesseis estados já introduziram a fitoterapia como alternativa de tratamento no sistema público de saúde (MINISTÉRIO DA SAÚDE, 2011). Ademais, essa prática foi regulamentada na portaria nº 5813

(BRASIL, 2006) com a adoção de uma Política Nacional de Práticas Integrativas e Práticas Complementares no SUS. Essa ampliação das opções terapêuticas oferecidas aos usuários do SUS incluem o acesso a plantas medicinais, medicamentos fitoterápicos e a serviços relacionados, tais como fitoterapia. Assim, segurança, eficácia e qualidade tornam-se estratégias importantes para melhorar a saúde, o desenvolvimento social e para a inclusão dos fitoterápicos de forma consciente no serviço público de saúde (MINISTÉRIO DA SAÚDE, 2011).

O Programa Nacional de Plantas Medicinais e Fitoterápicos, onde consta uma lista de plantas medicinais (RENISUS) que são recomendados pelo SUS onde contém 71 espécies de plantas medicinais que são popularmente utilizadas no país e que são consideradas potencialmente úteis para a produção de fitoterápicos e outros compostos. Esta lista também busca o cultivo, manejo, produção, comercialização e dispensação de plantas e ervas medicinais na forma de medicamentos, e se destina a orientar a pesquisa que pode apoiar o desenvolvimento de uma lista nacional de plantas e ervas medicinais, a fim de contribuir para o desenvolvimento e inovação na área de plantas medicinais e fitoterápicos. As plantas foram pré-selecionados por região, e a lista faz alusão a sua utilização por indicação e de acordo com as categorias da Classificação Internacional de Doenças (MINISTÉRIO DA SAÚDE, 2006).

3.4.1 Uso de Plantas medicinais na Odontologia

A Política Nacional de Práticas Integrativas e Complementares (PNPIC), do Ministério da Saúde, insere o uso das plantas medicinais e da Fitoterapia no SUS, tendo sido o reconhecimento do exercício da Fitoterapia pelo cirurgião-dentista regulamentado em 2008 pelo Conselho Federal de Odontologia (CFO).

Na odontologia, a utilização de plantas medicinais para tratar doenças bucais ou doenças sistêmicas com manifestações bucais ainda é pouco explorada (VARONI *et al*., 2012). Entretanto, pesquisas relacionadas a produtos naturais cresceram significantemente frente ao aumento pela busca por produtos com menor toxicidade, maior atividade farmacológica e biocompatíveis, além de custos acessíveis à população (FRANCISCO, 2010).

Estudos com chás mostram que estas infusões podem ser utilizadas para inibir o crescimento bacteriano e a aderência nas superfícies dentais e redução na produção de ácidos e polissacarídeos extracelulares (FRANCISCO, 2010). Espécies como cravo-da-índia, Romã, Malva, Sálvia e Camomila, são indicadas nos casos de gengivite, abscesso na boca, inflamação e aftas (OLIVEIRA *et al*., 2011). O alho (*Allium sativum*) tem mostrado conhecida propriedade antibacteriana, antifúngica e antiviral. Além de ser considerado um importante coadjuvante no tratamento de pacientes portadores de periodontites (JUIZ, 2010).

Óleos de cajueiro e do cravo podem ser usados em caso de odontalgias. No caso do açaí (*Euterpe oleracea*), este produz um evidenciador (corante) de placa dental com eficiência de 90% superior a produtos comercializados tais como o verde de malaquita, a fucsina e a eritrosina (FRANCISCO, 2010). Estudos analisaram o efeito antimicrobiano do extrato de romã *in vitro* e avaliação clínica de um dentifrício sobre microrganismos do biofilme dental, onde os pacientes que usaram a pasta apresentaram diminuição do número de *S. mutans* e do índice de sangramento gengival (PEREIRA *et al*., 2005).

Outro extrato com efeitos inibitórios sobre o crescimento das bactérias do biofilme dental e fungos da candidíase oral é o extrato de malva. Sugerindo a utilização dessa planta como meio alternativo na terapêutica odontológica (ALVES, 2009).

No trabalho de Ditterich e colaboradores (2007), foram avaliadas a atividade antimicrobiana de várias marcas comerciais de dentifrícios com componentes terapêuticos: Sorriso Herbal® (própolis), Malvatricin® anti-placa e anti-tártaro (tintura de malva), Colgate Herbal® (camomila), Gessy® (extrato de juá), Sorriso® (juá e própolis) e Paradontax® (tintura de mirra e camomila). Seus resultados mostraram que tanto *Staphylococcus aureus* como *Escherichia coli* foram sensíveis aos dentifrícios estudados.

Outro óleo que tem despertado interesse para uso na área odontológica é o óleo de Melaleuca por sua ação antibacteriana *in vitro* contra microrganismos de origem bucal, porém, pesquisas envolvendo o estudo do mecanismo de ação ainda são escassas e precisam ser realizadas (OLIVEIRA *et al*., 2011).

3.4.1.1 Mentha piperita

O gênero *Mentha* (família Lamiaceae) é originário da Europa, porém, sua grande área de aplicações garante seu cultivo em todo o mundo, tanto para fins medicinais como culinários, com predomínio da Índia, maior produtor de óleo *de Mentha*, chegando a 80% da produção mundial. Existem várias espécies de *Mentha*, porém, apenas três são aprovadas pela *International Standard Organization* (ISO) devido à dificuldade de classificação desse gênero, são elas *M. piperita, M. spicata* e *M.aquatica* (PEIXOTO, 2010). Os componentes químicos do óleo de *Mentha* variam de acordo com idade da planta, região geográfica, clima, variedade da espécie, e condições de processamento, entretanto, os principais elementos voláteis identificados são: mentol, mentona, isomentona, 1,8 cineol, acetato de metila, limoneno, β-mirceno, β-cariofileno, pulegonae carvona (PEIXOTO, 2010).

A hortelã pimenta (*M. piperita* L.), um híbrido natural entre *M. aquatica* e *M. spicata*, é uma das espécies produtoras de terpenoides mais exploradas comercialmente (ISCAN *et al*., 2002), sendo uma rica fonte de mentol (KEIFER *et al*., 2007). Segundo a medicina popular, espécies do gênero *Mentha* podem ser empregadas no tratamento de náuseas, cólicas gastrointestinais, flatulências, cálculos biliares, icterícia, ansiedade e expectoração. Além disso, pode ser empregada como aromatizante com propriedades antissépticas, na indústria farmacêutica, de tabaco e perfumaria. É também usada em infusões, como condimento e aroma de bebidas. Extratos originários de *M. piperita* têm evidenciado propriedades antifúngicas (MATAN *et al*., 2009; SOKOVIC *et al*., 2009), antiviral (ASTANI, REICHLING, SCHNITZLER, 2010) e antibacteriana (PRIYA *et al*., 2007; BAKKALI *et al.,* 2009; VANVUUREN, SULIMAN, VILJOEN, 2009).

Carretto (2010) avaliou a atividade inibitória do óleo essencial de *M. piperita* contra 39 cepas da levedura *Candida*, sendo a *C. albicans* de maior sensibilidade ao óleo essencial. Mahboubi & Haghi (2008) sugerem o uso do óleo essencial de *Mentha pulegium* como alternativa ao uso de antifúngicos convencionais, uma vez que leveduras do gênero *Candida* tem apresentado resistência microbiana. Além disso, os autores indicam que o uso de óleo de *Mentha* não leva a efeitos adversos indesejados, ao contrário de fármacos antifúngicos, associados principalmente a nefrotoxicidade.

Gonçalves e colaboradores (2007), também avaliaram atividade antimicrobiana do óleo essencial da *M. piperita* frente a *E. coli*, *S. aureus, Salmonella typhi* e *C. albicans*. Neste estudo, também foi reconhecido à atividade antimicrobiana do óleo essencial desta planta. Matos e colaboradores (2013), avaliaram a atividade *in vitro* do óleo essencial de diferentes acessos de *Mentha* sobre *S.mansoni* em sua forma adulta, seus resultados demonstraram uma moderada inibição no crescimento desses parasitas, indicando uma potencial atividade antiparasitária do óleo.

Figura 4. *M.piperita*

3.4.1.2 Bixa orellana

Bixa orellana (urucum) é uma árvore pequena pertencente à família Bixaceae, originária da América tropical, incluindo a Amazônia brasileira, com no máximo cinco metros de altura e de copa bem desenvolvida. As folhas são simples, glabras, medindo de oito a onze centímetros de comprimento. Suas flores são levemente rosas e o fruto é uma cápsula deiscente ovóide, com dois a três carpelos, cobertos por espinhos. Nas sementes encontra-se um óleo essencial rico em all-E-geranilgeraniol, monoterpenos e sesquiterpenos oxigenados, além dos carotenóides bixina e norbixina e em menor quantidade, alfa e beta-caroteno (LORENZI & MATOS, 2005; YOLMEH *et al*., 2014). É popularmente conhecida como urucu, urucum, urucuzeiro, açafrão, açafroa, açafroa-da-bahia, açafroa-do-brasil, açafroa-indígena, açafroeira-da-terra, anoto e colorau. O nome popular urucum tem origem na palavra tupi “uruku”, que significa “vermelho”.

Além de corante natural na culinária, sua utilização na medicina popular é bem pronunciada, principalmente para doenças coronarianas, afecções do

estômago e intestino, afecções respiratórias, queimaduras e como afrodisíaco (LORENZI & MATOS, 2005). O uso do urucum é muito pronunciado em diversos setores industriais em substituição a muitos corantes sintéticos, devido a sua baixa toxicidade e baixo custo de produção, tornando-o conveniente e atrativo (CARDARELLI, BENASSI, MERCADANTE, 2008).

O Brasil é o maior produtor e exportador de sementes e extratos de urucum, que são utilizados como corantes nas indústrias alimentícias, farmacêutica e de cosméticos (CARDARELLI, BENASSI, MERCADANTE, 2008). De acordo com o Instituto Brasileiro de Geografia e Estatística (IBGE) – (2009), a quantidade de semente de urucum produzida no Brasil em 2008 foi de 12.472 toneladas, sendo a maior parte oriunda da região norte (5.310 t), seguida do sudeste (3.507 t), nordeste (2.187 t), sul (1.121 t) e centro-oeste (221 t). Dentre os estados brasileiros, Rondônia é o maior produtor de sementes de urucum (23%), seguido do Pará (18%) e São Paulo (14%). No país, além do amplo emprego na indústria, a preparação comercial contendo 0,20-0,25% de bixina, conhecida como colorífico, é componente indissociável de inúmeros pratos da culinária brasileira. Este condimento é produzido a partir das sementes de urucum, previamente aquecidas a 70°C em óleo vegetal, seguido de abrasão com fubá ou farinha de mandioca ou pela mistura destas com urucum em pó, obtido por extração com solventes (COSTA & CHAVES, 2005).

As sementes de urucum são ricas em carotenoides, sendo o mais abundante a bixina, compreendendo cerca de 80% do total dos carotenoides (SMITH, 2006), pigmento responsável pela coloração vermelha, e várias por atividades biológicas dentre elas antioxidante e antimicrobiana (CARDARELLI, BENASSI, MERCADANTE 2008; SHAN *et al*., 2009; VIUDA-MARTOS *et al*., 2012). Estudos com o urucum relatam sua atividade antimicrobiana, demostrando a capacidade preservativa desta planta na conservação e agregação de qualidade aos alimentos (CARDARELLI, BENASSI, MERCADANTE 2008). Um extrato orgânico (etanólico 95%) de folhas de *B. orellana*, apresentou significativa ação antibacteriana, principalmente frente à bacterias Gram-positivas como: *Bacillus subtilis*, *S. aureus* e *Streptococcus faecalis* (VENUGOPALAN & GIRIDHAR, 2012).

Apesar do uso das sementes de urucum serem voltados principalmente para a indústria alimentícia para a utilização como condimentos e indústria de

coméstico na produção de loções bronzeadoras. Os estudos citados acima embasam a potencial atividade antimicrobiana do urucum.

Figura 5. Sementes de *B. orellana*

3.4.1.3 Tagetes minuta

Tagetes é um gênero de ervas e arbustos que engloba algumas espécies da família Asteraceae, nativo da América Central e Sul, sendo naturalizado em outras regiões nos trópicos e subtrópicos. No Brasil, é encontrada nos estados do Piauí, Pernambuco, Bahia, Mato Grosso do Sul, Goiás, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Santa Catarina, Rio Grande do Sul e no Distrito Federal (HATTORI, 2009; ROCA *et al*., 2009). É popularmente conhecida por cravo-de-defunto e algumas espécies são cultivadas como plantas ornamentais, tais como: *T.erecta*, *T.tenuifolia* e *T.patula* (MAROTTI *et al*., 2010).

Recentemente, muitas espécies desse gênero têm sido investigadas como fontes de diferentes atividades biológicas devido ao grande número de compostos que se formam nas folhas e flores, e que são acumulados em órgãos específicos da planta na forma de óleos essenciais, apresentando propriedades inseticidas, antimicrobianas e antioxidantes (VIEIRA *et al*., 2011; ALI *et al*., 2014; KARIMIAN, KAVOOSI, AMIRGHOFRAN, 2014).

O extrato metanólico utilizando as folhas de *T.minuta* demonstrou efeito antifúngico contra *C.albicans, C.krusei*, *Fusarium verticillioides*, *F.proliferatum*, *Aspergillus flavus*, A.*parasiticus*, *A.niger*, *Trichophyton mentagrophytes*, *Cryptococcus neoformans* e *Penicillium* spp (THEMBO *et al*., 2010; VIEIRA *et al*., 2011). O extrato hidroalcoólico, extrato metanólico, extrato aquoso e óleo essencial da planta demonstraram atividade antibacteriana contra *S.aureus*, *S.agalactiae*, *E.faecium*, *Salmonella* spp., e *Paenibacillus larvae* (WIEST *et al*., 2009; GONZÁLEZ & MARIOLI, 2010; VIEIRA *et al*., 2011). Trabalhos também

relataram que o extrato aquoso é nematicida em plantações (AMARAL *et al*., 2009; JUNGES *et al*., 2009).

Figura 6. Folha de *T. minuta*

PARTE III

CAPITULO I

Artigo científico

Cada capítulo desta dissertação será apresentado na forma de manuscrito a ser submetido a diferentes periódicos Internacionais. Este Capítulo corresponde ao periódico *Journal of Ethnopharmacology*
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Systematic review and technological overview of antimicrobial activity of *Tagetes minuta*, and future perspectives

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Abstract:

Ethnopharmacological relevance: The antimicrobial potential of *Tagetes minuta* was correlated with its traditional use as antibacterial, insecticidal, biocide, disinfectant, anthelminthic, antifungal, and antiseptic agent as well as its use in urinary tract infections.

Aim of the study: This study aimed to systematically review articles and patents regarding the antimicrobial activity of *Tagetes minuta* and give rise to perspectives on this plant as a potential antimicrobial agent.

