

**UNIVERSIDADE ESTADUAL DO CEARÁ
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS**

RAQUEL OLIVEIRA DOS SANTOS FONTENELLE

**EFEITO ANTIFÚNGICO DE ÓLEOS ESSENCIAIS DE *Lippia sidoides*
Cham., *Croton argyrophyllumoides* Muell., *Croton zenhtneri* Pax et Hoffm., *Croton
nepetaefolius* Baill. E DE SEUS PRINCIPAIS CONSTITUINTES CONTRA
DERMATÓFITOS E *Candida* spp. ISOLADOS DE CÃES**

**FORTALEZA-CE
2008**

Livros Grátis

<http://www.livrosgratis.com.br>

Milhares de livros grátis para download.

RAQUEL OLIVEIRA DOS SANTOS FONTENELLE

**EFEITO ANTIFÚNGICO DE ÓLEOS ESSENCIAIS DE *Lippia sidoides*
Cham., *Croton argyrophyllumoides* Muell., *Croton zenhtneri* Pax et Hoffm., *Croton
nepetaefolius* Baill. E DE SEUS PRINCIPAIS CONSTITUINTES CONTRA
DERMATÓFITOS E *Candida* spp. ISOLADOS DE CÃES**

Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do grau de Doutor em Ciências Veterinárias.

Área de Concentração: Reprodução e Sanidade Animal.

Linha de Pesquisa: Reprodução e sanidade de carnívoros, onívoros, herbívoros e aves.

Orientador: Dra. Selene Maia de Moraes

Co-orientador: Dr. Marcos Fábio Gadelha Rocha

**FORTALEZA-CE
2008**

F677a Fontenelle, Raquel Oliveira dos Santos

Efeito antifúngico de óleos essenciais de *Lippia sidoides* Cham., *Croton argyrophyllloides* Muell., *Croton zenhtneri* Pax et Hoffm., *Croton nepetaefolius* Baill. e de seus principais constituintes contra dermatófitos e *Candida* spp. isolados de cães./ Raquel Oliveira dos Santos Fontenelle. – Fortaleza, 2008.

163 p.; il.

Orientadora: Profa. Dra. Selene Maia de Moraes.

Tese (Doutorado em Ciências Veterinárias) – Universidade Estadual do Ceará, Faculdade de Veterinária.

1. *Croton* 2. *Lippia* 3. Atividade antifúngica. I. Universidade Estadual do Ceará, Faculdade de Veterinária. II. Título.

CDD: 636

**EFEITO ANTIFÚNGICO DE ÓLEOS ESSENCIAIS DE *Lippia sidoides* Cham., *Croton argyrophyilloides* Muell., *Croton zenhtneri* Pax et Hoffm., *Croton nepetaefolius* Baill. E DE SEUS PRINCIPAIS CONSTITUINTES CONTRA DERMATÓFITOS E *Candida* spp.
ISOLADOS DE CÃES**

Raquel Oliveira dos Santos Fontenelle

Tese Aprovada em: 17 de Julho de 2008

Conceito: Satisfatório

Nota: 9,5

Banca Examinadora

Selene Maia de Moraes, Profa. Dra.
Orientadora (UECE)

Marcos Fábio Gadelha Rocha, Prof. Dr.
Co-orientador (UECE)

Nilce Viana G. P. de Sousa Brasil, Profa. Dra.
Examinadora (UFC)

José Júlio Costa Sidrim, Prof. Dr.
Examinador (UFC)

Nilberto R. F. do Nascimento, Prof. Dr.
Examinador (UECE)

A Deus, meu fiel amigo,
dedico.

AGRADECIMENTOS

Agradeço a Deus e a todos aqueles que tanto me ajudaram durante todo o curso.

A meu marido, Joffre Fontenelle Filho, pelo grande incentivo e força. E minha filha, Ester dos Santos Fontenelle, pois seu sorriso me dá ânimo para seguir em frente.

Aos meus pais, Luís Gonzaga Rodrigues dos Santos, Edviegens Moreira Oliveira dos Santos e Maria de Fátima Barbosa Fontenelle, minha segunda mãe, pelo apoio e carinho. E aos meus irmãos, Rute Oliveira dos Santos e Moisés Oliveira dos Santos, pelo companheirismo.

À professora Dra. Selene Maia de Moraes, por sua orientação e dedicação na realização deste trabalho.

Ao professor Dr. Marcos Fábio Gadelha Rocha, pela co-orientação oferecida na execução desta tese, que foi de vital importância para o meu crescimento como pesquisadora.

Ao professor Dr. José Júlio Costa Sidrim e as professoras Doutoras Raimunda Sâmia Nogueira Brilhante e Rossana Aguiar Cordeiro, pelo acolhimento no Centro Especializado em Micologia Médica (CEMM) e pelos conhecimentos transmitidos.

A minha banca examinadora, composta pelas professoras Doutoras Selene Maia de Moraes e Nilce Viana Gramosa Pompeu de Sousa Brasil e pelos professores Doutores

Marcos Fábio Gadelha Rocha, José Júlio Costa Sidrim e Nilberto Robson Falcão do Nascimento.

Aos professores e funcionários do PPGCV, pela paciência e dedicação.

Ao professor José Ferreira Silva Bastos, pela correção gramatical deste trabalho.

As minhas amigas Elaine Vieira de Lima Leitão, Ana Lisalba da Silva Sousa, Benaíza Cristina Albuquerque dos Santos e Carolina Sidrim de Paula Cavalcante, sem as quais minha vida não teria este colorido especial.

A todos os meus amigos e irmãos da Igreja Batista Fonte de Vida, pois sei que sofreram e se alegraram comigo durante todo este percurso.

A todos os alunos dos laboratórios de Química em Produtos Naturais, em especial à aluna Ynayara, que muito me ajudou nos experimentos.

A todos os amigos do Centro Especializado em Micologia Médica, que me ajudaram na realização deste projeto e estiveram ao meu lado durante este período, em especial, à Érika Helena Salles de Brito, por todos os ensinamentos e amizade.

A todos os colegas do mestrado em Ciências Veterinárias do PPGCV, que fizeram parte da minha vida durante o período de mestrado e doutorado.

À FUNCAP, pelo financiamento da bolsa durante o período de dois anos e seis meses.

RESUMO

Produtos naturais à base de plantas medicinais vêm sendo estudados ao longo dos anos e a constatação de sua eficácia tem sido demonstrada por pesquisadores de todo mundo. As micoses constituem um sério problema, especialmente em lugares de clima tropical e subtropical, sendo os dermatófitos e as *Candida* spp os agentes mais freqüentes destas enfermidades. Os objetivos deste trabalho foram avaliar a atividade antifúngica dos óleos essenciais da *Lippia sidoides*, *Croton zehntneri*, *Croton argyrophyllumoides* e *Croton nepetaefolius*, bem com de seus principais constituintes contra cepas de *Microsporum canis*, *Trichophyton mentagrophytes* var. *mentagrophytes* e *Candida* spp.; determinar os constituintes majoritários; sintetizar derivados metilados a partir do eugenol e timol; testar a atividade antifúngica *in vitro* dos constituintes majoritários dos óleos e seus derivados metilados; e testar a atividade antifúngica *in vivo* do óleo essencial da *L. sidoides*. Para tanto, as plantas foram coletadas e identificadas. Posteriormente os óleos essenciais foram obtidos e seus constituintes analisados por cromatografia de gás e espectrometria de massa. As cepas fúngicas foram recuperadas da micoteca do Centro Especializado em Micologia Médica e a viabilidade das mesmas foi avaliada através de testes laboratoriais. A atividade antifúngica foi averiguada pelo método de difusão em ágar e microdiluição em caldo. Os óleos essenciais obtidos do *C. nepetaefolius* e do *C. argyrophyllumoides* demonstraram atividade apenas contra o *M. canis* ($\text{CIM} > 5.000 \mu\text{g ml}^{-1}$ e CIM variando de 9 a $19 \mu\text{g ml}^{-1}$, respectivamente) enquanto que os óleos essenciais do *C. zehntneri* e da *L. sidoides* foram efetivos contra todos os fungos testados, apresentando dose-dependência. O óleo essencial da *L. sidoides* apresentou melhor atividade antifúngica que os óleos essenciais das espécies de *Croton* com CIM variando de 4 a $10 \mu\text{g ml}^{-1}$ para cepas de *M. canis* e CIM variando de 620 a $2.500 \mu\text{g ml}^{-1}$ para cepas de *Candida* spp. Nas maiores concentrações testadas, o óleo essencial da *L. sidoides* inibiu completamente o crescimento dos fungos. A toxicidade aguda de todos os óleos essenciais até 3 g/kg por via oral foi desprovida de qualquer toxicidade. Desta forma, devido à alta eficácia e à baixa toxicidade, os óleos essenciais das espécies de *Croton* e da *L. sidoides* constituem fontes importantes de compostos antifúngicos que podem ser úteis no tratamento de micoses em animais.