Materials and Methods: A literature search of studies published between 1997 and 2015 was conducted over five databases: MedLine (PubMed), Web of Science, Scopus, Google Scholar, Portal de Periódicos Capes and SciFinder, grey literature was explored using the System for Information on Dissertations database, and theses were searched using the ProQuest Dissertations and Theses Full text database and the Periódicos Capes Theses database. Additionally, the following databases for patents were analysed: United States

Patent and Trademark Office (USPTO), Google Patents, National Institute of Industrial Property (INPI) and Espacenet patent search (EPO). The data were tabulated and analysed using Microsoft Office Excel 2010.

Results: After title screening, 51 studies remained and this number decreased to 26 after careful examinations of the abstracts. The full texts of these 26 studies were assessed to check if they were eligible. Among them, 3 were excluded for not having full text access, and 11 were excluded because they did not fit the inclusion criteria, which left 10 articles for this systematic review. The same process was conducted for the patent search, resulting in 4 patents being included in this study.

Conclusion: Recent advances highlighted by this review may shed light on future directions of studies concerning *Tagetes minuta* as a novel antimicrobial agent, which should be repeatedly proven in future animal and clinical studies. Although more evidence on its specificity and clinical efficacy are necessary to support its clinical use, *Tagetes minuta* is expected to be a highly effective, safe and affordable treatment for infectious diseases.

Keywords: antimicrobial, antifungal, botany, plant conservation, essential oil.

1. Introduction

Currently, there has been a return to the search for products called "natural," which actually never ceased to exist. The analysis of the composition of many drugs shows that almost 50% of those in clinical use are derived from natural compounds (Perecin, Bovi, and Maia, 2002). Medicinal plants have become the focus of many studies, in terms of a validation of their traditional uses by determination of their actual pharmacology. Many efforts have been made to discover new antimicrobial compounds from various kinds of plants.

To move forward in the discovery of natural plant metabolites, plant extracts were screened for potential candidates with antimicrobial activity (Crouse, 1998). Essential oils and extracts from aromatic plants have been known to possess biological activity, mainly antimicrobial and antioxidant properties (Zygadlo and Juliani, 2000). *Tagetes minuta* is a common weed of the family Asteraceae. It is erect, strong-smelling, often

robust, but variable in habit and very plastic in its response to crowding. Its leaves are pinnate with elliptic toothed leaflets and heads that are yellow (Boekaert*et al.,* 1989). *Tagetes* species originally were used as a source of essential oil (extracted from leaves, stalks and flowers) for flavouring in the food industry. The powders and extracts are rich in orange-yellow carotenoids and are used as food colouring. This is a commonly occurring plant all over the world and is well known for a wide range of biological properties. The foliar parts possess essential oils, known for antibacterial and insecticidal properties (Piccaglia*et al*., 1997), and thiophenes, which have a marked biocidal activity (Hulst*et al*., 1989). *T. minuta* has diuretic, disinfectant, antispasmodic, anthelminthic, antifungal, antiseptic, and antitussive actions and is used for urinary tract infections (Toursarkissian, 1980,Wang *et al*., 2006, Lacroix *et al*., 2011). *T. minuta* essential oil have significant antibacterial activity against both Gram-negative and Gram-positive bacteria (Héthélyi *et al*., 1986, Céspedes*et al*., 2006). Several studies have also described antifungal activities of the essential oils against species of *Candida*, *Penicillium* and *Aspergillus* (Dunkel *et al*., 2010; Thembo *et al*., 2010). *Tagetes* species have been reported in the literature to have insecticidal properties (Zoubiri and Baaliouamer, 2011), and studies have been conducted on the use of *T. minuta* extracts to reduce parasitic plant nematode populations and have reported a reasonably level of effectiveness (Cerruti,*et al*.,2010; Gakuya *et al*., 2013). Thus, the aim of this study was to systematically review articles and patents related to the antimicrobial activity of *T. minuta* to use as a strategic tool for the prospect of identifying new drugs with antimicrobial activity.

The rising incidence of multidrug resistance amongst pathogenic microorganisms has further necessitated the need to search for newer antibiotic sources (Veronika *et al*., 2006). Contrary to synthetic drugs, antimicrobials with plant origins are not associated with many side effects and have therapeutic potential to heal infectious diseases (Doughari, 2006). Although many plant species have been tested for antimicrobial properties, the majority have not been adequately evaluated (Balandrin and Klocke, 1988).

2. Methods

This systematic review was carried out according to the guidelines of the Cochrane Handbook for Systematic Reviews of Interventions (Higgins, 2011), following the four-phase flow diagram of the Preferred Reporting Items for Systematic Reviews

and Meta-Analyses (PRISMA) Statement (Moher *et al*., 2009). This report is based on the PRISMA Statement.

2.1. Search strategy and study selection

Studies written in English were identified in six international databases: Medline (PubMed), Scopus, Web of science, SciFinder, Periódicos Capes and Google Scholar. The grey literature was explored using the System for Information on Dissertations database, and theses were searched using the ProQuest Dissertations and Theses Full text database as well as the Periódicos Capes Theses database. The following databases were searched for patents: United States Patent and Trademark Office (USPTO), Google Patents, National Institute of Industrial Property (INPI) and Espacenet patent search (EPO). The last search was carried out in March 2016, with no date restriction. The search method used was appropriately modified for each database and is listed in Table 1. Literature search results were de-duplicated using EndNote X7 software (Thomson Reuters, New York, NY, USA). The titles of all identified studies were screened by two independent reviewers (D.S. and L.S.). Abstracts were carefully appraised when the title indicated inclusion. Articles considered eligible for the review (or in the case of doubt) were selected for full-text reading. Discrepancies were resolved by group discussion. References of all the included studies were also hand-searched for additional studies.

2.2. Eligibility criteria

Studies that evaluated the antimicrobial activity of essential oils or extracts of *T. minuta*, with no restriction on study populations, were selected. The following inclusion criteria were used: peer reviewed research articles from scholarly journals or theses written in the English language that referenced the use of *T. minuta*, evaluated the antimicrobial activity and contained a control group. When more than one possible control was reported in the study (e.g., untreated medium and a commercial reference of antibiotics), the first was selected as the control group. The following were reasons for exclusion: literature review articles or studies not related to the assessment of the antimicrobial activity of *T. minuta* or that lacked some kind of control group. Furthermore, a patent search was also performed using the International Patent Classification (IPC) with the following codes: A61P 31/10 (antimycotic), A61P 31/02 (antibacterial agent), A61P 31/02 (antiseptic site) and A61K 8/97 (vegetable origin). Just as in most applications, each patent may submit more than one IPC. This technology and

its applications are related to different areas of science and technology. The aim of identifying these codes is to create a specific tool for search and retrieval of documents.

Table 1. Electronic database search strategy

Database Search and/or Terms
Google Scholar Antimicrobial activity (Title) AND tagetes minuta
Portal de Periódicos CAPES Antimicrobial activity (Title) AND tagetes minuta (subject)
PubMed (MEDLINE) #1 Tagetes minuta #2 “antimicrobial” [MeSH Terms] OR “antimicrobial” OR “Agents, Anti-Infective” OR “Anti Infective Agents” OR “Antiinfective Agents” OR “Agents, Antiinfective” OR “Microbicides” OR “Antimicrobial Agents” OR “Agents, Antimicrobial” OR “Anti-Microbial Agents” OR “Agents, Anti-Microbial” OR “Anti-Microbial Agents” #3 “biofilms” [MeSH Terms] OR “biofilms” OR “Bacterial Adhesion” OR “Adhesins, Bacterial” OR “Biofouling” #4 “antibacterial” [MeSH Terms] OR “antibacterial” OR “Agents, Anti-Bacterial” OR “Anti Bacterial Agents” OR “Antibacterial Agents” OR “Agents, Antibacterial” OR “Antibiotics” OR “Bacteriocidal Agents” OR “Agents, Bacteriocidal” OR “Bacteriocides” OR “Anti-Mycobacterial Agents” OR “Agents, Anti-Mycobacterial” OR “Anti Mycobacterial Agents” OR “Antimycobacterial Agents” OR “Agents, Antimycobacterial” #5 “antifungal” [MeSH Terms] OR “antifungal” OR “Agents, Antifungal” OR “Therapeutic Fungicides” OR “Fungicides, Therapeutic” OR “Antibiotics, Antifungal” OR “Antifungal Antibiotics” #6 “antiparasitic” [MeSH Terms] OR “antiparasitic” OR “Agents, Antiparasitic” OR “Antiparasitic Drugs” OR “Drugs, Antiparasitic” OR “Parasiticides” OR “Antiparasitics” #1 AND #2 OR #3 OR #4 OR #5 OR #6
SciFinder antimicrobial activity of tagetes minuta

Scopus
#1 ("tagetes minuta") AND #2 ("antimicrobial activity") OR #3 ("biofilms") OR
#4 ("antibacterial") OR #5 ("antifungal") OR #6 ("antiparasitic")
Web of Science
#Topic: ("antimicrobial activity") OR #Topic: ("biofilms") OR #Topic: ("antibacterial") OR #Topic: ("antifungal") OR #Topic: ("antiparasitic") AND #Topic: ("tagetes minuta")
Search in patent
Patent search was also made using the International Patent Classification (IPC) with the following codes:
#A61P 31/10 (antimycotic)
#A61P 31/02 (antibacterial agent)
#A61P 31/02 (antiseptic site)
#A61K 8/97 (vegetable origin)

2.3. Data collection

When the study evaluated more than one plant extract or more than one chemical substance or antibiotic reference, data were extracted individually for each potential antimicrobial agent and antimicrobial assay performed. Authors of the studies were contacted in case of missing data (e.g., data provided in graphs); these studies were only included if the authors provided the missing information. Data extraction was conducted by consensus between the two researchers who conducted the collection.

2.4. Assessment of risk of bias

The risk of bias and the methodological quality for all the included studies were assessed adapted from another systematic review of antimicrobial monomers used in dental materials (Cocco *et al*., 2015). The parameters used for the evaluation of the methodology assays were discussed by the researchers involved, and judgement was carried out by group discussion.

3. Results

3.1. Study selection

The flowchart of the systematic review is shown in Figure 1. After the database screening and removal of duplicates, 95 studies were identified. After screening the 95 titles, 51 studies were initially included, and after careful examination of the abstracts, the full-texts of 26 studies were assessed to check if they were eligible. The full texts of these 26 studies were assessed to check if they were eligible. Among them, 4 were excluded for not having access to the full text, and 11 were excluded because they did not fit the inclusion criteria. In total, 84 studies (see Appendix) were excluded for the reasons reported in Figure 1. After title screening, 51 studies remained, this number decreased to 26 after careful examination of the abstracts, and based on the full texts of these 26 studies, 10 were used in this study. The patent search initially retrieved 80 patents, with 38 duplicates that were excluded and 15 that were excluded after reading the title and abstract (Figure 1). After, 23 were excluded because they did not fit the eligibility criteria since they were not related to antimicrobial activity. A total of 4 patents were included in the analysis.

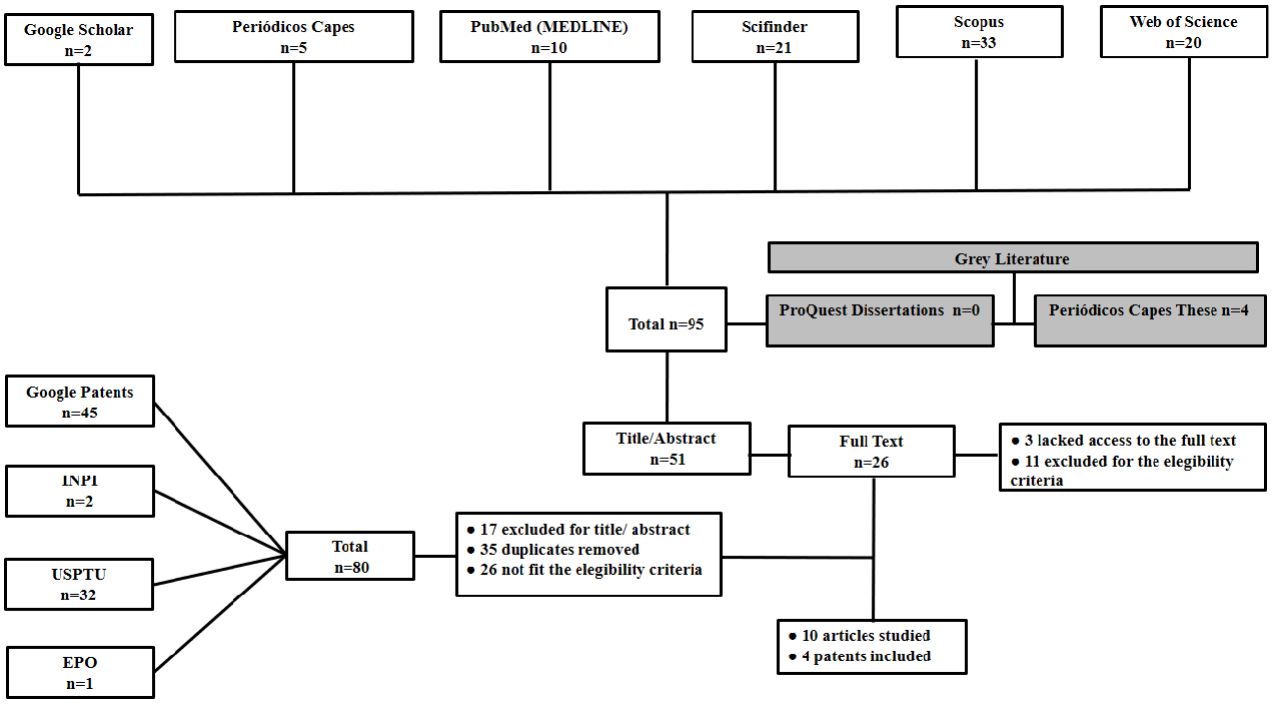

Fig. 1– Search flowchart for articles (as described in the PRISMA Statement)

3.2. Characteristics of the included articles

The characteristics of the ten selected studies are listed in Table 2.

Table 2. Demographic data and main results of the studies.

Study	Assay	Microorganisms tested	Types of extract	Antimicrobial assay	Exposure times in the antimicrobial test	Control groups	Sample size	Main results
Tereschuk *et al*.,1997	Antimicrobial activity of flavonoids from leaves of *Tagetes minuta*	*Lactobacillus rhamnosus* *L. plantarum, Pseudomonas aeruginosa Saccharomyces cerevisiae, Staphylococcus aureus* *S. epidermidis Zymomonas mobilis*	Methanol Chloroform Ethyl acetate Water	Agar-well diffusion method	12 h	Chloramphenicol	n=3	At higher concentrations (200–500 µg/ml^{-1}), total extracts of *T. minuta* showed the same inhibition of growth as 200–220 µg/ml^{-1} of chloramphenicol, except the chloroform fraction did not show any antimicrobial activity against all microorganisms tested.