ABSTRACT

Natural products from medicinal plants have been studied over the years and many have been found to be effective by researchers throughout the world. Mycoses are a serious problem, especially in tropical and subtropical regions. Dermatophytes and species of *Candida* spp are the most frequent causes of these ailments. The aim of this study was to examine the antifungal activity or the essential oils of *Lippia sidoides*, *Croton zehntneri*, *Croton argyrophyllumoides* and *Croton nepetaefolius*, as well as of their main constituents, against strains of *Microsporum canis*, *Trichophyton mentagrophytes* var. *mentagrophytes* and *Candida* spp.; to determine the majority constituents of these oils; to synthesize methylated derivatives from eugenol and thymol; to test the antifungal activity *in vitro* of the majority constituents of the oils and their methylated derivatives; and to test the antifungal activity *in vivo* of the essential oil of *L. sidoides*. After collecting and identifying the plants, we obtained the essential oils and identified their constituents by gas mass spectrometry. The fungal strains were obtained from the collection of the Specialized Medical Mycology Center and their viability was evaluated by laboratory tests. The antifungal activity was ascertained by diffusion in agar and microdilution in broth. The essential oils of *C. nepetaefolius* and *C. argyrophyllumoides* demonstrated activity only against *M. canis* ($\text{MIC} > 5.000 \mu\text{g ml}^{-1}$ and CIM ranging from $9 - 19 \mu\text{g ml}^{-1}$, respectively), while the essential oils of *C. zehntneri* and *L. sidoides* were effective against all the fungi tested, showing dose dependence. The essential oil of *L. sidoides* was the most potent of the oils of the *Croton* species with MIC ranging from $4 - 10 \mu\text{g ml}^{-1}$ for *M. canis* and MIC ranging from $620 - 2.500 \mu\text{g ml}^{-1}$ for *Candida* spp. At the highest concentrations tested, the essential oil of *L. sidoides* completely inhibited the growth of the fungi. There was no acute toxicity of any of the essential oils up to 3 g/Kg administered orally. Therefore, due to the high efficacy and low toxicity, the essential oils of the *Croton* and *L. sidoides* species can be important sources of antifungal compounds that can be useful in treating mycoses in animals.

LISTA DE FIGURAS

Figura 1: Fotografia de arbustos de <i>Croton nepetaefolius</i> Baill	11
Figura 2: Fotografia de folhas de <i>Croton nepetaefolius</i> Baill	12
Figura 3: Fotografia de arbustos de <i>Croton argyrophyloides</i> Muell Arg	13
Figura 4: Fotografia de folhas de <i>Croton argyrophyloides</i> Muell Arg	13
Figura 5: Fotografia de arbustos de <i>Croton zenhtneri</i> Pax et Hoffm	16
Figura 6: Fotografia de folhas de <i>Croton zenhtneri</i> Pax et Hoffm	16
Figura 7: Fotografia de arbustos de <i>Lippia sidoides</i> Cham	19
Figura 8: Fotografia de folhas de <i>Lippia sidoides</i> Cham	19
Figura 9: Fotografia de colônia de <i>Microsporum canis</i>	30
Figura 10: Colônia de <i>T. mentagrophytes</i> var. <i>mentagrophytes</i>	31
Figura 11: Colônias de <i>Candida</i> spp	33
Figura 12: Blastoconídios de levedura	33

LISTA DE ABREVIATURAS

CFM	Concentração fungicida mínima
CIM	Concentração inibitória mínima
CLSI	<i>Clinical Laboratory Standards Institute</i>
DL ₅₀	Dose letal média
DMSO	Dimetil-sulfóxido
K.I.	Índice de retenção na coluna do cromatógrafo
IT	Inibição total
NCCLS	<i>National Committee for Clinical Laboratory Standards</i>
OELs	Óleo Essencial da <i>Lippia sidoides</i>
OECn	Óleo Essencial do <i>Croton nepetaefolius</i>
OECa	Óleo Essencial do <i>Croton argyrophyllumoides</i>
OECz	Óleo Essencial do <i>Croton zehntneri</i>
OE	Óleo Essencial
RPMI	Solução nutritiva desenvolvida no Instituto <i>Roswell Park Memorial</i>
TW	Tween
UFC/mL	Unidades Formadoras de Colônia por mililitro
VCM	Volume corpuscular médio

SUMÁRIO

1 INTRODUÇÃO	2
2 REVISÃO BIBLIOGRÁFICA	4
2.1 Plantas medicinais: generalidades	4
2.1.1 Importância das plantas medicinais	4
2.1.2 Óleo essencial	7
2.2 Plantas medicinais estudadas nesta tese	9
2.2.1 <i>Croton</i> spp.	9
2.2.1.1 <i>Croton nepetaefolius</i> Baill.	10
2.2.1.2 <i>Croton argyrophyllumoides</i> Muell Arg.	12
2.2.1.3 <i>Croton zenhtneri</i> Pax et Hoffm.	14
2.2.2 <i>Lippia</i> spp.	17
2.2.2.1 <i>Lippia sidoides</i> Cham.	17
2.3 Principais constituintes das plantas estudadas	20
2.3.1 Anetol e estragol	20
2.3.2 Timol	21
2.3.3 Eugenol	22
2.3.4 Metil-eugenol	23
2.4 Estudos toxicológicos utilizados no screening de plantas medicinais: um enfoque para os métodos empregados na pesquisa com <i>Croton</i> spp. e <i>Lippia sidoides</i>	24
2.5 Fungos: abordagem geral sobre dermatófito e leveduras	25
2.5.1 Dermatófitos	28

2.5.2 Gênero <i>Candida</i>	31
2.6 Métodos de estudo da atividade antifúngica <i>in vitro</i>	33
2.7 Métodos de estudo da atividade antifúngica <i>in vivo</i>	35
3 JUSTIFICATIVA	38
4 HIPÓTESE	39
5 OBJETIVOS	40
5.1 Objetivo geral	40
5.2 Objetivos específicos	40
6 Capítulo I	41
Chemical composition, toxicological aspects and antifungal activity of essential oil from <i>Lippia sidoides</i> Cham	41
7 Capítulo II	61
Antifungal activity of essential oils of <i>Croton</i> species from the Brazilian <i>Caatinga</i> biome	61
8 Capítulo III	87
Antifungal Activity of Alquifenois against <i>Candida</i> spp. and <i>M. canis</i>	87
9 Capítulo IV	105
Experimental dermatophytosis in the pavilhão auditivo of Guinea Pigs: a model that mimetiza the natural infection	105
10 Capítulo V	118
Antifungal effect of essential oil of <i>Lippia sidoides</i> against <i>Trychophyton</i> <i>mentagrophytes</i> in a Guinea Pigs model	118
11 CONCLUSÕES GERAIS	130

12 PERSPECTIVAS 131

13 REFERÊNCIAS GERAIS 132

1 INTRODUÇÃO

A fitoterapia pode ser historicamente definida como a ciência que trata dos problemas de saúde utilizando os vegetais (fitocomplexos), sendo contemporânea ao início da civilização. As plantas são tradicionalmente usadas por populações de todos os continentes no controle de doenças e pragas desde a antigüidade até os dias atuais.

No Brasil, o comércio de ervas medicinais começou com os índios e, atualmente, em qualquer cidade, é possível comprar plantas, pós e ungüentos em mercados e também nas ruas. Essa alternativa é utilizada tanto dentro de um contexto cultural, na medicina popular, quanto na forma de fitoterápicos, pelo fato de essas plantas serem fontes importantes de produtos naturais biologicamente ativos, muitos dos quais constituem modelos para a síntese de um grande número de fármacos, revelando nestes produtos alta diversidade em termos de estrutura e de propriedades físico-química e biológica.

Os óleos essenciais obtidos de diversas plantas têm ganhado popularidade e despertado interesse de pesquisadores de todo o mundo. Na medicina tradicional, muitos óleos essenciais têm apresentado atividade contra fungos patógenos, por essa razão, pesquisas nesta área que comprovem esta atividade de acordo com protocolos científicos modernos e adequados tornam-se cada vez mais necessárias.

Os fungos são microrganismos que constituem um grupo diversificado e abundante na natureza, fazendo parte de vários nichos no ambiente, incluindo a microbiota de homens e animais. São caracterizados por estruturas unicelulares ou multicelulares e classificados de acordo com sua morfologia em filamentosos, leveduras e dimórficos. Na clínica veterinária de pequenos animais, os fungos patogênicos mais freqüentemente isolados são os filamentosos, especialmente os dermatófitos, seguidos das leveduras. As doenças infecciosas causadas por fungos constituem um problema crítico para a saúde pública, especialmente em regiões tropicais e subtropicais em desenvolvimento

Nos últimos anos, tem ocorrido um aumento de pesquisas por novos compostos com atividade antifúngica. Isso tem acontecido, principalmente, pelos vários efeitos colaterais ligados à terapia e pela resistência de cepas fúngicas ao limitado arsenal de fármacos utilizados para o tratamento destas infecções.

Na região da Caatinga, no Nordeste brasileiro, diversas espécies de *Crotons* são utilizadas pela medicina tradicional. Algumas atividades farmacológicas destas plantas têm sido comprovadas, tais como: atividade antimicrobiana do *C. nepetaefolius* e *C. zenhtneri*. Atividade larvicida contra *Aedes aegypti* nos óleos essenciais do *C. zenhtneri*, *C. nepetaefolius*, *C. argyrophyllloides* e *C. sonderianus*. *Lippia sidoides*, planta que também é encontrada no Nordeste brasileiro, é utilizada como antiséptico tópico natural e estudos anteriores têm registrado atividade antimicrobiana, *in vitro*, bem como efeito larvicida contra *Aedes aegyptii*.

No entanto, para uma investigação eficiente da atividade de plantas e seus principais compostos contra espécies de dermatófitos e leveduras, faz-se necessária a realização de testes de sensibilidade *in vitro*, correlacionando com resultados *in vivo*. Nesta tese foi realizada análise da atividade antifúngica dos óleos essenciais do *Croton zenhtneri*, *Croton nepetaefolius*, *Croton argyrophyllloides* e da *Lippia sidoides* contra os dermatófitos *Microsporum canis* e *Trychophyton mentagrophytes*, e as leveduras *Candida albicans* e *Candida tropicalis*, oriundas de animais.

2 REVISÃO BIBLIOGRÁFICA

2.1 Plantas medicinais

2.1.1 Importância das plantas medicinais

O uso de produtos naturais com propriedades terapêuticas é tão antigo quanto a civilização humana e, por um longo tempo, minerais, plantas e produtos animais foram as principais fontes de drogas (Bajpai et al., 2008). O documento médico mais antigo conhecido é sumeriano e data de 4.000 anos atrás. Este documento menciona remédios a base de plantas utilizados no tratamento de diversas doenças (Morais & Braz-Filho, 2007). O papiro de Ebers, espécie de farmacopéia faraônica, escrita em torno em 1.550 a.C., já se referia aos medicamentos de origem vegetal e mencionava cerca de 700 remédios, entre eles, o bulbo da cila, o óleo de rícino e a genciana (Gurgel et al., 2005). Os egípcios já faziam uso medicinal dos vegetais, e algumas das espécies que eles utilizavam continuam sendo empregadas até os dias de hoje, tais como: *Papaver somniferum* (papoula), *Scilla maritima* (scila), *Aloe vera* (babosa) e *Ricinus communis* (óleo de rícino) (Lavabre, 1993; Motsei et al., 2003; Yayli et al., 2005; Magwa et al., 2006; Morais & Braz-Filho, 2007; Abdelgaleil et al., 2008).

As plantas medicinais são um importante elemento da medicina indígena. Na Amazônia, por exemplo, os índios usam, pelo menos, 1.300 espécies de plantas. No Sudeste da Ásia, os curadores tradicionais utilizam 6.500 plantas diferentes para tratar malária, úlceras de estômago, sífilis e outras doenças (Morais & Braz-Filho, 2007).

Das drogas prescritas mundialmente, cerca de 25% provêm de plantas, 121 dos compostos ativos são de uso corriqueiro. Das 252 drogas consideradas como básicas e essenciais pela Organização Mundial de Saúde, 11% são exclusivamente originárias de plantas, como, por exemplo: digoxina de *Digitalis* spp, vincristina de *Catharanthus roseus*, entre outras (Rates, 2001). Para enfatizar o benefício de certas plantas com propriedades medicinais, basta recordar

que o princípio ativo do comprimido símbolo da alopatia, a aspirina, ácido acetil-salicílico (AAS), foi sintetizada por Gilm, em 1859, baseado na salicina, substância ativa de *Salix alba* L. (Calixto et al., 2000; Morais & Braz-Filho, 2007).

A automedicação “milagrosa” com plantas medicinais, a qual chegou ao extremo de substituir terapias tradicionais em doenças graves, é prática bastante utilizada tanto em populações de baixa renda, como único lenitivo para seus males, bem como nas camadas mais privilegiadas que gozam de facilidade sanitária e pronto atendimento médico, somente por puro modismo ou sob influência de grande exploração comercial. Assim, é necessária extrema atenção ao uso não controlado das plantas medicinais e seus devidos estudos de toxicidade para garantir o uso sem riscos na espécie humana (Dikbas et al., 2008).

A grande biodiversidade de espécies vegetais presentes no Brasil constitui uma de suas maiores riquezas e se destaca como fonte para obtenção de novas substâncias com finalidade terapêutica (Kordali et al., 2008). A utilização de plantas no tratamento de diversas enfermidades, infecciosas ou não, é, na prática, bastante utilizada (Maciel & Viana, 2005), visto que o Brasil possui sérios problemas de saúde pública, principalmente na região Nordeste, em razão do baixo poder aquisitivo de grande parte da população local. O alto custo dos remédios torna-os inacessíveis até mesmo nos mais simples casos, como dores agudas e/ou crônicas, febres e gripes. Portanto, o uso popular das plantas medicinais foi o primeiro passo para o desenvolvimento de novos agentes terapêuticos de baixo custo para uma região tão carente de recursos financeiros e, por outro lado, tão rica em flora, quanto reúne milhares de espécies vegetais distintas (Rabelo, 2003).

Apesar do aumento dos estudos sobre plantas medicinais, somente de 15% a 17% foram estudadas quanto ao seu potencial medicinal. Considerando a grande biodiversidade do Nordeste brasileiro, esse número poderia ser bem maior (Almeida et al., 2006). No Ceará, inúmeras pesquisas vêm sendo desenvolvidas com o objetivo de comprovação científica de produtos naturais extraídos de plantas utilizadas pela medicina tradicional, dentre os quais citam-se os trabalhos de Oliveira (2001), Lahliou et al. (2000), Bertini et al. (2005), Morais et al. (2006), Almeida et al. (2006) e Botelho et al. (2007).

É válido ressaltar, contudo, que a planta dita medicinal, muitas vezes, chega às mãos do usuário despojada de qualquer informação fidedigna, visto que há desconhecimento total da época da coleta, do especialista (se foi um especialista) que a colheu, dos cuidados tomados durante os processos de armazenamento, secagem e moagem, da ausência de contaminação por fungos e outros microrganismos, se é tóxica ou não. Tudo isso torna impossível a previsão de eventuais efeitos colaterais que a planta possa provocar, porém, nada haveria a objetar ao receituário de plantas integrais ou de seus extratos brutos, desde que se tratasse de produtos obtidos sob rigoroso controle (Almeida et al., 2006).