Senatore *et al*., 2004	Antibacterial activity of *Tagetes minuta* L. (Asteraceae) essential oil with different chemical composition	*Bacillus cereus* *B. subtilis* *Staphylococcus aureus* *Streptococcus faecalis* *Escherichia coli* *Proteus mirabilis* *Pseudomonas aeruginosa* *Salmonela Typhi 2*	Essential oil	Broth macrodilution method	24 h	Cultures containing only sterile Tris buffer were used as positive controls	n=3	Antimicrobial activity was greater in oils from the UK than in oils from Egypt or South Africa, irrespective of whether the UK plants had been grown in a greenhouse or in the field. The MICs for the oil from UK greenhouse-grown plants were 6.25-25 μg/mL for Gram-positive bacteria and 25–50 μg/mL for Gram-negative bacteria, with the lowest MIC of 6.25 μg/mL against S. faecalis.
Hamza *et al*., 2006	Antifungal activity of some Tanzanian plants used traditionally for the treatment of fungal infections	*Candida albicans* *C. glabrata* *C. parapsilosis* *C. krusei* *C. tropicalis*	Methanol	Broth microdilution method	48 h (*Candida sp.*) and 72 h (*C. neoformans*)	Amphotericin B	n=2	Methanol extract showed effect against these organisms with MICs ranging from 500 g/ml to 1000 g/ml for *C. tropicalis*, *C. cruzei* and *C. neoformans*, respectively.

Oyedemi *et al*., 2008	Compositions and comparisons of antimicrobial potencies of some essential oils and antibiotics against selected bacteria	*Cryptococcus neoformans* *Bacillus cereus* *B. subtilis* *Listeria monocytogenes Streptococcus pyogenes* *Escherichia coli* *Klebsiella pneumoniae* *Proteus vulgaris* *Pseudomonas fluorescens* *Shigella flexneri Alcaligenes faecalis*	Essential oil	Agar diffusion method	18 h	Chloramphenicol Penicillin G Tetracycline Streptozotocin Rifampicin Norfloxacin Actinomycin D Chromomycin A_3 Arugomycin Netropsin	n=3	The MIC of *T. minuta* varied from 1.0 to 1.5 mg/ml for all tested drugs, while the mic of the medicaments ranged from 0.02 to 1 mg/ml
Thembo *et al*., 2010	Antifungal activity of four weedy plant extracts against selected mycotoxigenic fungi	*Fusarium verticillioides*, *F. proliferatum, Aspergillus flavus* *A. parasiticus*	Methanol Hexane Dichloromethane	Broth microdilution method	24, 48,72, 96 and 120 h	Amphotericin B Cantus	n=3	The methanol extract of *T. minuta* exhibited growth inhibition activity against *F. proliferatum*; however, it lost its activity after 72–96 h, implying a fungistatic effect. The hexane extracts lost their activity to some extent over the 120 h incubation period, although not to the same

							.	extent as the methanol extract. The dichloromethane extract initially showed activity against all the isolates of *F. proliferatum* and *F. verticilliodes* that was lost after 72–96 h. No inhibitions of the *Aspergillus* spp. tested were observed relative to the growth controls.
Macedo *et al*., 2013	In vitro effects of *Coriandrum sativum, Tagetes minuta,* *Alpinia zerumbet* and *Lantana camara* essential oil son *Haemonchus contortus*	*Haemonchus contortus*	Essential oil	Egg Hatch Test Larval development test	48 h	Thiabendazole	n=3	The essential oil inhibited 98.1% of *H. contortus* larvae hatching at a concentration of 2.5 mg/mL^{-1}. The effective concentration to inhibit 50% (EC_{50}) of Egg Hatch Test was 0.53 mg/mL^{-1}. In the larval development test, a concentration of 10 mg/mL^{-1} inhibited 99.5% of *H. contortus* larval development, presenting EC_{50} values of 1.67 mg/mL^{-1}.

Kyarimpa *et al*., 2014	Essential oil and composition of *Tagetes minuta* from Uganda. Larvicidal activity on *Anopheles gambiae*	*Anopheles gambiae*	Essential oil	Larvicidal activity	24 h	Ethanol	n=3	After 2h of exposure, the LC_{50} was 2.9 mg/L, while the LC_{90} was 3.29 mg/L. After 6 h of exposure, the LC_{50} and LC_{90} were 2.31 mg/L and 2.68 mg/L, respectively, while after 12 h, they was 1.49 and 1.82 mg/L, respectively. After 24 h, the LC_{50} and LC_{90} were not determined because the mortality was 100%.
Shirazi	Chemical compositions and antioxidant, antimicrobial and	*Salmonela Typhi* *Escherichia coli* *Staphylococcus aureus*		Broth microdilution method	24 h	Gentamicin, Ampicillin, Ketoconazole	n=3	MICs against *S. typhi*, *E. coli*, *S. aureus*, *B. subtilis*, *A. niger*, and *C. albicans* were 150±8, 165±9, 67±8, 75±7,

et al., 2014	cytotoxic activities of *Tagetes minuta* and *Ocimum basilicum* essential oils	*Bacillus subtilis* *Aspergillus niger* *Candida albicans*	Essential oil					135±15, and 115±8 µg/mL, respectively, for essential oil of *T. minuta*
Shahzadi, Mohammd, 2015	Acylated flavonol glycosides from *Tagetes minuta* with antibacterial activity	*Micrococcus luteus,* *Staphylococcus aureus* *Bacillus subtilis* *Pseudomonas picketti* *Salmonella Setubal*	Methanol n-hexane Chloroform Ethyl acetate n-butanol.	Agar well-diffusion method	24 h	Ampicillin	n=3	All the samples showed significant antibacterial activities against *M. leteus, S. aureus, B. subtilis* and *P. pikettii* principily but were inactive against *E. coli* and *S. setubal*.
Martin Muthee Gakuubi *et al*., 2016	Chemical Compositions and Antibacterial Activities of Essential Oils of *Tagetes minuta* (Asteraceae) against selected plant pathogenic bacteria	*Pseudomonas savastanoi* pv. *Phaseolicola* *Xanthomonas axonopodis* pv. *Phaseoli* *pv.manihotis*	Essential Oil	Agar disc-diffusion method	24 h and 48 h	Dimethyl sulphoxide And Enrich BM	n=3	The MICs of essential oils were in the range of 95 to 190 mg/mL for *X. axonopodis* pv. p*haseoli* and *P. savastanoi*, pv. *phaseolicola* had the lowest MBCs (95 mg/mL), while *X. axonopodis* pv. *manihotis* had the highest (190 mg/mL).

The characteristics of the ten selected patents are listed in Table 3.

Table 3. Patents data and claims related to *T. minuta*.

Patent	Country	Title	Year	Inventor	Company	Claimed
WO 1999020258 A1	France	Polymers containing antimicrobial agents and methods for making and using them	1999	Samuel G. Seabrook, Jr., William E. Craver III	Magellan Companies, Inc.	Antimicrobial polymers
US20020136789A1	United States	Use of *Tagetes minuta* oil and its components as antiviral agents	2001	Bikram Singh, Virendara Joshi, Raja Ram, Anupama Sharma, Aijaz Zaidi	Council of Scientific and Industrial Research (CSIR)	Oral Antiviral
WO 2009045952A1	France	Oral compositions containing botanical extracts	2009	Diane Cumins	Colgate Palmolive Company	Oral antimicrobial
US 20120107258 A1	United States	Omega-cyclohexylalkan-1-oles and use thereof as antimicrobial actives to combat body odour	2012	Walter Kuhn, Ingo Wöhrle, Erich Dilk, Christian Ewering, Jörg Mampel, Michael Krohr and Holger Zinke	Symrise AG	Body Spray

4. Discussion

4.1. Effectiveness of antimicrobial activity of *T. minuta*

With this systematic review, it was possible to demonstrate that *T. minuta* is a possible antimicrobial agent. In this manuscript, it was hypothesized that these substances could minimize or reduce bacterial growth. A total of ten *in vitro* studies for antimicrobial activity were obtained, and 35 microorganisms were tested in these studies (two larvicidal, 12 Gram-negative, ten Gram-positive and 11 fungal). Of the antimicrobial trials, four used the agar diffusion method, four used the broth dilution method, and two were larvicidal tests, conducted specifically in isolated samples of microorganisms in planktonic models. The sample size of the test groups used in these antimicrobial assays are generally small. The samples per group are usually processed in triplicate. Additionally, the duration of the exposure times in most of these antimicrobial tests are usually 24h (1 day). These facts may be limitations of the study designs of these experiments.

Six studies used evaluated the antimicrobial activity of the oil of *T. minuta*. The antimicrobial mechanism of action of the essential oil can be associated with either attacking phospholipids present in cell membranes, which causes increased permeability and leakage of cytoplasm, or to their interaction with enzymes located on the cell wall (Paparella *et al*., 2008). Thus, the resistance of Gram-negative bacteria to the essential oils likely came from the protective role of their cell wall lipopolysaccharide or outer membrane proteins, which restrict diffusion of hydrophobic compounds through the lipopolysaccharide layer (Garcia *et al*., 2011). Essential oils have the ability to disrupt the lipid structure of the cell wall of bacteria, leading to destruction of the cell membrane, cytoplasmic leakage, cell lysis and ultimately cell death (Xu *et al*., 2008).

T. minuta has a significant antibacterial effect against both Gram-positive and Gram-negative bacteria (Cespedes *et al*., 2006). Four studies evaluated a crude extract of *T. minuta*; however, only two used isolated compounds of this extract. It was observed that methanol extraction was the most frequently used. The inhibitory effects of the plant extracts depend mainly on the specific plant in the study and the solvent used, as well as on the fungal isolate. A diversity of molecules with distinct polarities were extracted from plants when considering the different solvent systems used in the extraction process. For example, methanol tends to extract a diversity of compounds such as polyphenols,

glycosides and flavonoids to some extent (Rauha *et al*., 2000), which may be linked to antimicrobial activity. These flavonoids were selected for testing antimicrobial activity because quercetagetin and its derivatives are characteristic of the *Tagetes* species. The flavonoid quercetagetin-3-arabinosyl-galactoside did not show antimicrobial activity, while quercetagetin-7-arabinosyl-galactoside showed a very high level of activity against all microorganisms tested. Similar results were found in the work of Shahzadi and Mohammad (2015); among the isolated flavonols, only three were found to possess significant antibacterial activities against *M. luteus*, which may mean that there is a synergistic interaction among the components of the crude extract that is different from when it is purified.

Two studies were carried out on the larvicidal action of *T. minuta* essential oil, and both the results were satisfactory. Kyarimpa *et al*., (2014) evaluated the larvicidal activity of essential oils from *T. minuta* against *A. gambiae* (mosquito larvae). The oil was tested on the 3rd and 4th instar to determine the larvicidal activity. Six different concentrations of this essential oil were studied and compared with methyl parathion, a synthetic organophosphorus insecticide, for 24 h. Larvicidal activity was evaluated according to the WHO protocol (Coles *et al*., 1992). Essential oil was tested at 3, 6, 12, 24, 48 and 96 mg/L concentrations in triplicate. The oil was dissolved in ethanol (99.8%) and then diluted in 249 mL of distilled water to obtain the desired concentrations. The control was prepared adding 1 mL of ethanol to 249 mL of distilled water. To each of the beakers containing the essential oil, ethanol and water, 25 late third instar larvae of *A. gambiae* were introduced. The experiment was observed over a 24 h period during which no food was given to the larvae. The lethal concentrations (LC_{50} and LC_{90}) were then calculated by Camurça-Vasconcelos *et al* (2007). After 2 h of exposure, the LC_{50} was 2.9 mg/L, while the LC_{90} was 3.29 mg/L. After 6 h of exposure, the LC_{50} and LC_{90} were 2.31 mg/L and 2.68 mg/L, respectively, while after 12 h, they was 1.49 and 1.82 mg/L, respectively. After 24 h, the LC_{50} and LC_{90} were not determined because the mortality was 100%. Macedo *et al*., 2013 evaluated the effects of essential oils of *C. sativum, T. minuta, A. zerumbet* and *L. camara* on the egg hatching and larval development of *Haemonchus contortus*, a highly pathogenic and prevalent nematode parasite in small ruminants. Two *in vitro* assays on *H. contortus* were completed: the Egg Hatch Test (EHT) described by Irobi, Moo-Young and Anderson (1996) and the larval development test (LDT) (Eloff, 1998). *T. minuta* essential oil exhibited a dose-dependent effect in the EHT, inhibiting 98.1% of *H. contortus* larvae hatching at a concentration of 2.5 mg mL^{-}

[1]. The effective concentration to inhibit 50% (EC_{50}) in the EHT was 0.53 mg mL^{-1} of *T. minuta* essential oils. In the LDT, *T. minuta* at 10 mg mL^{-1} inhibited 99.5% of *H. contortus* larval development, presenting an EC_{50} value of 1.67 mg mL^{-1}. Based on the results presented in this *in vitro* model, it may be possible to use essential oil for the control of gastrointestinal nematodes per the author, and essential oils are composed of a mixture of chemical substances whose interaction can result in compounds that can interfere with nematode metabolism, inhibiting or disorganizing vital functions from the initial stages of development onward, and can furthermore interfere with drive mechanisms due to possible damaging of the nervous system (Oka *et al*., 2000). It is easier for lipophilic anthelmintics to cross the external surface of helminths than it is for hydrophilic compounds (Geary, Sangster, and Thompson, 1999). The low density of plant oils and their rapid diffusion across cell membranes can enhance the targeting of the active components of essential oils into endoparasites (Anthony, Fyfe and Smith, 2005).

Only the study of Shirazi *et al*., (2014) assessed the *in vitro* cytotoxic activity of the essential oil on two tumour cell lines, viz., Nasopharyngeal cancer (KB) and liver hepatocellular carcinoma (HepG2) cell lines were examined using a modified MTT assay (Nouri et al., *2000)*. The MTT assay results indicated that the essential oil of *T. minuta* had no effect on KB or HepG2 viability at low concentrations (<50 µg/mL). However, at higher concentrations (50–200 µg/mL), cell viability was significantly reduced in a concentration-dependent manner, with the maximum effect at concentrations >200 µg/mL. The IC_{50} values for KB and HepG2 were 75±5 and 70±4 µg/mL, respectively. Cytotoxic activities of the ethanol extract of *T. eracta* roots against prostate (PC-3) and HeLa cancer cell lines were investigated by Gupta et al. using an MTT assay. The extract revealed noticeable cytotoxicities against both PC-3 and HeLa cell lines with IC_{50} values of 407 and 164 µg/mL, respectively (Gupta *et al*,. 2012). The small number of studies that carried out cytotoxicity assays has led to the need for the effective use of this plant to be investigated more in depth. In analysing the emerging data from the current review and emphasizing the demonstrated effects, we can conclude that all of the studies have used *in vitro* experimental assays, which demonstrates the need for *in vivo* studies to prove the true efficacy of the plant in question.