As principais causas do aumento na procura de produtos alternativos são: a medicina convencional pode ser ineficiente, abusiva e/ou, pelo uso incorreto de drogas sintéticas com efeitos secundários, uma grande parte da população mundial não tem acesso ao tratamento farmacológico convencional (Rates, 2001; Kotan et al., 2008). Diante destes riscos, as plantas apresentam-se como uma alternativa natural, visto que possuem, contra o ataque de patógenos, mecanismos de defesa baseados na produção de compostos específicos que lhes conferem resistência (Dikbas et al., 2008; Martos et al., 2008).

2.1.2 Óleos Essenciais

Os óleos essenciais (OE) são produtos de composição complexa extraídos de plantas por vários processos, sendo o mais utilizado a destilação por arraste em vapor d'água (Craveiro et al., 1976; Bruneton, 1995). As plantas têm um conteúdo de óleo essencial em torno de 0,1% a 0,5% e, raramente, de 1% a 5% do peso verde (Bruneton, 1995).

De forma geral, os óleos essenciais são misturas complexas de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas. Também podem ser chamados de óleos voláteis, óleos etéreos ou essências. Essas denominações derivam de algumas de suas características físico-químicas, como, por exemplo, a de serem geralmente líquidos de aparência oleosa a temperatura ambiente, advindo daí a designação de óleo. Sua principal característica, entretanto, é a volatilidade, diferindo dos óleos fixos, misturas de substâncias lipídicas, obtidos geralmente

de sementes. Outra característica importante é o aroma agradável e intenso, sendo, por isso, chamados de essências (Simões & Spitzer, 1999; Giordani et al., 2008).

Embora sua maior utilização ocorra nas áreas de alimentos (condimentos aromatizantes de alimentos e bebidas), cosméticos (perfumes e produtos de higiene) e também em farmácias, drogas vegetais ricas em óleos essenciais são empregadas *in natura* para a preparação de infusões e/ou sob a forma de outras preparações simples (Deba et al., 2008; Dikbas et al., 2008).

Vários estudos têm comprovado o efeito de compostos isolados, extraídos de óleos essenciais de plantas, que atuam como fungicidas naturais, inibindo a atividade fúngica, dentre os quais, um número significativo destes constituintes se mostrou eficaz (Abdelgaleil et al., 2008; Chang et al., 2008; Kordali et al., 2008). Os constituintes químicos desses óleos aromáticos variam desde hidrocarbonetos terpênicos, álcoois simples, fenóis, aldeídos, éteres, ácidos orgânicos, ésteres, cetonas, lactonas, cumarinas, até compostos contendo nitrogênio e enxofre. Podem ser utilizados para a síntese de vitaminas, hormônios, antibióticos e anti-sépticos (Simões et al., 2004). A composição do óleo essencial de uma planta é determinada geneticamente, sendo geralmente específica para um determinado órgão e características para o seu estágio de desenvolvimento (Morais & Braz-Filho, 2007), entretanto, fatores ambientais, tais como temperatura, umidade relativa, duração total de exposição ao sol, regimes de ventos, grau de hidratação do terreno e presença de micronutrientes neste terreno, podem influenciar a composição dos óleos essenciais. A investigação da composição química de óleos essenciais foi iniciada no século dezenove e levou à descoberta de alguns hidrocarbonetos isoméricos de fórmula C₁₀H₁₆, os quais foram chamados terpenos (Morais & Braz-Filho, 2007).

2.2 Plantas medicinais de interesse nesta pesquisa

2.2.1 *Croton* spp.

O gênero *Croton*, cujo nome significa “carrapato”, é o segundo maior gênero da família das Euforbiáceas, pertencendo à subfamília Crotonoideae e tribo Crotoneae. É encontrado principalmente como árvore de pequeno porte e arbusto. Plantas da família das Euforbiáceas têm sido usadas, ao redor do mundo, para tratar muitas doenças (Magalhães et al., 2004). Esta família possui cerca de 300 gêneros e 5000 espécies de árvores, arbustos e ervas conhecidas. Os 300 gêneros de Euforbiaceas estão agrupados em 52 tribos e 5 famílias, com diversas destas tribos divididas em subtribos. A distribuição geográfica dos gêneros das Euforbiaceas concentrava-se, primitivamente, na África e Madagascar, mas algumas subfamílias se dispersaram em centros neotropicais (Webster, 1994).

O gênero *Croton* é um dos maiores da família das Euforbiaceas, com cerca de 800 espécies distribuídas nas regiões tropicais e subtropicais. É notavelmente bem representado na América do Sul, e no Brasil estão registradas cerca de 300 espécies (Amaral, 2004). Espécies do gênero *Croton* usualmente são monóicas, as inflorescências apresentam flores femininas na base e masculinas no ápice, caracterizam-se ainda por possuírem flores masculinas com filamentos flexionados no botão e as flores femininas com pétalas reduzidas ou ausentes (Suarez, 2003).

Amplamente distribuído na flora do Nordeste brasileiro, principalmente nas caatingas, o gênero *Croton* é muito importante pela sua utilidade ao indivíduo que vive afastado dos grandes centros populacionais para o tratamento de diversas doenças, incluindo micoses (Souza et al., 2003; Duarte et al., 2005; Cruz et al., 2007). As espécies de *Croton* podem ser agrupadas, de acordo com as denominações populares, em canelas silvestres, marmeleiros, velames, dentre outras (Fernandes et al., 1971).

Diversas espécies de *Croton* vêm sendo estudadas e os resultados destas pesquisas têm trazido grandes contribuições para o campo científico. Silva et al (2001) identificaram a presença de princípios tóxicos e atividade leishimanicida, antinociceptiva, antiulcerogênica, hipoglicêmica e hipolipidêmica do *C. cajucara*. Guerrero (2002), relatam a presença da atividade anti-hipertensiva e vasorelaxante do *C. schiedeanus*. Nardi (2003), averiguaram atividade antiinflamatória e antioxidante do *C. celtidifolius*.

Algumas espécies de *Croton* sp. possuem atividade antimicrobiana relatada na literatura, dentre as quais podem ser citadas o *C. antisyphiliticus* e o *C. perdicipes*, por seu uso popular em feridas e úlceras, e o *C. urucurana*, que é usado como antiséptico (Gurgel et al., 2005; Fenner, 2006). Neste trabalho analisou-se a atividade antifúngica de três espécies do gênero *Croton*: *C. nepetaefolius*, *C. argyrophylloides* e *C. zenhteneri*.

2.2.1.1 *Croton nepetaefolius* Baill

O *Croton nepetaefolius* Baill., popularmente conhecido como marmeiro vermelho ou marmeiro sabiá, é uma árvore de pequeno porte, muito abundante na região Nordeste do Brasil, inclusive no Ceará (Canuto, 2005). Pode ser encontrado nos cerrados, matas litorânicas e principalmente na caatinga (Magalhães et al., 2004).

Possui folhas com limbo triangular (Figuras 1 e 2), palmatinérvico, oval, agudo, de base cordato-subtruncado ou levemente cordado, providas de glândulas estipitadas e de margens duplamente serradas; na página superior são vilosopubescente, tornando-se depois hirtopubescente. As flores são pequenas, reunidas em cachos e os frutos são cápsulas tricocas (Magalhães et al., 2004).