Patents were filed between 1998 and 2008. Of the four patents selected in this study, three used a mixture of medicinal plants including *T. minut*a, and one patent was found using only *T. minuta* as an antimicrobial agent. With regard to the technological development of the sector, the patents came from the United States or French. The patents

filed in these underdeveloped countries are mostly foreign-owned. The patent document is an essential source of information for technological analysis, considering the wide variety of content available only in this type of document (CLSI, 2006). Moreover, each patent office uses a different tool that allows the recovery of documents, which makes it very difficult to collect and find interesting information. Therefore, there is a need for obtaining software program licenses that facilitate technological monitoring by institutions and companies, such as Questel Orbit (Paris, France) or Vantage Point (Search Technology, Inc., Norcross, GA, USA). From this review, it was possible to obtain a scientific and technological overview of the antimicrobial activity of *T. minuta*. By combining and analysing scientific and technological information, the design of this study can provide strategic information to drive new projects and support for scientific and technological development.

4.2. Future prospects for *T. minuta*

The present investigation provides support for the *in vitro* evidence of the antimicrobial effectiveness of essential oils and other extracts of *T. minuta* against pathogenic bacteria that are resistant to current antibiotics. However, animal models and clinical trials are required to justify and further evaluate the potential of these extracts from *T. minuta* as reliable antibacterial agents. Additionally, more detailed studies of the mechanisms of action of these extracts would be beneficial for reaching their full potential in the pharmaceutical, cosmetics and aromatherapy industries. Furthermore, the food industry is actively seeking natural preservatives and other agents that can replace the synthetic compounds now used in fresh and processed food.

5. Conclusion

When analysing the results of the articles and patents selected for this systematic review, we conclude that both the essential oil and the different organic extracts of *T. minuta* have antimicrobial activity *in vitro*, as proven against both fungi and bacteria. Therefore, *in vivo* studies are necessary to prove this biological effect.

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CAPITULO II

Artigo científico

Cada capítulo desta dissertação será apresentado na forma de manuscrito a ser submetido a diferentes periódicos Internacionais. Este Capítulo corresponde ao periódico *Asian Pacific Journal of Tropical Disease*

Status: Submetido

Antimicrobial activity of *Bixa orellana*: a systematic review

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Abstract:

The aim of this study was to systematically review articles related to antimicrobial activity of *B. orellana*. Studies written in English were identified in six international databases: Google Scholar, Medline (PubMed), Scopus, Scifinder and Web of Science. The following databases for patent search were analyzed: United States Patent and Trademark Office (USPTO), Google Patents, National Institute of Industrial Property (INPI) and Espacenet (European Patent Office, EPO). The last search was carried out in July 2016 with no date restriction. The grey literature was searched using the ProQuest Dissertations and Periódicos Capes Theses database. After analyzing the 13 studies included in the present investigation, it was concluded that despite the large number of *in vitro* studies that prove the antimicrobial activity of essential oil and extracts of *B. orellana*, further assays related to the cytotoxicity and *in vivo* studies are necessary for this confirmation.

Keywords: *B. orellana*, antimicrobial agents, patents, systematic review

1. Introduction

The use of natural products in the therapeutic management against diseases caused by microorganisms presents advantages over synthetic drugs. This is due to the low side effects of these drugs regarding their toxicological and pharmacological activity when compared to those synthetic ones from industrial sources[1]. In this context, medicinal herbs, due to their wide biological activities, can serve as a potential source for the development of new drugs, including antimicrobial agents.

Annatto seed has been used as a natural colorant in many foods found in Asia. Annatto ranks second place worldwide in economic importance among all natural colorants and its extract shows antimicrobial and antioxidant properties[2]. The color of the pigment from the outer layer of annatto seeds ranges from yellow to red and is affected by the concentration of the color compounds. The main color pigments of annatto seeds are bixin and nor-bixin, extracted from the outer coating of the seeds[3].

The antimicrobial activity of ethanol extracts from *B. orellana* leaves were also determined by Viuda-Martos *et al*[4] and the plant demonstrated inhibitory activity against *P. aeruginosa* and *B. cereus*. In addition, one study has shown that *B. orellana* ethanolic extracts exhibit anti-leishmanial activity[5]. *Bixa Orellana* (achiote, orlean, roucou or annatto), belongs to the Bixaceae family, and is a native plant from Central and South America, which contains several carotenoid derivatives (bixin and norbixin), terpenoids, tocotrienols and flavonoids in its seeds and leaves[6]. Gomez *et al.*[7] demonstrated that a leaf ethanol extract exhibited higher antimicrobial activity against *Bacillu*s sp., *Staphylococcus aureus* and *Streptococcus faecalis* (Gram-positive microorganisms) than against *Escherichia coli and Pseudomonas aeruginosa* (Gram-negative microorganisms) and the fungi *Candida albicans* and *Aspergillus niger*.

The only systematic review available in the literature on *B. orellana* discusses the plant assignments in general. However, it is not focused on the antimicrobial potential of this plant[8]. Thus, the aim of this study was to systematically review articles and search patents related to the antimicrobial activity of *B. orellana* in order to use this information as a strategic tool for prospecting new investigations with this medicinal plant.

Figure 1. Leaves and seeds of *B. Orellana*

2. Materials and Methods

This systematic review was carried out according to the guidelines of Cochrane Handbook for Systematic Reviews of Interventions[9], following the four-phase flow diagram of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement[10].

2.1. Search strategy and study selection

Studies written in English were identified in six international databases: Google Scholar, Medline (PubMed), Scopus, Scifinder and Web of Science. The following databases for patent search were analyzed: United States Patent and Trademark Office (USPTO), Google Patents, National Institute of Industrial Property (INPI) and Espacenet (European Patent Office, EPO). The last search was carried out in July 2016 with no date restriction.

The grey literature was found using the ProQuest Dissertations and Periódicos Capes Theses database. The search used was appropriately adapted for each database and is listed in Table 1. The results were de-duplicated using EndNote X7 software (Thomson Reuters, New York, NY, USA). The titles of all identified studies were screened by two independent reviewers (D.S. and L.S.). Abstracts were carefully appraised when the title indicated the inclusion of such studies and articles considered eligible for the review (or in case of doubt) were selected for full-text reading. Discrepancies were resolved by group discussion. References lists of all included studies were also hand-searched for additional studies.

2.2. Eligibility criteria

Investigations that evaluated the antimicrobial activity of essential oil or extracts of *B. orellana*, with no restrictions of study, were selected. The following inclusion criteria were: peer reviewed research articles from scholarly journals or theses written that referenced the use of *B.orellana* plant, some assessment of its antimicrobial activity, as well as the existence of a control group in the methodology assays.

The following reasons were considered for exclusion: articles of literature review, studies not related to the assessment of the antimicrobial activity of *B. orellana*, or with the absence of control group. Furthermore, a patent search was also made using International Patent Classification (IPC) with the following codes: A61P 31/10 (antimycotic), A61P 31/02 (antibacterial agent), A61P 31/02 (antiseptic site) and A61K 8/97 (vegetable origin).

Table 1. Terms used in the search strategy

Electronic database
Search and Terms
Google Scholar
Antimicrobial activity (Title) AND bixa orellana
PubMed (MEDLINE)
#1 Bixa orellana
#2“antimicrobial” [MeSH Terms] OR “antimicrobial” OR “Agents, Anti-Infective” OR “Anti Infective Agents” OR “Antiinfective Agents” OR “Agents, Antiinfective” OR “Microbicides” OR “Antimicrobial Agents” OR “Agents, Antimicrobial” OR “Anti-Microbial Agents” OR “Agents, Anti-Microbial” OR “Anti-Microbial Agents”
#3“biofilms” [MeSH Terms] OR “biofilms” OR “Bacterial Adhesion” OR “Adhesins, Bacterial” OR “Biofouling”
#4“antibacterial” [MeSH Terms] OR “antibacterial” OR “Agents, Anti-Bacterial” OR “Anti Bacterial Agents” OR “Antibacterial Agents” OR “Agents, Antibacterial” OR “Antibiotics” OR “Bacteriocidal Agents” OR “Agents, Bacteriocidal” OR “Bacteriocides” OR “Anti-Mycobacterial Agents” OR “Agents, Anti-Mycobacterial” OR “Anti Mycobacterial Agents” OR “Antimycobacterial Agents” OR “Agents, Antimycobacterial”
#5“antifungal” [MeSH Terms] OR “antifungal” OR “Agents, Antifungal” OR “Therapeutic Fungicides” OR “Fungicides, Therapeutic” OR“Antibiotics, Antifungal” OR “Antifungal Antibiotics”

#6 “antiparasitic” [MeSH Terms] OR “antiparasitic” OR “Agents, Antiparasitic” OR “Antiparasitic Drugs” OR “Drugs, Antiparasitic” OR “Parasiticides” OR “Antiparasitics”

#1 AND 2# OR3# OR 4 #OR 5# OR #6

Scifinder

antimicrobial activity of bixa orellana

Scopus

#1("bixa orellana") AND#2("antimicrobial activity") OR #3("biofilms") OR #4("antibacterial") OR #5("antifungal") OR #6("antiparasitic")

Web of Science

#Topic: ("antimicrobial activity") OR #Topic: ("biofilms") OR #Topic: ("antibacterial") OR #Topic: ("antifungal") OR #Topic: ("antiparasitic") AND #Topic: ("bixa orellana")

Search in patent

Patent search was also made using International Patent Classification (IPC) with the following codes:

#A61P 31/10 (antimycotic)

#A61P 31/02 (antibacterial agent)

#A61P 31/02 (antiseptic site)

#A61K 8/97 (vegetable origin).

2.3. Data collection

When the study had more than one group for the same treatment (e.g. three plant extracts), but only one control group, data for all of the experimental groups were included. Data extraction was carried out by consensus among the two researchers who conducted the extraction.

2.4. Assessment of risk of bias

The risk of bias of all studies which were included was assessed based on The Cochrane Collaboration’s tool for assessing risk of bias[9] and the methodological quality was adapted from another systematic review about antimicrobial monomers used in dental materials[11]. Risk of bias was assessed according to the description of the methodologies in the articles included, the rules which were followed are: sample size, positive control group, negative control group, and microorganisms tested. The parameters used were discussed by the researchers involved and judgment was carried out by group discussion.

3. Results

3.1. Study selection

The flowchart of the systematic review is shown in Figure 1. After the database screening and removal of duplicates, 100 studies were identified. Then, it was removed duplicate of 70 studies, 50 titles were screened and a careful examination of the abstracts was performed. Next, the full-texts of 31 studies were selected and assessed to check if they were eligible for this systematic review.

As a result, 18 studies were excluded because they did not fit the inclusion criteria. The patent research initially showed 54 patents. 17 duplicates were removed and 24 patents were excluded after the title and abstract reading (Figure 1). After that, 13 full texts were excluded because they they were not related to antimicrobial activity. 16 studies were extracted from the grey literature and after careful examination of the abstracts, the full-text of only one study was assessed to check if it would be eligible.

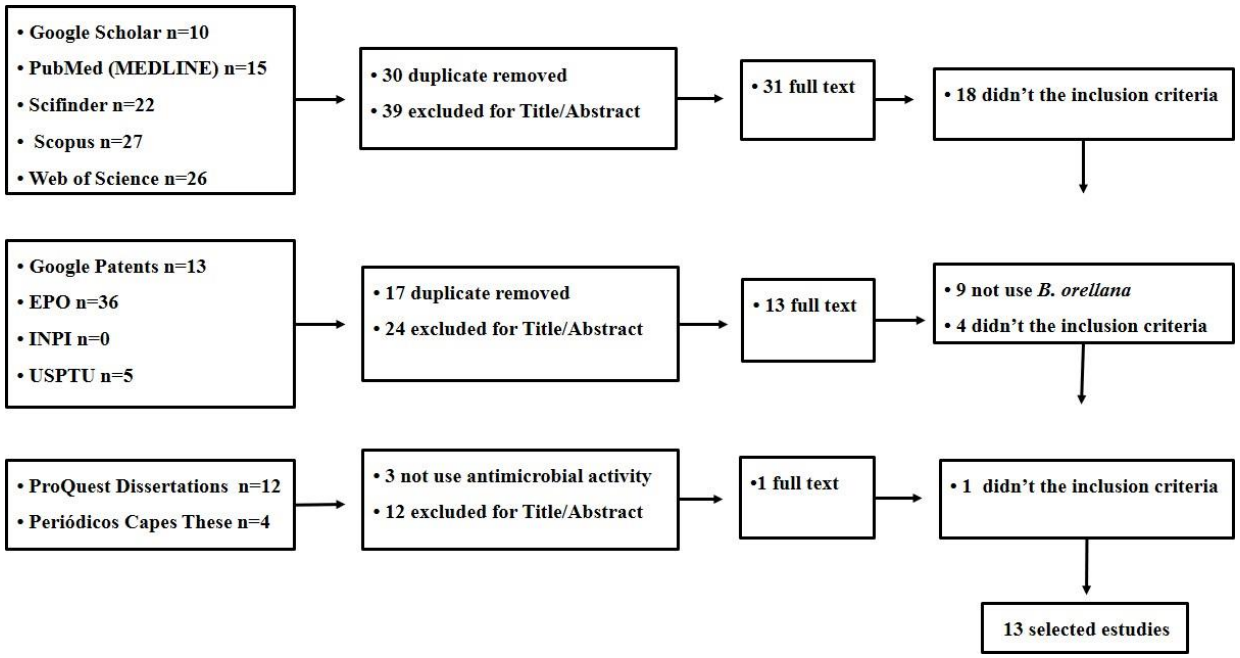

Fig. 1– Flowchart of the selection process of studies according to the PRISMA statement

3.2. Characteristics of included articles

The characteristics of the 13 selected studies are listed in Table 2.

Study	Assay	Microorganisms tested	Types of extract	Antimicrobial assay	Exposure times in the antimicrobial test	Control groups	Sample size	Main results
Cáceres *et al*.[12]	Antigonorrhoeal activity of plants used in Guatmala for the treatment of sexualy transmitted diseases	*Neisseria gonorrhoeae*	Ethanol	Agar disc-diffusion method	24 h	Ethanol	n=5	The root and leaf extracts from *B. orellana* were both effective against *N. gonorrhoeae* penicillin-resistant strains isolated from symptomatic patients with gonorrhoea, inhibiting 6.0±0. and 17.4±0.5 respectively.
Navarro *al*.[13]	Antifungal activities of nine traditional Mexican medicinal plants	*Trichophyton mentagrophytes Trichophyton rubrum* *Aspergillus niger Candida albicans*	n-hexane Methanol	Agar difusion	*C.albicans* (24 h) *A. niger* (48h and 72h)	Ketoconazole Nystatin	n=3	The results showed that the hexane and methanol extracts obtained MIC values above 8 mg/ml against all tested microorganisms, except for *T. mentagrophytes.*
Irobi; M. Moo-Young and Andeson [14]	Antimicrobial activity of annatto (*Bixa orellana*) extract	*Bacillus subtilis* *Staphylococcus aureus*	Ethanol	Broth macrodilution and	24 h	Chloramphenicol Phenol	n=2	The zones of inhibition obtained against the susceptible bacteria were 15-17mm while those obtained in assays with Chloramphenicol and

		Enterococcus faecalis *Escherichia coli* *Serratia marcescens Candida utilis* *Aspergillus niger*		Agar diffusion				phenol positive controls were 12 18mm and 10-28mm, respectively. The MIC of the extract was 4-16 mg/ml while its bactericidal action (MBC) was exerted at higher doses (16-64 mg/ml).
Zollo *et al*.[15]	Aromatic plants of tropical Central Africa. Part XXXII. Chemical composition and antifungal activity of thirteen essential oils from aromatic plants of Cameroon	*Candida albicans* *Cryptococcus neoformans* *Aureobasidium pullulans* *Trichoderma viride, Microsporum gypseum* *Trichophyton rubrum*	Essential oil	Broth microdilution	2d (dermatophytes) and 7d (filamentous)	Culture medium	n=2	*B.orellana* presented MIC values greater than 15µl for all tested microorganisms
Rojas *et al*.[16]	Screening for antimicobial activity of ten medicinl plants used in Colombian folkloric medicine: a	*Staphylococcus aureus* *Staphylococcus hemolític* *Bacillus cereus*	Ethanol and n-hexane	Agar-well diffusin method	24h	Gentamycin sulfate Clindamycin Nystain	n=3	*B. orellana*, presented the lowest MICs against E. coli (0.8µg/ml) compared to gentamycin sulfate (0.98g/ml) and exhibited a better MIC against *B. cereus* (0.2 µg/ml) than gentamycin sulfate (0.5 µg/ml). For the rest of the microrganisms tested, the controls

	possible alternative in the treatment of non nosocomial infectios	*Pseudomonas. Aeruginosa* *Escherichia coli* *Candida albicans*						showed better antimicrobial activity than the extracts.
Sumathi and Parvathi [17]	Antibacterial potential of the aqueous and organic extracts of *Bixa orellana* l.	*Escherichia coli* *Klebsiella pneumoniae* *Salmonela typhi* *Staphylococcus aureus*	Methanol, Dmethyl Suphoxide Ethanol Aetone	Agar diffusion	24h	Streptomycin	n=2	The extracts of dimethyl sulphoxide and methanol from empty seed also showed effective antibacterial activity against *S.aureus* and *S. typhi* at concentrations of 3200, and 800 and 3200µg/ml. Moderate growth inhibition zone of *S. typhi* and *S. aureus* in dimethyl sulphoxide was noticed at high concentrations of 6400µg/ml
Tamil Selvi A *et al*.[18]	Leaf and Seed extracts of *Bixa orellana* L. exert antimicrobial activity against bacterial pathogens	*Staphylococcus aureus* *Salmonella typhi* *Klebsiella pneumoniae* *Pseudomonas aeruginosa*	Methanol	Agar disc diffusion and Agar-well diffusion	C. albicans (24h) A. niger 48h) and 72 h for the dermatophytes (72h)	Dimethyl sulphoxide Streptomycin	n=3	Leaf extract of *B. orellana* at 1000µg/ml concentration showed significant inhibition against all the microrganims tested with highest inhibition zone (18±0.3mm) against *S. typhi*, *Acinetobacter* sp., *T. mentagrophytes* and *T. rubrum*. Seed extract was less efficacious in most of the tested pathogens, except *Brucella* sp. which was zonne inhibited

		Enterococcus fecalis *Vibrio cholera* *Moraxella* c*atarrhalis* *Acinetobacter* sp. *Brucella* sp. *Candida. albicans* *Aspergillus niger* *Trichophyton mentagrophytes* *Trichophyton rubrum*						(15±0.1mm). MIC of leaf extract was determined as 15.62µg/ml against *S. aureus* and 31.25µg/ml for *K. pneumoniae*, *P. aeruginosa*, *E. fecalis* and *S. typhi*, on average. Approximately78.2% of inhibition was seen against *T. mentagrophytes* and *T.rubrum*.
Venugopalan and Giridhar [19]	Bacterial growth inhibition potential of annatto plant parts	*Escherichia coli* *Klebsiella pneumoniae* *Pseudomonas aeruginosa* *Bacillus subtilis* *Bacillus cereus* *Staphylococcus aureus*	Acetone, Dimethyl sulphoxide, Ethanol and Methanol	Agar diffusion	24h	Tetracycline	n=3	Aqueous extracts of plant parts did not show any antibacterial activity against the selected organisms. However, the degree of inhibition varied according to different concentrations of various solvents on the test organisms.Acetone extract of the leaf was found to be highly effective against (>14 mm diameter) *S. aureu*s from 750µg to 6000µg. In other concentrations (100-500µg), moderate level (10-14 mm) of antibacterial activity was noticed.

Gómes *et al*.[7]	Ethanolic extract from leaves of *Bixa orellana* l.: a potential natural food preservaive	*Escherichia coli* *Bacillus cereus* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Salmonella typh* *Shigella sonnei Listeria monocytognes* *Candida albicans Saccharomces cerevisiae* *Aspergillus niger* *Penicillium chrysogenum Byssochlamys fulva*	Ethanolic	Colorimetric broth microdilution	24h	Nisin	n=3	The ethanolic extract exhibited a broad spectrum of antimicrobial activity for both Gram positive and Gram negative bacteria with MIC values from 256 to1024ppm. For MIC values between 1 and 512ppm, fungi showed greater sensitivity to extract than bacteria. Nisin used as positive control caused growth inhibition of all bacteria tested, with MICs between 2 and 1024ppm. In contrast, fungi were not inhibited by nisin.
Viuda-Martos *et al*.[4]	*In vitro* antioxidant and antibacterial activities of extracts from	*Listeria innocua* *Aeromonas hydrophila* *Bacillus cereus*	Ethanol	Broth macrodilution	5h 26h 30h	Nisin	n=3	Bixin and norbixin content of polar extract seed was a stronger inhibitor ($P < 0.05$) than bixin and norbixin content of polar extracts of leaves. In a comparison with nisin, which is the

	annatto (*Bixa orellana* l.) leaves and seeds	*Pseudomonas aeruginosa*						only bacteriocin widely accepted as a natural food preserver, the MIC values were found to be 1,024, 256, 512 and 256 mg/mL for *P. aeruginosa*, *B. cereus*, *L. innocua* and *A. hydrophila*, respectively. As regards seed, the MIC values were 128 and 1,024mg/mL for *P aeruginosa* and *B. cereus*, respectively. As in the case of leaves, seed did not inhibit the growth of *L. innocua* and *A. hydrophila* in the concentrations tested.
Suneetha *et al*.[20]	Comparative studies on antimicobial and antifungal efficacy from *Bixa orellana* l., *Lantana camara* l., *Stachytarpheta jamaicesis* (l.) *Vahl., hyptis suaveolns* (l.) poit. with triclosan	*Staphylococcus aureus* *Bacillus substitis* *Escherichia coli* *Pseudomonas fluorescens* *Aspergillus niger* *Aspergillus flavus* *Mucorsp*	Phenolic	Agar disc-diffusion	18h and 24h	Triclosan	n=2	The extract containing 70µg of phenolic substance was found to have more inhibitory effect on *A. niger* and *Mucor* Sp., whereas slightly higher concentration was required to inhibit the growth of *A. flavus*. The extract showed the zones of inhibition (mm) 17, 11, 22, 11 for *B. substilis*, *E. coli*, *P. fluorescence* and *S. aureus* respectively. The Triclosan obtained values of 17, 23, 22 and 22, respectively.

Miller *et al*.[21]	The antibacterial and antifungal activity of essential oils extracted from Guatemalan medicinal plants	*Staphylococcus aureus* *Escherichia coli* *Streptococcus mutans* *Lactobacillus acidophilus* *Candida albicans*	Essential oil	Broth macrodilution	24h	Gentamycin Nystatin	n=3	The extract containing 70µg of phenolic substance was found to have more inhibitory effect on *A. niger* and Mucor Sp., where as slightly higher concentration was required to inhibit the growth of *A. flavus*. The extract showed the zones of inhibition (mm) 17, 11, 22, 11 for *B. substilis*, *E. coli*, *P. fluorescence* and *S. aureus* respectively.The Triclosan obtained values of 17, 23, 22 and 22, respectively.
Flores *et al*.[22]	Antibacterial activity of *Bixa orellana* L. (achiote) against *Streptooccus mutans* and *Streptooccus sanguinis*	*Streptococcus mutans* *Streptococcus sanguinis*	Methanol	Agar diffusion and Broth microdilution	72h	Chlorhexidine	n=3	For *S. mutans* the seed extract produced an inhibition zone of 15.11mm and the leaves extract an inhibition zone of 19.97mm. Moreover, *S. sanguinis* showed an inhibition zone of 16.15mm and 19.97mm for seeds and leaves extract, respectively. In both bacterial cultures a larger inhibition zone was observed in the leaves methanolic extracts. The MIC for *S. mutans*, we observed a MIC between 25 and 50mg/mL for the seeds extract and a MIC between 50 and 75mg/mL for the leaves extract. On the other hand, for the S. *sanguinis* strains a MIC of 125mg/mL was

observed for the seeds extract and a lower MIC between 50 and 75mg/mL was observed for the leaves extract.

4. Discussion

To the total of this systematic review, 13 studies were found regarding the *B. orellana* antimicrobial activity. Searching the international scientific literature and patent databases, it was observed that *B.orellana* is widely referred in researches to discuss about its use as a natural reddish orange dye. There is a large number of studies and patents that use its pigment as a condiment in the food industry, or even as tanning lotion produced by the pharmaceutical industry. From 13 papers included in this review, 11 assessed *B.orellana* leaves extract. Two out of the 13 articles also evaluated the antimicrobial activity of the seeds of this plant and only two studies dealt with essential oil leaves. This low number of articles that discuss about essential oil may be associated with the low yield of the oil, since *B. orellana* is a plant used mainly in the food and cosmetics industry, to use methods that use extracts and not oils, become more viable in terms of income.

From the methods empoyed in the studies for the evaluation of the antimicrobial activity of *B. orellana*, six used the agar diffusion technique, four the broth macrodilution method and three, the broth microdilution assay. It is noteworthy that only four papers used more than one antimicrobial assay. Yet, only one of the works analyzed used the methodology of microdilution by colorimetry. In total, 36 microorganisms were tested in these studies. Of these, 14 were fungi, 13 Gram negative bacteria and 6 Gram positive bacteria. The main difference of the present review compared to previous reviews on the literature about *B. orellana* is the systematic assessment of the existing evidence based on a large number of scientific and patent databases. We provide a detailed methodological quality assessment of each study that was mentioned in this review. Moreover, in the literature there is no a systematic review on the antimicrobial potential of *B. orellana* specifically. Until now, there are only narrative reviews that, for the most part, report the use of the dye obtained through the seeds of *B. orellana* in the culinary and food industry.

The lack of studies related to the cytotoxicity of the plant in question as well as the lack of *in vivo* studies related to *B. orellana*'s antimicrobial activity may justify the lack of patents related to this subject, since the patents found in the present search were all related to the use of this plant either as a natural dye, either as a condiment, either related to cosmetic industry, or as tanning lotion. Thus, more studies are needed to reinforce *B. orellana* as a truly antimicrobial plant.

5. Conclusion

Despite the large number of studies that prove the *in vitro* antimicrobial activity of essential oil and extracts of *B. orellana*, further assays on cytotoxicity and *in vivo* studies are necessary for this confirmation.

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CAPITULO III

Cada capítulo desta dissertação será apresentado na forma de manuscrito a ser submetido a diferentes periódicos Internacionais. Este Capítulo corresponde ao periódico *Journal of Endodontics*

Status: A ser submetido

Physicomechanical and antimicrobial properties of experimental endodontic sealers containing *Bixa. orellana, Mentha. piperita* and *Tagetes. minuta* extracts.

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Abstract

In order to achieve a successful root canal treatment, the use of endodontic sealers with antibacterial properties may be useful for the reduction and elimination of persistent microorganisms. The aim of this study was to evaluate the effect of addition of oil-base compound obtained from *Bixa orellana*, *Mentha piperita* and *Tagetes minuta* on antimicrobial activity and phisico-mechanical performance of experimental resin based cements. The groups were divided as follows: cement with Mentha pipperite oil, cement with *Tagetes minuta* oil, cement with *Bixa orellana* oil, cement without oil for control and cement Real Seal as commercial control. The following tests were performed with the experimental groups: chemical characterization of essential oils and plant extracts, antimicrobial assay using MIC and TCD against *Streptococcus mutans* ATCC 25175, *Enterococcus faecalis* ATCC 4083 and *Candida albicans* ATCC 62342 and, finally, physical-mechanical tests to evaluate the effectiveness of the new experimental sealers according to ISO 6876. It was possible to demonstrate the antimicrobial potential of these

three different plant species in experimental endodontic sealers. Although the antimicrobial activity of the tested species decreased when added to the novel materials, it was still possible to identify antimicrobial activity among the experimental groups tested.

Key Words: endodontic sealers, antimicrobial activity, medicinal plants

Introduction

The ultimate goal of root canal therapy might be considered to be the complete elimination of all microorganisms from the root canal system. Unfortunately, this appears to be impossible in typical clinical situations,[1] because evidence suggests that persistent microorganisms after endodontic treatment can survive and interfere with healing and repair[2] Also, the mouth is host to a variety of potentially damaging bacteria like *Streptococcus mutans*, which is the primary species associated with dental caries[3] and *Entecoccocus Faecalis*, which one due its ability to attach to dentin walls, invade into dentinal tubules, and form biofilm, represents a common bacteria in persistent endodontic infections.[4] In addition, numerous studies have revealed incidence of *Candida albicans* in infected root canals.[5,6]

In order to achieve a successful root canal treatment, a suitable technique of sealing through restauration of endodontic channels systems is mandatory for longevity of treated teeth. Despite the importance of clinical restorative protocol and material selection, the use of endodontic sealers with antibacterial properties may be useful for the reduction and elimination of persistent microorganisms.[7] In this context, plants with wide variety of chemical constituents offer some promising sources of new antimicrobial agents with general as well as specific activities.

Reports abound in the literature on the presence of antimicrobial compounds in various plants.[9,10] The *Mentha piperita* L (Lamiaceae) is a cultivated natural hybrid of *Mentha aquatica* L. and *Mentha spicata* L. Although a native genus of the Mediterranean region, it is cultivated all over the world for its use in flavor, fragrance, medicinal, and pharmaceutical applications. Peppermint oil is one of the most widely produced and consumed essential oils.[11] Many of these peppermint's medicinal uses have been recently verified by scientific trials and it has been commonly used in many pharmaceutical and industrial products due to their wide range of demonstrated pharmacological properties

including antioxidant, antitumor, antiallergenic, antiviral, fungicide, insecticide and antibacterial activities.[12, 13]

Tagetes species (Asteraceae) originally has been used as a source of essential oil for the flavoring in the food industries. The powders and extracts of *Tagetes* are rich in the orange-yellow carotenoid and are used as a food colorant in foods such as pasta, vegetable oil, margarine, mayonnaises, salad dressing, baked goods, confectionery, dairy products, ice cream, yogurt, citrus juice, mustard and as colorant in poultry feed.[14,15,16] *Tagetes* species have been reported in literature to have many of the above properties and special emphasis has been placed on *Tagetes minuta* as having insecticidal, atioxidant and antibacterial properties.[17,18]

Bixa orellana or Annatto ranks second place in economic importance worldwide among all natural colorants and its extract shows antimicrobial and antioxidant properties.[19] The color of the pigment from the outer layer of annatto seeds ranges from yellow to red and is affected by the concentration of the color compounds.[20] *B. orellana* possesses various pharmacological activities like anti-diarrheal, anti-inflammatory, antioxidant, hypoglycemic and antibacterial. Scientific evidences show that it possesses antioxidant, antimicrobial, anticonvulsant, antidiabetic and cardio-protective activity. [21,22]

Therefore, the objective of this work was to evaluate the antimicrobial activity of extracts and essential oils of *B. orellana*, *M. piperita* and *T. minuta* included in formulation of experimental root canal sealers in order to develop new products of dental use with potential antimicrobial activity against microorganisms that cause infections of the canal root system.