A espécie produz um óleo essencial com aroma agradável e característico, que apresenta os seguintes constituintes químicos: α -pineno, canfeno, β -pineno, 1,8-cineol, cânfora, α -cubebeno, α -copaeno, α -cubebeno, α -elemeno, metil-eugenol, *E*-cariofileno, α -Z-bergamoteno,

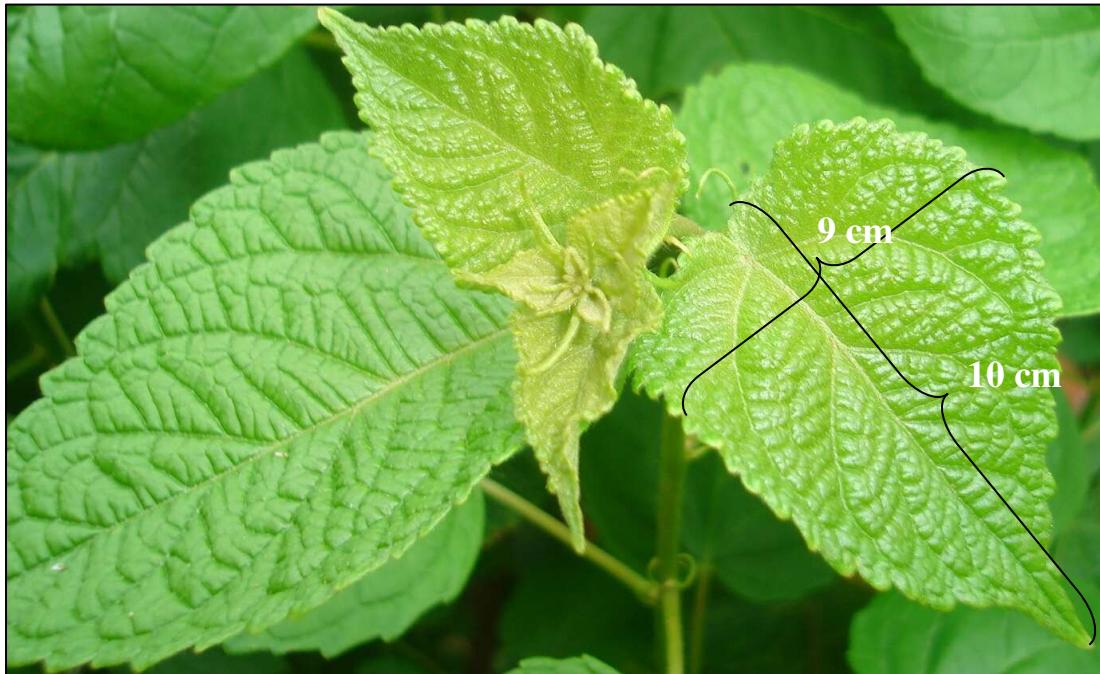
α -guaieno, α -humuleno, biciclogermacreno, α -cadineno e o óxido de cariofileno. O teor desses constituintes pode variar dependendo do horário em que a planta foi coletada (Morais et al., 2006).

Esta planta é popularmente usada para aliviar distúrbios gastrintestinais, na forma de chás e infusões. Seu óleo essencial tem o efeito antiespasmódico comprovado sobre a musculatura lisa intestinal (Coelho de Souza et al., 1997; Morais et al., 2006). Também exerce efeito anti-hipertensivo, que é mais potente em animais com hipertensão genética ou experimental, o qual se deve a uma ação direta, que é o relaxamento miogênico do músculo liso vascular, e a um efeito indireto, via atuação no sistema nervoso autônomo (Catunda Jr., 2003). Ademais, o óleo essencial tem atividade antimicrobiana e antiinflamatória, também relatada, sugerindo que o mesmo tenha também atuação sobre as funções imunológicas do organismo. Essa hipótese é reforçada por dados da literatura, que mostram que o metil-eugenol, um importante constituinte deste óleo, tem propriedade imunomoduladora e antiinflamatória (Franchomme & Penoel, 1995; Catunda Jr., 2003; Magalhães et al., 2004).



(Fonte: Fontenelle, 2008)

Figura 1: Fotografia de arbustos de *Croton nepetaefolius* Baill



(Fonte: Fontenelle, 2008)

Figura 2: Fotografia de folhas de *Croton nepetaefolius* Baill

2.2.1.2 *Croton argyrophyllumoides* Muell Arg.

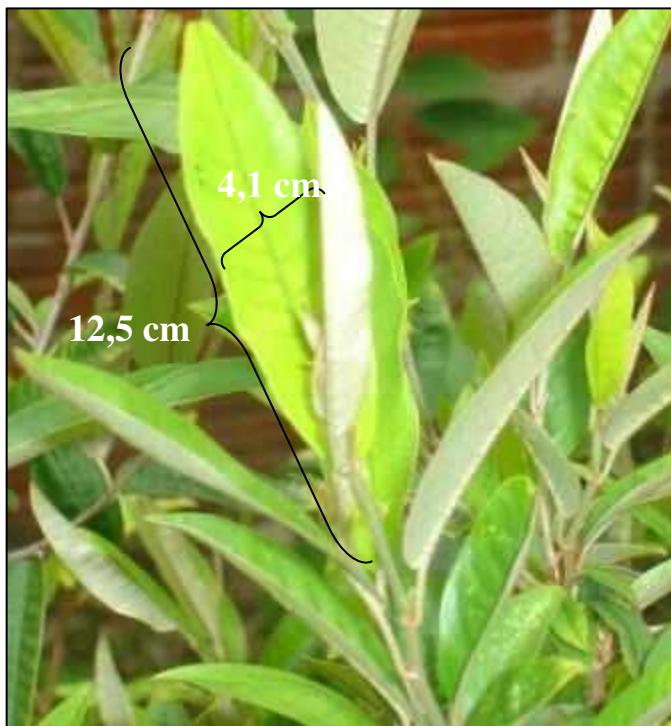
O *Croton argyrophyllumoides* Muell. Arg., conhecido como marmeleiro prateado, é um arbusto de ramos delgados, cilíndricos, cinerescentes e escabros. É nativo da caatinga do Nordeste do Brasil (Catunda Jr., 2003). De acordo com Morais et al. (2006), esta espécie não é usada popularmente por causa do aspecto não atrativo de suas folhas. Alguns trabalhos recentes mostram resultados importantes sobre a atividade antioxidante e antibacteriana do seu óleo essencial contra *Streptococcus* spp., *Serratia* spp., *Staphylococcus* spp., *Proteus mirabilis*, *Enterobacter* spp. e *Pseudomonas aeruginosa* (Canuto, 2005).

O fracionamento dos extratos hexânicos e alcoólicos do lenho do caule e do extrato hexânico da casca do caule do *C. argyrophyllumoides* levaram ao isolamento de um ácido carboxílico diterpênico e seu éter metílico. O estudo químico do seu óleo essencial mostrou a presença de α -pineno e β -guaieno (Craveiro, 1976; Catunda Jr., 2003).



(Fonte: Fontenelle, 2008)

Figura 3: Fotografia de arbustos de *Croton argyrophyllloides* Muell. Arg.



(Fonte: Fontenelle, 2008)

Figura 4: Fotografia de folhas de *Croton argyrophyllloides* Muell. Arg.

2.2.1.3 *Croton zehntneri* Pax et Hoffm

O *Croton zehntneri* Pax et Hoffm., conhecido popularmente como canela de cunhã, é uma planta subarbustiva e caducifólia do Nordeste brasileiro (Morais et al., 2006), de ocorrência nos cerrados, matas de tabuleiro litorâneos, matas pluviais e principalmente nas caatingas (Oliveira, 2005). Suas folhas e talos são dotados de um aroma que lembra uma mistura de erva-doce e cravo-da-índia (Morais et al., 2006).

Apresenta-se como um arbusto delicado, perene, muito aromático e com ramos que são revestidos com pêlos estrelados. As inflorescências mostram-se em racemos contínuos, densifloras de três a oito centímetros de comprimento. O fruto mostra cápsula subglobosa, trilobulada, medindo de quatro a cinco centímetros de comprimento (Oliveira, 2005). As folhas e os ramos contêm com relativa abundância (1,5 a 3% do peso verde) um óleo essencial de aroma agradável e sabor adocicado (Barreto, 2005), o qual é usado em perfumes e como adoçantes em comidas e bebidas (Siqueira et al., 2006).

De acordo com Morais et al. (2006), em experimentos realizados para determinar a concentração dos constituintes químicos mais abundantes do óleo essencial, esse se mostra variável entre exemplares desta planta coletados em diferentes regiões do Nordeste. Assim, distinguem-se, para esta espécie, quatro tipos de óleos essenciais de acordo com seus constituintes químicos majoritários, estes são:

Tipo I. Estragol: exemplares coletados em Tianguá (CE) e Granja (CE);

Tipo II. Anetol: exemplares coletados em Fortaleza (CE) e Viçosa (CE);

Tipo III. Eugenol: exemplares coletados em Areia Branca (RN) e Quixadá (CE);

Tipo IV. Metil-eugenol: exemplares coletados em Ipu (CE) e Oeiras (PI).

A medicina popular emprega esta planta para diversos fins, sendo alguns desses já validados farmacologicamente. Por exemplo: o tipo químico anetol é antidiáspéptico e estomáquico; o tipo metil-eugenol é usado popularmente, em forma de chás e infusões, contra a

insônia e como sedativo, embora seu uso ainda não esteja validado cientificamente, mesmo estando comprovado que estas propriedades podem ser atribuídas ao metil-eugenol; o tipo eugenol é utilizado como anti-séptico (Craveiro et al., 1976).

Estudos das propriedades farmacológicas do óleo essencial de *C. zehntneri* mostram que este tem diversas atividades biológicas em tecidos, órgãos e sistemas animais. Administrado por via oral o óleo mostrou grande atividade antiinflamatória em doses iguais e superiores a DL₅₀ calculada até 2,5 g/kg (Oliveira, 2005). É agente depressor do sistema nervoso central de roedores, antiespasmódico em músculo liso intestinal (Oliveira, 2005) e possui efeito antinociceptivo (Barreto, 2005), antiedemogênico (Canuto, 2005) e antimicrobiana (Lopes Júnior, 2005). O óleo essencial do *C. zehntneri* interfere com os efeitos neuro-moduladores das prostaglandinas e em músculo detrusor, induz contrações tônicas não mantidas, aumentando a amplitude e a freqüência de suas movimentações espontâneas (Oliveira, 2005).



(Fonte: Fontenelle, 2008)

Figura 5: Fotografia de arbustos de *Croton zehntneri* Pax et Hoffm



(Fonte: Fontenelle, 2008)

Figura 6: Fotografia de folhas de *Croton zehntneri* Pax et Hoffm

2.2.2 *Lippia* spp

O gênero *Lippia* pertence à família Verbenaceae, a qual é distribuída nas regiões tropicais e subtropicais de todo o mundo. No Brasil existem cerca de 120 espécies dessa família (Mendonça, 1997) e são caracterizadas como ervas eretas, procumbentes ou receptantes, até subarbustos e arbustos perenes e anuais. As folhas apresentam sua corola com colorido que varia de branco até rubescente, são pequenas e até mesmo diminutas, reunidas em inflorescências de diversos tipos, predominando as racemosas (Medonça, 1997).

As diversas espécies do gênero *Lippia* apresentam-se, quase sempre, aromáticas e ornamentais. Dados de estudos biológicos mostram, para muitas espécies, atividades sobre o SNC, além de propriedades antivirais, analgésicas, entre outras (Soares, 2001).

2.2.2.1 *Lippia sidoides* Cham

A *Lippia sidoides*, conhecida popularmente como alecrim-pimenta, é um arbusto de folhas caducas, bastante aromático e próprio da vegetação nordestina, pertencente à família Verbenaceae. Esta espécie de planta se destaca pelos elevados rendimentos de seu óleo essencial, de até 6%, (Mendonça, 1997), sendo este rico em timol (43,5%), que é o responsável pelo alto poder antiséptico de suas folhas. Outros constituintes de seu óleo são: α-felandreno (22,4%), β-cariofileno (9,7%), α-cimeno (8,6%), mirceno (6,5%) e carvacrol (4,3%) (Sousa et al., 2004).

Além de ser considerada um poderoso anti-séptico e germicida natural, devido ao timol presente, esta planta apresenta um odor mais aprazível que aquelas ricas em ácido carbólico ou fenol comum (Mendonça, 1997). Possui inúmeras aplicações na medicina popular, sendo muitas delas comprovadas cientificamente. É usada para combater infecções da garganta e da boca, para o tratamento de ferimentos na pele e no couro cabeludo, para o tratamento de acne, sarna infectada, pitiríase versicolor, dermatomicoses, caspa, mau cheiro

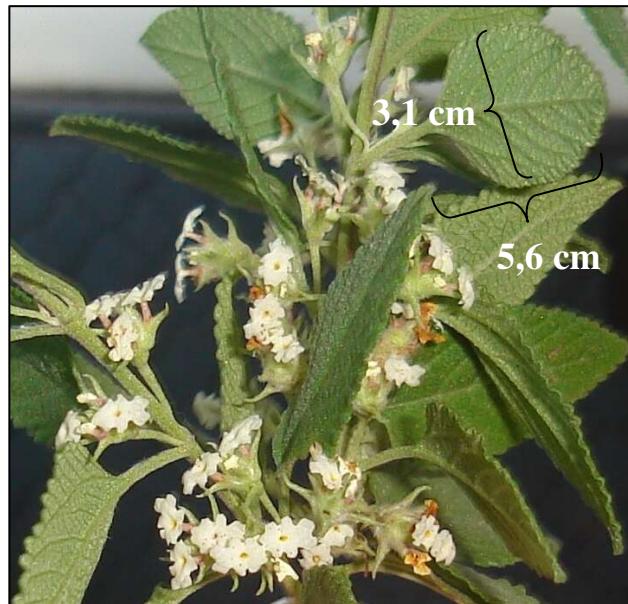
nos pés, nas axilas e virilha (Matos, 2000). Botelho et al. (2007) testaram extratos de *Lippia sidoides* contra bactérias causadoras de cárie, encontrando eficácia por parte desses extratos.

Trabalhos já registrados na literatura mostram atividade bactericida e fungicida contra diferentes espécies microbianas, bem como larvicida (Botelho et al., 2007; Kordali et al. 2008). O óleo essencial desta planta mostra forte atividade sobre os microrganismos que vivem na pele dos pés e especialmente sobre o *Corynebacterium xerosis*, bactéria responsável pelo odor característico das axilas (Costa, 2001). Apresentou ação contra *E. coli*, *S. aureus*, *P. aeruginosa*, *Bacillus subtilis*, *Mycobacterium smegmatis* e os fungos *Saccharomyces cerevisiae*, *Cryptococcus neoformans* e *Aspergillus flavus* (Costa, 2001).



(Fonte: Fontenelle, 2008)

Figura 7: Fotografia de Arbustos de *Lippia sidoides* Cham



(Fonte: Fontenelle, 2008)

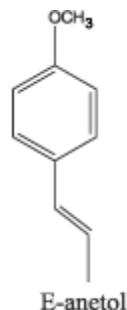
Figura 8: Fotografia de folhas de *Lippia sidoides* Cham

2.3 Principais constituintes das plantas estudadas

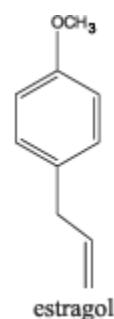
Uma planta é classificada como medicinal por possuir substâncias que têm ação farmacológica. Estas substâncias são denominadas princípios ativos e, na maioria das vezes, não se sabe quais destes realmente estão atuando na terapêutica (Barraca, 1999). Todos os óleos essenciais constituem uma mistura de princípios químicos muito complexa e variam amplamente em sua composição (Matos, 1989; Kordali et al., 2008). Na mistura, tais compostos apresentam-se em diferentes concentrações, normalmente um deles é o composto majoritário, existindo outros em menores teores e alguns em baixíssimas quantidades (Simões et al., 2004). Tais substâncias podem ser utilizadas para a síntese de vitaminas, hormônios, antibióticos e antissépticos (Lavabre, 1993; Bruneton, 1995).