Materials and Methods

Plant material

Aerial parts of *M. piperita*, *T. minuta* and seeds for *B. orellana* were collected in the Brazilian Agricultural Research Corporation, Embrapa Temperate Climate, Monte Bonito, RS, Brazil. The essential oils were obtained according to the Brazilian Pharmacopoeia,[23] using a *Clevenger* type apparatus for 3h. The essential oil was then stored at 4^{o}C in amber glasses vials until analysis. The extract of the annatto seeds was obtained through the technique described by Lorenzi & Matos,[24] with modifications, where 100g of seeds were placed in 1000mL of cereal ethanol at 70° GL. After 15 days,

the extract was filtered to separate the filtered extract (supernatant) from the residue and then the supernantant was lyophilized for the obtation of the dried extract. The extract then was stored in a desiccator protected from light room temperature untilits use.

GC-analysis

Identification of the *T. minuta* and *M. piperita* essentials oils compounds were done using a Gas Chromatograph coupled to a GC/MS-QP 2010SE (Shimadzu, Japan) Mass Detector (GC-MS) equipped with AOC-20 in auto injector. Separation of the compounds was performed on a RTX-5MS capillary column (Restek, USA) having dimensions of 30mx0.25mmx0.25μm in the following chromatographic conditions: initial temperature of 40^0C rising at 5^0C/min to 280^0C; Remaining at this temperature for 10min; Volume injected: 1μL; Interface: 300^0C; Injector temperature: 280°C; Entrainment gas: helium; Linear gas flow: 1.27mL.min^{-1}; Split: 1:10; Purge flow: 3.0 mL.min^{-1}, running in scan mode; m/z range of 40 to 700 Da and filament voltage in 70 eV. Quantifications were done by normalized area and the identifications of the compounds by the mass spectra using the *GC Solution* Program and the NIST 8 library.

Determination of phenolic compounds of *B. orellana* extract

The methodology described by Swains & Hillis[25] was used, with minor modifications. Five grams of sample was added in 20mL of methyl alcohol, which were shaken in ultraturrax (TURRATEC TE-102) for 2min and centrifuged at 3420g for 20 min. To perform the reaction, 4mL of distilled water, 150μL of methyl alcohol, 100μL of the extract and 250μL of 0.25M Folin-Ciocalteau solution were added to a falcon tube and allowed to react for 3min. After this period, 500μL of 1M Sodium Carbonate was added, allowing to react for 2h, and then the spectrophotometer (JENWAY 6705 UV/Vis.) It was read at 725nm. for the quantification of the phenolic compounds a standard curve prepared with gallic acid was used, the results expressed in mg of gallic acid equivalent.

Determination of carotenoids from *B. orellana* extract

The method described by Rodriguez-Amaya[26] was followed with minor modifications. Five grams of sample and 2g celite were weighed, ice-cold acetone was added, the contents being shaken ultraturrax (TURRATEC TE-102) for 2min. The material was filtered and washed with acetone until the extract became colorless. The

filtrate was transferred to a separatory funnel, where 30mL of petroleum ether and 30mL of distilled water were added. The bottom phase was discarded, distilled water was added again and the procedure was repeated 4 times for complete removal of the acetone. The top extract was transferred to a 50mL volumetric flask and the volume was quenched with petroleum ether. The reading was carried out in a spectrophotometer (JENWAY 6705 UV/Vis) at 470nm and the results expressed in μg of sample lycopene g^{-1}.

For the determination of carotenoids, the following classification was used:

$$C = \frac{ABS \times 50\ mL \times 1000000}{2592 \times 100 \times g} \quad \text{(eq.1)}$$

Where:

C = Carotenoid content of the sample

ABS = Absorbance

g = sample grams

Antimicrobial Assay

The reference strains used in this study were chosen based on their pathological effects on dentistry. The strains were *Streptococcus mutans* ATCC 25175, *Enterococcus faecalis* ATCC4083 *and Candida albicans* ATCC 62342. The microorganism used in this study consisted from the American Type Culture Collection (ATCC) kept in the collection of the Research Laboratory of Microbiology of Dentistry School at Federal University of Pelotas (UFPel).

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) were determined in triplicate by using the broth microdilution technique modified version of the reference documents (CLSI, 2006)[27] and (CLSI, 2008).[28] The susceptibility test was performed on 96-well microplates. 0.10mg of lyophilized extract were diluted in 100μl of esteril water and essential oils were dissolved in ethanol at 0.5g/mL. This diluted into the 96-well microtiter plates in culture medium (RPMI for fungi and Mueller-Hilton for bacteria) in concentration of 2500μl-4.88μl. Then, aerobic and microaerophilic bacteria were incubated for 24h, and fungal for 48h. *S. mutans* was incubated at 37°C in an environment of 5-10% CO_2 (Anaerobac - Probac do Brasil produtos Bacteriológicos Ltda., Santa

Cecília, SP, Brazil) produced in anaerobic jars (Probac do Brasil Produtos Bacteriológicos Ltda). Two replicates in triplicate were performed for each concentration of the compounds and the MIC was defined as the lowest concentration of the antimicrobial agent that changed from purple to pink after 10µl of resazurin in incubition for 20 min.

Modified direct contact test

The modified direct contact test was described by Danlar *et al.* [29]. Was tested in 96-well microtiter plates in two diferents moments. The first assay using only the plant products in the following concentration 0.5%. After comproved atimicrobial activity, the assay was accomplished again for antimicrobial activity of the endodontic sealer and were used 10µL microrganism suspension (approximately 1×106 cells) that was placed in the times of 1h and 24h in incubation in a moist atmosphere at 37°C. After the time, 240µL of Brain Heart Infusion (BHI) for bacterial and Agar Sabouroud Dextrose for fungal was added to each pit. The solutions were gently mixed with a micropipette for 1min, and the Microbial suspensions from each well were serially diluted in BHI and Saboroud agar plates. After incubation for 24 h at 37° C, the colonies on the plates were counted. The viable bacteria counts (CFU) were converted to $\log_{10}$ values, and the results were expressed in CFU/mL. Microplate cavities with only the natural oil or extracts were used as negative control and cavities with only the microorganisms suspensions (without the test extracts or oils) were used as positive control. Each test was performed in triplicate.

Formulation of experimental endodontic sealers

The experimental dual cured endodontic sealers were formulated as two paste materials. The compositions of the experimental materials are summarized in Table 1.

Table 1. Composition of the experimental endodontic sealers.

Materials	% Weight
Base paste	
Ethoxylated Bisphenol-A dimethacrylate 30	40
Exothane 8	10
Polyethylene glycol 400 dimethacrylate	10
Triethylene glycol dimethacrylate	10

Camphorquinone	0.4
Ethyl 4-(dimethylamino)benzoate	0.6
Aerosil 380	4
Yterrbium Fluride	25
Catalyst paste	
Ethoxylated Bisphenol-A dimethacrylate 30	40
Exothane 8	20
Triethylene glycol dimethacrylate	10
Benzoyl peroxide	0.4
p-Toluenesulfonic acid	0.6
Aerosil 380	4
Yterrbium Fluride	25

Three different base pastes were prepared adding extract powders of *T. minuta* (G1), *M. piperita* (G2) and *B. orellana* (G3) at a mass concentration of 0.5%. This concentration was chosen according to a previously screening concentration test. A base paste without natural extract addition was used as control. The physicochemical properties of the endodontic sealers were evaluated by assessing their dimensional stability, water sorption, water solubility, flow, film thickness and setting time according to the ISO 6876 (2012). To perform photoactivation, a light-emitting diode activation unit (Radii Cal, SDI, Bayswater, Victoria, Australia) with irradiation value of 800 mW/cm^2 was used.

Flow

A total of 0.05 mL (± 0.005) of each experimental sealer was dispensed on a glass slab with 40x40x5 mm. One hundred and eight seconds after mixing started, another slab with a mass of 20g (± 2) and a load of 100g was placed on top of the material. Ten minutes after start of mixing, the material was photoactivated for 20s and the load was removed. The major and minor diameters of the compressed material were measured. The test was conducted in triplicate for each group (n=3).

Film thickness

Two glass slabs with surface area of 200,0 mm^2 and 5,0 mm thickness were placed together and their thickness was measured by a . 0.05mL of each experimental endodontic sealer was mixed and placed on the surface of the first slab, and the second slab was placed on the top of the material. One hundred and eight seconds after the start of mixing, a load of 150 N was applied on top of the glass slab. Ten minutes after the start of mixing, the load was removed and the material photoactivated for 20s. After the photopolimerization process, the thickness of the two glass plates and the interposed sealer film was measured. The film thickness was determined by the difference in the thickness of the plates with and without sealer. The test was carried out in triplicate for each group (n=3).

Dimensional stability

The dimensional stability of the endodontic sealers was evaluated according to the method described by Carvalho-Junior *et al.*[30]. Eight cylindrical specimens (3.58mm height x 3mm diameter) were fabricated using a silicon mold. After removing the specimens from the mold, flat surfaces of each specimen were polished with a 600-grit wet sandpaper and its initial length was measured with a digital caliper (Mitutoyo Sul Americana Ltda, Santo Amaro, São Paulo, Brazil). Then, they were stored in flasks containing distilled water at 37°C for 30 days. After the storage time, the specimens were removed from the flasks, dried, and their final lengths were measured. The percentage of dimensional change was calculated as follows:

$$DS = [(L30 - L)/L] \times 100$$

Where L is the initial length of the specimen in mm and L30, the length in mm after 30 days.

Setting time

The experimental endodontic sealers were mixed and inserted into stainless-steel moulds (10mm diameter x 1mm height). Determination of setting time was performed using an indenter with a head weight of 200g. Each hour, the indenter was carefully lowered vertically on to the horizontal surface of the sealer. The setting time of each sealer was established by calculating the mean time elapsed from mixing until the indenter failed to leave an indentation on the surface of the specimens.

Degree of conversion

The degree of conversion of the experimental resin sealers (n = 3) was evaluated using FTIR spectroscopy (RT-FTIR Shimadzu Prestige 21 Spectrometer, Shimadzu, Japan) with an attenuated total reflectance device. The mixed sealer was placed on the diamond crystal and covered with a Mylar® strip and a glass slide. An infrared spectrum of the uncured and cured material was obtained. For each spectrum, it was determined the height of the aliphatic C=C peak absorption at 1638 cm^{-1} and the aromatic C=C peak absorption at 1609 cm^{-1}. The aromatic C=C vibration was used as an internal reference. The degree of conversion was determined in accordance with the following equation:

$$ouble\ bond\ conversion\ (\%) = 100\,[1 - \frac{(\frac{A_{1638}}{A_{1609}})\ polymer}{(\frac{A_{1638}}{A_{1609}})\ monomer}]$$

Radiopacity

A bipartite metallic matrix with 5mm of internal diameter and 1mm of thickness (n = 5) was used to make the samples. The sealers samples were positioned on an occlusal phosphor plates of the VistaScan Plus® digital system (Dürr Dental AG, Bietigheim-Bissingen, Germany) and radiographed with a x-ray unit (Ion 70x®, Procion, Ribeirão Preto, São Paulo, Brazil) with 70 kVp, 8mA, exposure time of 0.2s and a focal length of 40cm. On this phosphor plates were also be placed a aluminum stepwedge, with purity greater than 98%, with 50 x 20mm and thickness varying in step form every 1mm. The 5 samples of each group experimental and the aluminum stepwedge were placed on the occlusal phosphor plates and were made 5 radiographs processed with the software of the VistaScan Plus (DBSWIN Imaging Software®, Dürr Dental AG, Bietigheim-Bissingen, Germany) and saved in Joint Photographic Experts Group (JPEG) format. These images were exported to the software Photoshop CC® (Adobe Systems Incorporated, São Jose, California, USA) and the radiopacity of the sealers and the steps of the aluminum stepwedge were measured using the histogram tool. With a standardized circle of 20x20 pixels, were made three measure in each sample of the experimental sealers and the steps of the aluminum stepwedge, in all five radiographs, and after were obtained the average and the standard deviation. The measures were acquire in pixel intensity and to sealers were transformed in millimeters of aluminum (mmAl), too.In order for the developed cement to comply with the ISO specification, the radiopacity of the material must be equal to or greater than the 3-mm aluminum radiopacity.

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics 20 Software (Armonk, NY, USA). The data were evaluated to check distribution normality and variance homogeneity. Analysis of variance (ANOVA) was used to evaluate the effect experimental variable (extract powder) on flow, film thickness, dimensional stability, degree of conversion and radiopacity. Data from direct contact test was transformed by Log10 and then subjected to ANOVA on ranks test. The level of significance was set for $p<0.05$.

Results

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of essentil oil of *M. piperita* and *T. minuta* were done to confirm the specific chemotype is presented in Table 2. Fifteen compounds was identificated the *T. minuta* essential oil, being 74.38% of D-Carvone compounds. The *M. piperita*, 12 indentificated compounds: Trans-beta Ocimene 30.47% and Cis- Tagetone 18.81%.

Table2. Identification of the total compounds (%) and carotenoids (mg) of the essential oils and vegetal extracts of *M. piperita*, *T. minuta* and *B. Orellana.*

Chemical compounds (%)	***M. pipperita***	***T. minuta***
Anethol	-	1.06
Alpha.-Caryophyllene	-	0.98
Alpha-Humulene	0.66	-
Beta- cis Ocimene	0.61	-
Caryophyllene	-	3.96
Cis-Carveol	-	2.27
Cis- Tagetone	18.81	-
D-Carvone	2.46	74.38
Dihydrocarveol	-	4.61
Dihydrocarvone	-	1.56
D-Limonene	7.41	1.35
Elemol	-	0.73
Elixene	0.57	-
Germacrene B	0.65	1.46
Myrtenal, dihydro	0.49	-

ND	-	1.71
ND	-	0.92
ND	21.71	-
ND	0.61	-
ND	1.62	-
ND	1.79	-
ND	1.19	-
ND	1.14	-
Pulegone	-	0.57
Terpene-4-ol	-	3.36
Trans-beta Ocimene	30.47	-
Trans-Carveol	-	1.08
Trans-Tagetone	7.55	-
3-7 Dimethil 2-3ª4,5,6-hexahydro-1-benzofuran	1.31	-
3-Ethoxy-4-methoxyphenol	0.95	-
Carotenoids*	276.47mg/100g	in β-caroteno
Phenolic compounds*	44.3mg/g	in acid gallic

ND= not identifield; * *Bixa orellana*

In Table 3 the MIC values are expressed against the three microorganisms tested.