2.3.1 Anetol e estragol

O anetol é um derivado do fenilpropano que ocorre com bastante freqüência nos óleos essenciais de um grande número de plantas (Craveiro et al., 1976). Apresenta-se como líquido incolor ou levemente amarelado em uma temperatura de 23°C. Possui um sabor adocicado e um odor aromático de anis. É praticamente insolúvel em água, mas solúvel em benzeno, acetona, éter de petróleo e prontamente miscível em éter e clorofórmio. O anetol, juntamente com o estragol, constitui os principais componentes do óleo essencial do *Croton zenhtneri*, cuja atividade antifúngica já se encontra descrita por vários estudos (Lopes Júnior, 2005).



Muitos efeitos bioquímicos e farmacológicos do anetol foram descritos. Coté et al. (1951) descreveram uma nítida inibição da formação do glicogênio pelo anetol, que é reversível e que, provavelmente, se verifica por uma competição pela glicose disponível, uma vez que este composto é eliminado conjugado ao ácido glucorônico como demonstrado por Le Bourhis et al. (1973).



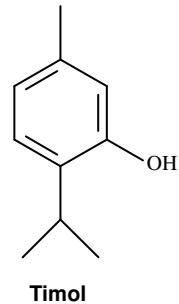
O anetol possui várias atividades farmacológicas importantes, tais como a indução de movimentos rítmicos, contração iônica e redução de tônus muscular, ação estrogênica, ação

depressiva no sistema nervoso central, propriedades psicolépticas, ação inseticida e antimicrobiana (Marcus et al., 1979; Lopes Júnior, 2005).

O estragol, isômero do anetol, apresenta efeito anestésico, miorrelaxante e anticonvulsivante (Dallmeir & Carlini, 1981), mostrando, ainda, atividade hipnótica e depressora do sistema nervoso central (Sell & Carlini, 1976).

2.3.2 Timol

O timol, também conhecido como ác. tímico ou isopropilmetacresol ($C_{10}H_{14}O$). É encontrado em diversas plantas como *Thymus eriocalyx*, *Thymus x-porlock* e principalmente no óleo essencial da *Lippia sidoides*. Apresenta-se sob a forma de cristais incolores grandes ou pó cristalino branco com aroma irritante, lembrando tomilho. Pouco solúvel em água, mas muito solúvel em álcool. É irritante da mucosa gástrica, gordura e álcool aumentam sua absorção. Possui atividade antimicrobiana, que é diminuída na presença de proteínas. Medidas apropriadas devem ser tomadas para evitar a contaminação no acondicionamento e nas diluições (Botelho et al., 2007).

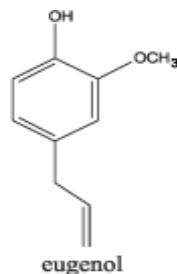


Timol

É absorvido no trato gastrointestinal e excretado na urina em sua forma pura e como glicuronídeo. É um antisséptico fenólico com atividade antibacteriana, antifúngica e é o mais potente dos fenóis, porém seu uso é limitado por causa da sua pouca solubilidade em água e sua ação irritante (Botelho et al., 2007), sendo usado principalmente como antisséptico bucal, associado à glicerina, em três vezes o seu volume, em água morna. O timol tem sido usado topicalmente no tratamento de enfermidades da pele e por inalação, associado a outras substâncias voláteis, para tratar enfermidades respiratórias (Kordali et al., 2008).

2.3.3 Eugenol

O eugenol é uma substância aromática natural, farmacologicamente muito ativa, presente nos óleos essenciais de algumas plantas do Nordeste brasileiro. Dentre estas plantas destacam-se: *Eugenia caryophyllus*, o “cravo-da-índia”; *Dicipelium cariophyllum*, “o craveiro do Maranhão ou cravinho”; *Ocimum gratissimum*, a “alfavaca-cravo”; e o *Croton zenhtneri*, a “canela-de-cunha” (Craveiro et al., 1981; Wu et al., 1994). Também conhecido como ácido eugênico ou cariofilico, o eugenol é um fenol monoterpenóide derivado do fenilpropano, popularmente denominado de essência de cravo (Escobar, 2002).



O eugenol se apresenta à temperatura ambiente como um líquido oleoso, incolor ou amarelo claro, mas quando em contato prolongado com o ar, torna-se mais espesso e de cor vermelho escuro. Em baixas temperaturas (- 9°C), encontra-se no estado sólido. É amplamente solúvel em clorofórmio, éter, gorduras e álcool etílico e pouco solúvel em água pura. Sua alta lipossolubilidade possibilita uma fácil absorção através das membranas lipídicas e rápido acesso ao local de ação. Apresenta odor característico, semelhante ao cheiro do cravo-da-índia, além de possuir sabor ardente e picante. É bastante irritante às mucosas e causa forte sensação de queimação quando em contato com a mucosa oral (Escobar, 2002).

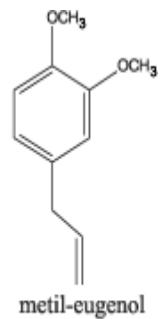
Devido a sua estrutura molecular complexa, o eugenol possui diversas ações farmacológicas comprovadas, sendo utilizado em práticas odontológicas como anti-séptico tópico, analgésico e anestésico local, além de conferir propriedades farmacológicas aos cimentos obturadores de canais, visto ter ação bactericida, portanto, eficaz no tratamento de algumas enfermidades infecciosas na cavidade bucal (Markowitz et al., 1992; Escobar, 2002). Também é utilizado como cimento provisório em cavidades dentárias, quando associado ao óxido de zinco (Nagababu & Lakshma, 1994).

Possui atividade antifúngica contra *Candida albicans* (Rakotonirainy & Lavédrine, 2005); antiviral, contra HSV-2 (vírus da herpes simples tipo 2) (Bourne et al., 1999); apresenta também efeito anti-edematógeno local, anti-inflamatório e antinociceptivo (Wright et al., 1995). Em tecido nervoso, apresenta ação anestésica geral, pois bloqueia tanto a condução do potencial de ação em nervos periféricos quanto na junção neuromuscular (Cruz, 2001).

Por suas diversas propriedades farmacológicas, o eugenol tem sido aplicado para diversos fins, seus efeitos, dependentes da concentração de eugenol livre e do tempo de exposição ao tecido, são complexos e muitos deles ainda não estão completamente esclarecidos. Com a evolução do conhecimento a cerca desse composto, seu uso ficou mais comum, específico e seletivo, sendo utilizado em diferentes propostas, principalmente na área odontológica (Escobar, 2002).

2.3.4 Metil-eugenol

O metil-eugenol é um dos análogos do eugenol que podem ser encontrados como constituinte de um grande número de plantas do Nordeste do Brasil, dentre as quais estão *Croton zehntneri*, ou “canela-de-cunhã” e *Croton nepetaefolius*, ou “marmeiro sabiá” (De Vincenzi et al, 2000). O metil-eugenol tem cor amarelo ouro, odor marcante e forte, insolúvel na água e solúvel em clorofórmio e éter, também é conhecido como 1,2 dimetoxialbenzeno; 1,3,4-eugenol-metil-éter; 4-alilveratrol dentre outros (De Vincenzi et al, 2000).



Esse composto é usado na indústria de cosméticos na fabricação de sabões, xampus e como agentes flavorizantes nas geléias, em bebidas não alcoólicas, goma de mascar e sorvetes (Council of Europa, 2001). A exposição humana ao metil-eugenol pode ocorrer pelo uso de cosméticos diretamente aplicados na pele, ou por via oral através de substâncias flavorizantes contidas nos alimentos (De Vincenzi et al., 2000).

Muitas ações farmacológicas têm sido relatadas, dentre as quais a atividade bactericida (Franchomme & Penoel, 1995); efeito na indução de hipertensão, além de ser miorelaxante, antiespasmódico, anticonvulsivante e anestésico (Magalhães et al., 1998; Lima et al., 2000; Sayyah et al., 2002). O metil-eugenol parece atuar diretamente na miofibrila lisa, exercendo, além do efeito relaxante, um efeito antiespasmódico inespecífico para as contrações induzidas por acetilcolina ou histamina, que é amplamente independente de alterações no potencial transmembrana (Lima, 1998).