Table 3. Determination the MIC of the *B. orellana*, *M. piperita* and *T. minuta*

Natural product	**Microrganisms tested**		
	C. albicans	*E. faecalis*	*S. mutans*
B. orellana (seed)	50mg/mL	6.25mg/mL	3.12mg/mL
M. piperita(leaves)	0.25µl/mL	0.100µl/mL	0.100µl/mL
*T. minut*a (leaves)	6.25µl/mL	6.25µl/mL	12.5µl/mL

In TCD (Figure 1) using only essential oils and extracts, it was observed that there was a statistically significant difference ($p<0.05$) at times of 1h and 24h. Against *E. faecalis*, *M. piperita* and *T. minuta* demonstrated higher values of antimicrobial activity in the 24h time when compared to the positive control. *B. orellana* did not show a

statistically significant difference when compared to the control group. At 1h, only *T. minuta* showed antimicrobial activity with values smaller than the control group. For *C. albicans*, *B. orellana* and *T. minuta* showed total inhibition of the microrganism in the interval of 1h. In the 24h interval, all tested products showed lower values than the control group. The group of *M. piperita* showed no difference in its antimicrobial activity when compared to the time variable. *T. minuta* demonstrated complete inhibition at the two time intervals analyzed in this work against *S. mutans*. *B. orellana* and *M. piperita* were equal to the positive control in the two times. All groups of experimental sealants demonstrated antibacterial activity against *E. faecalis*. *T. minuta* was able to inhibit the total growth of *E. faecalis* in the time of 1h. And demonstrated strong antibacterial activity together with *B. orellana* within 24h. *M. piperita* also demonstrated antibacterial activity against *E. faecalis* in the two times tested, when compared to the positive control group, however, in the 24h period, *M. piperita* and the exercise base showed similar values. The tested materials did not demonstrate antifungal activity against *C. albicans* in the interval of 1h. However, within 24h, *T. minuta* demonstrated antifungal activity. For *S. mutans*, the experimental groups also did not show antibacterial activity in the 1h interval, however, in the 24h interval. *T. minuta* and *B. orellana* demonstrated considerable antibacterial activity when compared to the positive control group.

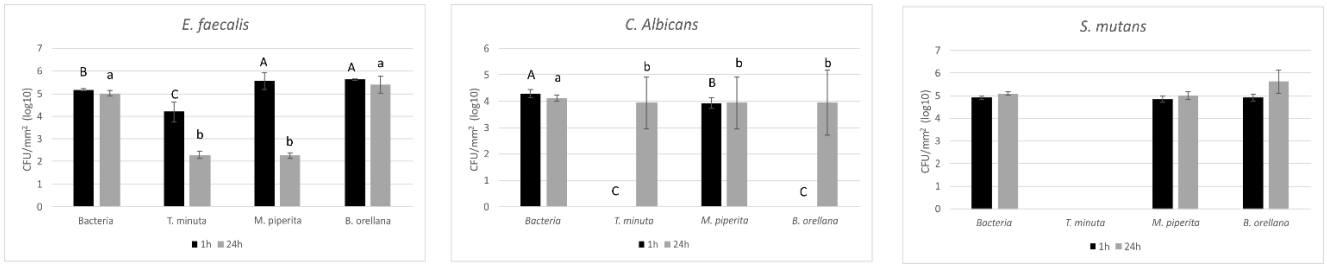

Figure 1. Antimicrobial effects of the essential oils and extract of the plants.

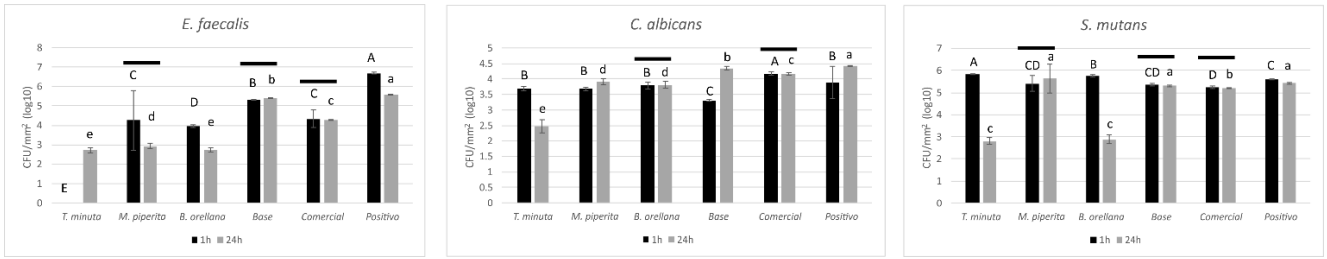

Figure 2. Antimicrobial effects of the test materials.

Table 4 shows flow, film thickness, dimensional stability, setting time, water sorption, solubility and degree of conversion results. Considering the mean values, the

greatest flow was observed for the *T. minuta* group, while the lowest was observed for Real Seal®, however, no statistical differences were detected among the materials. All materials were in accordance with ISO 6876 (lembre-se que é uma referência e deve ser citada como referencia no final do trabalho). Experimental groups had film thickness values higher than the value indicated by the ISO 6876 (50µm). Only Real Seal® and *B. orellana* meet this criterion. Among experimental materials, *T. minuta* and *M. piperita* groups had the highest values of film thickness. The experimental material formulated with *B. orellana* showed the lowest value of film thickness ($p<0.05$). Regarding dimensional stability, all endodontic sealers analyzed presets statistically similar values ($p>0.05$). *T. minuta* group had the higher linear expansion (8.7%), while the Real Seal® group presented the lower linear expansion (5.9%).

Experimental materials with natural extracts presented the longest setting time (48h). As shown in Table 3, Real Seal® showed the shortest final setting time. Degree of conversion results showed that experimental sealer containing *B. orellana* extract showed the highest degree of conversion ($p<0.05$). Conversely, Real Seal® material presented the lowest degree of conversion values ($p<0.05$).

Table 4. Physicochemical and mechanical properties evaluated in the different endodontic sealers.

Group	Flow (mm)	Film thickness (µm)	Dimensional stability (%)	Setting time (h)	Degree of conversion (%)
T. minuta	21.06 (0.18)[a]	100.00 (0.00)[a]	8.71 (3.02)[a]	48	97.83 (0.41)[a]
M. piperita	20.86 (0.23)[a]	83.33 (11.54)[a]	8.38 (3.43)[a]	48	87.89 (0.86)[b]
B. orellana	20.96 (0.23)[a]	106.66 (15.28)[a]	8.00 (2.27)[a]	48	90.08 (0.20)[b]
MES	20.95 (0.11)[a]	83.33 (5.77)[a]	7.44 (1.48)[a]	40	74.53 (0.91)[c]
Real Seal	20.93 (0.00)[a]	36.67 (5.77)[b]	6.00 (2.68)[a]	24	53.84 (2.52)[d]

Common corresponding letters (a–d) in a given column indicate no significant difference($p<0.05$)

Figure 3 shows that there was a statistically significant difference ($p <0.0001$) in the radiopacity of the different experimental materials evaluated, comparing them and also with the aluminum stepwedge. The base material and the material composed of B. orellana reached higher values of radiopacity compared to the materials composed of M. piperita and T. minuta. However, none of the tested materials reached the radiopacity equivalent to 3-mmAl, recommended as minimum for sealer materials, in accordance with ISO 6876.

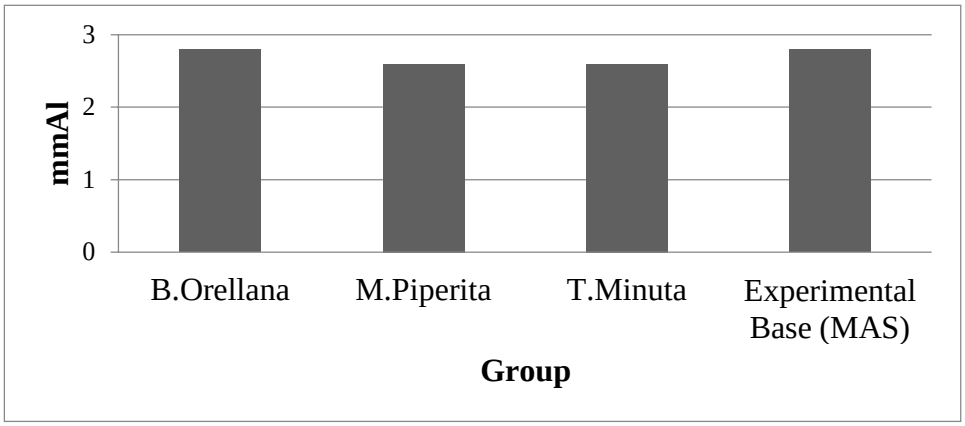

Figure 3. Radiopacity of the experimental materials in mmAl

Discussion

The main objective of this study was to investigate the influence of the addition of natural extracts into the antibacterial, chemical and mechanical properties of experimental endodontic sealers. Some properties these experimental materials were compared with Real Seal cement because all sealers (experimental and commercial) are resin-based materials.

Antimicrobial activity of root filling materials may be advantageous when the pulp canal system is infected. In this study, the antimicrobial activity of root canal sealers with different natural products, demonstrating all materials exerted varying degrees of antibacterial activity, which generally tended to increase with time. Residual microrganisms resisting may cause treatment failure in endodontic therapy. The major chemical component found in the essential oils of *M. piperita* and *T. minuta* were Trans-beta Ocimene and D-Carvone, respectively. These results highlight the differences in concentrations of chemical components that may vary from plant to plant, because it is known that the chemical components of plants vary with plant maturity, variety, geographical region and processing conditions.[7] The method of extracting the seeds of B. *orellana* is a method that guarantees greater withdrawals of carotenoids and phenol groups.[24] The antimicrobial activity of the tested compounds has already been well cited in the literature[12,13,14,19], but lacks a study evaluating the antimicrobial activity of these plant species in a sealer material.

The use of a root canal sealer having good antimicrobial activity is essential for long-term success of endodontic therapy. The most commonly used assays to evaluate antimicrobial activity of sealers and dental materials are the agar diffusion test and the direct contact test. The agar diffusion test is a relatively insensitive and semiquantitative method; the results are depend on the solubility and diffusibility of test agent in the

agar.[31] It is difficult to determine the true antimicrobial activity of water-insoluble materials using the agar diffusion test, because a less soluble and diffusible material could result in a smaller size of inhibition zone.[32] On the other hand, the direct contact test is a quantitative and reproducible assay which relies on direct contact to the test micro-organisms with the test material for a controlled period of time and independent of the diffusion and solubility properties of the material tested and media[23] and *E. faecalis. C. albicans* and *S. mutans* were chosen as the test organism because of its presence in persistent endodontic infections and its use in numerous previous studies examining the effectiveness of disinfecting agents in endodontics.[34, 35]

Endodontic sealers must meet several requirements in order that increased properties of the materials are related to high quality root filling. Physicomechanical tests in this study were performed according to ISO 6876, which ensures reproducibility and allows comparing our experimental materials with others. Flow is one of the most important properties of endodontic sealer materials. This property reflects the capacity of a material to penetrate into small irregularities and ramifications of the root canal system and dentinal tubules[36] and plays an important role in allowing sealer penetration. According to the results, no statistically differences were founded among the materials. In addition, all materials meet what is stablished in ISO 6876. Natural plant extracts addition did not impair this property in the model endodontic sealer which ensure for all materials a correct penetration into confined areas of the root canal system. [37]

Film thickness is a test which provides information about the volume occupied by the endodontic sealer in the root canal system after filling.[38] A thin film thickness is required to ensure a correct wetting of the dental substrate, and thus providing a better seal.[39] According to the results, the addition of *B. orellana*, *M. piperita* or *T. minuta* did not affect the film thickness when compared to the model experimental sealer, however, the values exceed what is stablished in ISO6876 (50µm). Film thickness depends by the composition of the sealer, specially the size of the filler particles.[40] In this study, experimental materials were formulated using a mixture of Aerosil 380 and Ytterbium fluoride. According to manufacturer, Ytterbium fluoride has a mean particle size of 14µm, so it was expected that experimental material had a film thickness around this value. However, it is possible that another material characteristics, as viscosity and particle size distribution, has higher influences on this property.[41]

Dimensional change test was performed to investigate the performance of the endodontic sealers. According to the results, all materials suffered expansion after water

storage which suggest highly hydrophilicity of the materials evaluated. This behavior could be explained due the characteristics of the organic matrix of the materials, which is composed of Bis-EMA 30, Exothane 8, PEG 400 DMA and TEGDMA. With exception of Exothane 8, whose chemical structure is unknown, all monomers used in the formulation have hydrophilic ether linkages which absorbs water within the polymer network.[42] In addition, the expansion of the materials could improve the sealing ability of the materials evaluated.[43]

Higher degree of conversion values for resin-based endodontic sealers is a highly desirable characteristic because the presence of uncured material in the tooth apex could promote an inflammatory reaction and consequently, a failure of the endodontic treatment.[44] All experimental materials presented a higher degree of conversion than Real Seal®. Experimental materials formulated in the present study contains Exothane 8 monomer as part of its organic matrix. This monomer has proved to reach higher degrees of conversion.[45] Molecular formula or weight is not supplied for this monomer, and consequently, limits the understanding of its properties. In addition, higher degree of conversion values results in materials with higher stability,[46] which possibly leads to an increase in the outcome of the endodontic treatment. The addition of natural extract plants did not decrease the degree of conversion values, which proves that any of the components of the plant extract impair the free-radical polymerization of the materials.

It is very important that an endodontic sealer presents radiopacity to identify teeth with and without root canal treatment. According ISO 6876, the endodontic sealers must have radiopacity equal to or grater than the equivalent radiopacity of 3-mmAl. In this study, the experimental base sealer without natural extract and the sealer with *B. orellana* presented the highest radiopacity values, but they do not reach the radiopacty recommended by ISO. The Ytterbium fluoride is the material that promotes radiopacity to the experimental materials tested. Thus, its is possible that the amount of Ytterbium fluoride incorporate to the sealer has been insufficient to give the adequate radiopacity to the experimental sealers. However, we need to be careful with the incorporation of a greater amount of Ytterbium fluoride in the composition of the sealers, because this component interferes in the thickness of the experimental materials.

Conclusion

The present study demonstrated the antimicrobial potential of three different plant species in experimental endodontic sealers. Although the antimicrobial activity of the

tested species decreased when placed in the test material, it was still possible to identify antimicrobial activity among the experimental groups tested. Although more tests are necessary. It can be concluded that this work was an initial step for searches of materials with antimicrobial potential for future use in endodontics.

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7. CONCLUSÃO

Ao finalizar este trabalho, concluímos que os óleos essenciais e extrato vegetais estudados demonstraram obter potencial antimicrobiano contra os microrganismos aqui testados, como comprova a literatura científica.

Além disso, quando incorporados aos cimentos endodônticos experimentais, esse potencial antimicrobiano, apesar de diminuído, continuou presente nos grupos experimentais. Foi observado também que este potencial antimicrobiano parece aumentar conforme exposição ao microrganismo, necessitando assim, de ensaios que englobem tempos de exposição maiores que os intervalos testados na presente dissertação.

Ainda, torna-se necessário estudos *in vitro* para avaliar o potencial citotóxico destas plantas, afim de identificar possíveis concentrações que possam apresentar citotoxicidade ao organismo.

Quanto aos testes físicos-mecânicos, os materiais desenvolvidos neste trabalho obtiveram resultados satisfatórios e de acordo com a ISO 6876 (referente aos materiais seladores do canal radicular), porém, ainda há ajustes que possam ser feitos, na formulação destes seladores, afim que possam estar de acordo com esta norma.

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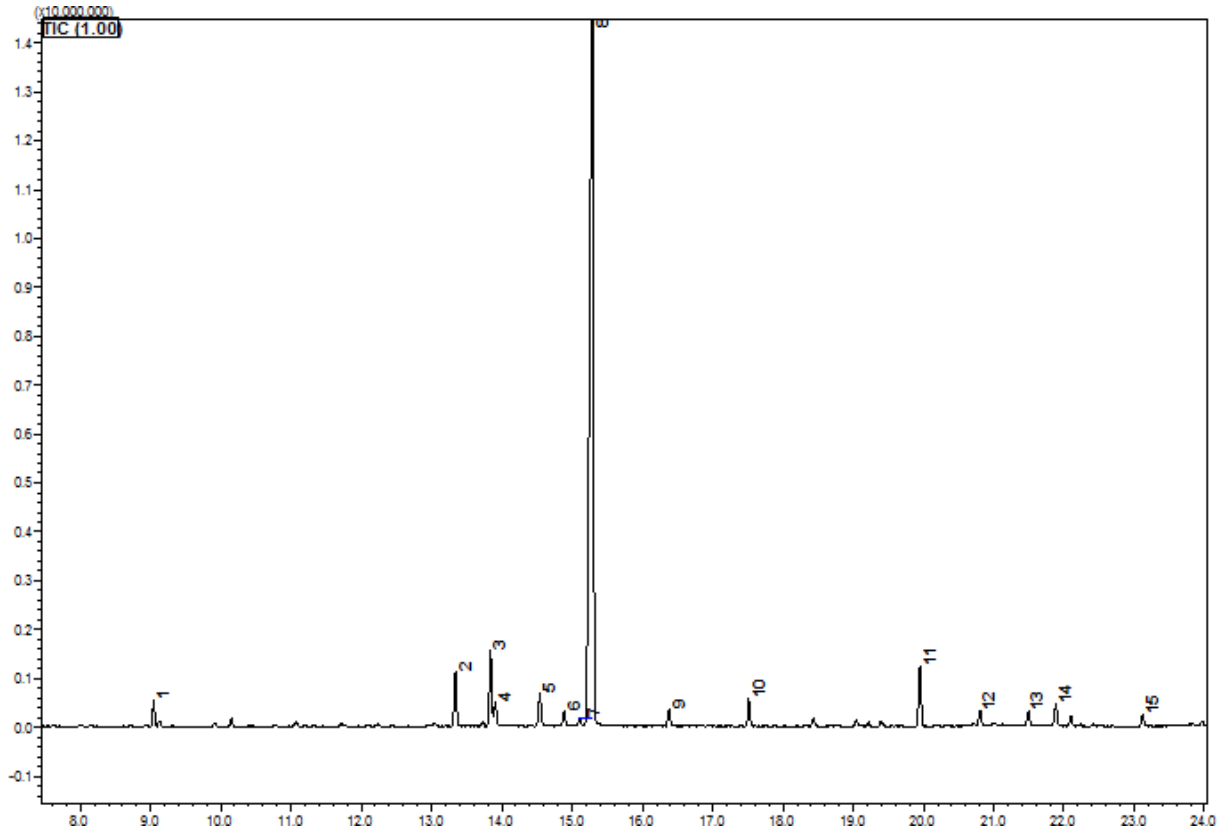
ANEXOS

ANEXO 1 - Análises por Cromatografia Gasosa

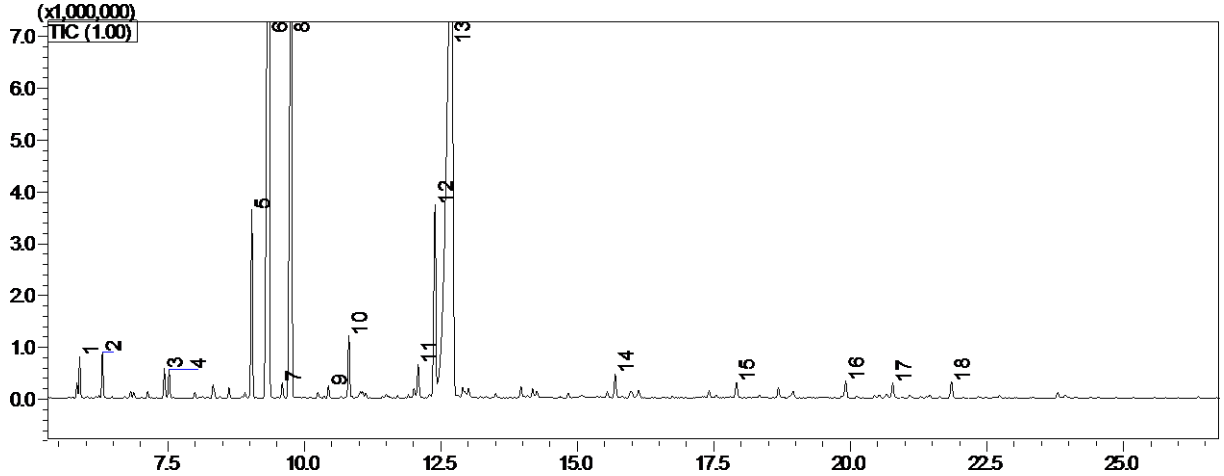
Amostra: Óleo essencial de *T. minuta*



Pico	Tempo de retenção	Área	Altura	Porcentagem	Nome
1	9.048	1039092	521849	1.35	D-Limonene
2	13.338	2577479	1109069	3.36	Terpene-4-ol
3	13.840	3541821	1544467	4.61	Dihydrocarveol
4	13.906	1198877	503609	1.56	Dihydrocarvone
5	14.541	1743533	684103	2.27	cis-Carveol
6	14.887	828957	312145	1.08	trans-Carveol
7	15.108	440708	166035	0.57	Pulegone
8	15.291	57097699	15517593	74.38	D-Carvone
9	16.380	816637	341037	1.06	Anethol
10	17.516	1312122	571595	1.71	ND
11	19.948	3039733	1232679	3.96	Caryophyllene
12	20.804	755849	310298	0.98	alpha.-Caryophyllene
13	21.490	708073	282002	0.92	ND
14	21.884	1119872	457905	1.46	Germacrene B
15	23.118	556695	231979	0.73	Elemol

Análises por Cromatografia Gasosa

Amostra: Óleo essencial de *M. piperita*



Pico	Tempo de retenção	Área	Altura	Porcentagem	Nome
1	5.883	1267089	793419	1.62	ND
2	6.299	1484931	877318	1.79	ND
3	7.433	1125165	582438	1.19	ND
4	7.519	1097156	558346	1.14	ND
5	9.034	7472963	3628542	7.41	D-Limonene
6	9.354	50467724	14927237	30.47	Trans-beta Ocimene
7	9.593	598125	299804	0.61	Beta- cis Ocimene
8	9.763	31806974	10637008	21.71	ND
9	10.439	481860	238922	0.49	Myrtenal, dihydro
10	10.812	2657521	1206248	2.46	Carvenone
11	12.082	1407688	641436	1.31	3-7 Dimethil 2-3ª4,5,6-hexahydro-1-benzofuran
12	12.389	9731147	3701008	7.55	Trans-Tagetone
13	12.684	74346516	9213967	18.81	Cis- Tagetone
14	15.691	1102218	466636	0.95	3-Ethoxy-4-methoxyphenol
15	17.911	774547	296912	0.61	ND
16	19.911	836002	323479	0.66	Alpha-Humulene
17	20.773	719813	281620	0.57	Elixene
18	21.850	868803	320165	0.65	Gemacrene B

ANEXO 2 - NORMAS DOS PERIÓDICOS PARA ENVIO DOS ARTIGOS DA DISSERTAÇÃO

Guide for Authors (*Journal of Ethnopharmacology)*

Use of word processing software

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

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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

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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*, *Aim of the study* , *Materials and Methods*, *Results*, and *Conclusions***.

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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). 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.

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 http://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.).

Formatting of funding sources

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

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

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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, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown '
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Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

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

Reference to a website:
Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13.03.03).

Reference to a dataset:
[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T., 2015. Mortality data for Japanese oakwilt disease and surrounding forest compositions. Mendeley Data, v1. http://dx.doi.org/10.17632/xwj98nb39r.1.

9.2. Guide for Authors (*Asian Pacific Journal of tropical disease)*

Authors

All authors should have participated sufficiently in the work to take public responsibility for the content. Written approval signed by all authors should be presented with the manuscript. The sequence of author's name(s) represent(s) the degree of contribution. Co-authorship should be identified in the manuscripts.

Manuscript

The manuscript should be submitted online through our email, later on through our website, and the text should be double spaced and have wide margins. The manuscript should be arranged in the following order: 1) Title Page, 2) Keywords, 3) Abstract, 4) Corresponding author, 5) Introduction, 6) Materials and Methods, 7) Results, 8) Discussion, 9) Acknowledgments, 10) References, 11) Tables, 12) Figure Legends, and 13) Figures. Please indicate the page of your manuscripts to facilitate reviewing.

Short communications not exceeding two printed pages, including title, author affiliations, references, and one figure or table, are acceptable for rapid publication if requested by the authors.

Criticisms or comments of less than 500 words and five references are welcome. Preference is given to letters related to articles published in the Asian Pacific Journal of Tropical Disease.

Title page

The title page should contain, on separate lines, the title of the manuscript, a running title of no more than 40 letters, the name(s) and affiliation(s) of the author(s), and the mailing address, telephone and fax numbers, and Email address of the corresponding author. The title must be informative, specific, and concise. Serialization of articles into parts is not permitted; such articles may be submitted independently with self-sufficient titles.

Keywords and Abstract

Provide 3-10 key words or phrases for indexing purposes, using terms from the latest US National Library of Medicine's Medical Subject Headings (MeSH) browser list at (http://www.nlm.nih.gov/mesh/meshhome.html). If appropriate MeSH terms are not available, other suitable terms may be used. Full-length submissions should include an abstract of up to 250 words in structured form, consisting of an Objective, Methods, Results, and Conclusion.

Text

The text should include the following sections. The Introduction summarizes the rationale, provides a concise research background (not an exhaustive review) and states in one sentence the objective of the study. Do not include any results or the conclusions of the study. The Materials and Methods provide technical

information about the study. Do not describe methodological details that have been published previously. Specifications(including the manufacturer, city, and the country) should be given for the main drugs, chemicals, and instruments. Indicate the statistical methods used and identify statistical significance using superscripts(a and b) following the data($^aP<0.05$, $^bP<0.01$). The Results are the findings, using SI units. In a sample, the number of effective digits is determined by the variation within the sample, that is, one-third of the standard deviation. Digits may be separated into groups of three by a small space. The Discussion deals with the interpretation of the results and their comparison with those of other studies. Do not repeat the results, do not review the literature, do not repeat textbook knowledge and do not cite references that do not have a close relationship with the present results. End with a brief conclusion linking back to the aim of the study.

Abbreviations

The use of abbreviations, except for units of measure, is discouraged. At the first appearance in the abstract and the text, abbreviations should be preceded by words for which they stand.

Tables

Tables must be concise and cited consecutively using Arabic numerals in the text (Table 1, Table 2, etc.). Each table should be typed on a separate sheet. The title of the table should clearly indicate the nature of the contents and sufficient details should be included in the footnote to facilitate interpretation without reference to the text. Use horizontal rules only.

Figures

Figures (photographs, drawings, diagrams and charts) should be clear, easily legible and cited consecutively using Arabic numerals in the text (Figure 1, Figure 2, etc.). Please supply figures 1.5 to 2 times the size at which they will be finally reproduced. For line work, submit black-ink drawings of professional quality. Micrographs or other glossy photographs must be of the highest quality. If a figure comprises more than one glossy photograph, these should be marked A, B, C, etc. Figure legends should be marked clearly with the correspond letter. Legends should contain sufficient details to permit figure interpretation without reference to the text. Scale markers should be indicated in the photographs. Color plates are also welcome. The choice of cover art illustration will be made by the Editor.

References

The Journal advocates the citation of new papers; old references are better replaced with updated ones. The authors are responsible for the correctness of references. References are numbered consecutively in the order in which they appear in the text. Citation should be labelled in superscript parenthesis and should appear in front of the period or comma at the end of the sentence/clause. All references should be cited.

Unpublished data, personal communications, abstracts at meetings and manuscripts submitted for publication are not acceptable as references. Information from such sources may be cited in the text with the sources given in parentheses. References should be listed in numerical order in the Reference section. Journal titles should be abbreviated according to the list of Journals Indexed in Index Medicus or MEDLINE (www.nlm.nih.gov). Please note that no periods are used after the authors-initials or journal abbreviations. A period is used at the end of each reference. The type and punctuation of references is consistent with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals of the International Committee of Medical Journal Editors (http://www.ICMJE.org). Some examples are as follows:

Journal:
Journal article up to six authors (list all authors)
Prasad A, Kumar R, Ramanan H, Khandige N, Prabhu K. Rhabdomyolysis due to multiple fire ant bites a case report. Asian Pac J Trop Dis 2012; 2(5): 417-18.

Journal article more than six authors (list first six and add et al.)
Doroodgar A, Sayyah M, Doroodgar M, Mahbobi S, Nemetian M, Rafizadeh S, et al. Progressive increasing of cutaneous leishmaniasis in Kashan district, central of Iran. Asian Pac J Trop Dis 2012; 2(4): 260-63.

Book:
Waugh N, Royle P, Craigie I, Ho V, Pandit L, Ewings P. Screening for Cystic Fibrosis-Related Diabetes: A Systematic Review. Southampton (UK): NIHR Evaluation, Trials and Studies Coordinating Centre (UK); 2012, p.15-8.

Chapter in a book:
Gubler DJ. Dengue/Dengue haemorrhagic fever: history and current status, in new treatment strategies for dengue and other flaviviral disease. In: Bock G, Goode J, editors. Novartis foundation symposium 277. Chichester: John Wiley& Sons, Ltd; 2008.

Acknowledgments
Authors should obtain written permission for everyone acknowledged by name, since readers may infer their endorsement of the paper and its conclusions.

9.3. Guide for Authors (*Journal of Endodontics*)

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. Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lowercase 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 the e-mail address is given and that contact details are 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 50 references. There are no limits in the number of references for review articles.