2.4 Importância dos estudos toxicológicos no screening de plantas medicinais: um enfoque para os métodos empregados na pesquisa com *Croton spp.* e *Lippia sidoides*

Os testes toxicológicos, aplicados em animais de laboratório e sob condições previamente estabelecidas, permitem determinar os possíveis efeitos das substâncias em humanos ou animais expostos a estas (Barros & Davino, 1996). No Brasil, a resolução 1/78 (D.O. 17/10/78), do Conselho Nacional de Saúde, estabelece os seguintes tipos de ensaios de toxicidade: aguda, subaguda (subcrônica), crônica, teratogenia e embriotoxicidade e estudos especiais (Vasconcelos, 2002).

A toxicidade aguda de um composto químico é definida como os efeitos adversos que ocorrem dentro de um período curto após administração de dose única ou doses múltiplas administradas dentro de 24 horas (Barros & Davino, 1996). É expressa pela quantidade necessária, em mg/kg de peso corpóreo, para provocar a morte em 50% de um lote de animais submetidos à experiência, sendo representada pela dose letal 50 (Eaton & Klaasson, 1995; Barros & Davino, 1996; Repetto, 1997). Os valores de dose letal 50 (DL_{50}) são obtidos a partir de índices de mortalidade observados, empregando-se diversos métodos estatísticos. Entre estes métodos, destaca-se o método de PROBITOS (termo derivado de PROBITS = probability units) que, além de determinar o valor de toxicidade aguda e seus limites fiduciais, possibilita a obtenção de uma reta “dose-resposta” (Barros & Davino, 1996).

Os resultados obtidos a partir dos estudos de toxicidade aguda servem também para conhecimento do mecanismo de ação da substância, identificar possíveis órgãos ou sistemas sensíveis e determinar se os efeitos são reversíveis ou não. Ainda mais, os resultados obtidos dos estudos de toxicidade aguda são empregados para o delineamento dos estudos de toxicidade subcrônica e crônica, particularmente no que se refere à escolha de doses (Barros & Davino, 1996).

Os estudos de toxicidade subcrônica são realizados para obter informações sobre a toxicidade de substâncias químicas, em humanos ou animais, após exposições repetidas a estas (Barros & Davino, 1996). Os estudos de toxicidade subcrônica e crônica, que objetivam determinar o efeito tóxico após exposição prolongada a doses cumulativas da substância em teste, não se diferenciam na sua essência, mas, sim, em sua extensão (Repetto, 1997). Parâmetros hematológicos e bioquímicos devem ser avaliados no início e final do estudo. Exames histopatológicos, também, devem ser realizados no final do experimento (Barros & Davino, 1996). Para realização desses testes são feitos através de a administração cumulativa de uma substância e envolvem análise hematológica, análise bioquímica e histopatológica dos animais. Além destes parâmetros, devem ser feitas observações diárias para verificar o comportamento dos animais tratados (Vasconcelos, 2002).

2.5 Conhecendo os fungos: uma abordagem geral sobre dermatófitos e *Candida* spp.

Os fungos são microrganismos que constituem um grupo diversificado e abundante na natureza, fazendo parte de vários nichos no ambiente, incluindo a microbiota de homens e animais. São caracterizados por estruturas unicelulares ou multicelulares e classificados de acordo com sua morfologia em filamentosos, leveduras e dimórficos (Prado, 2007). São ainda seres eucarióticos, isto é, apresentam uma membrana nuclear que envolve os cromossomos e o nucléolo. São classificados como seres heterotróficos por não possuírem pigmentos fotossintéticos, capazes de absorver energia luminosa e utilizá-la para síntese de compostos orgânicos, e aproveitarem à energia contida nas ligações químicas de vários nutrientes (Sidrim & Rocha, 2004).

Nos últimos anos, a Micologia Médica evoluiu muito como ciência básica. Diversos fungos foram reclassificados e as infecções fúngicas foram alvo de sensíveis alterações. Essas mudanças taxonômicas não tiveram bases e/ou razões apenas teóricas e especulativas, já que encerram conotações práticas nos setores clínico, laboratorial e terapêutico (Sidrim & Rocha, 2004). O reino *Fungi* (*Eumycota*) está atualmente subdividido em sete filos: *Microsporidia*, *Chytridiomycota*, *Blastocladiomycota*, *Neocallimastigomycota*, *Glomeromycota*, *Basidiomycota* e *Ascomycota*, sendo estes dois últimos incluídos no sub-reino *Dikarya* (Hibbet et al., 2007).

Além da capacidade de produzir doença em seres humanos, os fungos também causam doenças em animais e vegetais, destroem a madeira e materiais sintéticos e compartilham com as bactérias um importante papel na decomposição de restos orgânicos do solo (Mendes-Giannini & Melhem, 1996).

Doenças fúngicas ocorrem com relativa freqüência em clínicas de pequenos animais, sendo que, nos casos crônicos, o tratamento se torna mais difícil e, às vezes, frustrante. A problemática do tratamento pode ser justificada pelo limitado arsenal de drogas antifúngicas comparadas com as drogas antibacterianas, pelo aparecimento de vários efeitos colaterais à terapia com medicamentos convencionais e pela seleção de cepas resistentes aos fármacos utilizados (Sidrim & Rocha, 2004).

As infecções causadas por fungos, denominadas micoses, parecem ser acidentais, ou seja, sua grande maioria não é contagiosa, mas adquirida por exposição do indivíduo a uma fonte natural de ocorrência do fungo. Existem na natureza mais de 250 mil espécies fúngicas conhecidas atualmente. Dentre estas, apenas aproximadamente trezentas foram identificadas, pelo menos uma vez, em processos patológicos em seres humanos ou animais (Sidrim & Rocha, 2004).

As micoses podem ser classificadas clinicamente em sistêmicas, subcutâneas e superficiais, de acordo com o grau de envolvimento no tecido e o sítio de instalação do agente infeccioso no hospedeiro (Tortora et al., 2003).

As micoses superficiais se destacam dentre as infecções fúngicas devido à freqüência de casos reportados em humanos e pequenos animais. As micoses superficiais podem ser classificadas em micoses superficiais estritas e dermatofitoses. As micoses superficiais estritas possuem a característica de acometerem a camada mais superficial do estrato córneo de humanos e animais, e, diferentemente das dermatofitoses, não induzem nenhuma resposta inflamatória no hospedeiro. As dermatofitoses são micoses superficiais causadas por fungos denominados de dermatófitos (Crespo et al, 2000; Cafarchia et al., 2006).

Nos últimos anos, em decorrência de uma demanda crescente na Micologia médica, houve grande aumento no arsenal terapêutico antifúngico. Em decorrência, porém, de serem os fungos seres eucarióticos aliado a outros fatores, as drogas antifúngicas surgiram tardeamente. Dessa forma, em 1903, a terapêutica antifúngica se restringia à utilização do iodeto de potássio, fármaco ministrado até os dias atuais (Sidrim & Rocha, 2004).

As drogas utilizadas no tratamento das dermatofitoses, como a griseofulvina, o cetoconazol e itraconazol, exibem alguns efeitos colaterais e possuem eficácia limitada (Gupta et al., 2000). Sendo este um dos motivos da necessidade do descobrimento de drogas antifúngicas mais seguras e de maior eficácia (Gurgel et al., 2005).

Na clínica veterinária cetoconazol, itraconazol e fluconazol são bastante utilizados. Em se tratando de leveduras, estes fármacos são citados para o tratamento de infecções por *Candida* spp. em cães, tais como cistite e dermatomicoses, e dermatites por *Malassezia* em cães e gatos (Mueller et al., 2002; Rochette et al., 2003; Ozawa et al., 2005).

O aumento da incidência de infecções por fungos aliado à resistência que estes agentes têm desenvolvido aos antimicóticos, bem como ao elevado custo dos fármacos antifúngicos, tem levado a uma busca constante por alternativas terapêuticas eficazes que possam oferecer melhores e mais acessíveis opções de tratamento aos doentes (Fontenelle et al., 2007). Surge então um interesse especial aos fungicidas derivados de plantas, baseado no conhecimento de que as plantas possuem sua própria defesa contra fungos.

Na presença de patógenos (fungos, bactérias e vírus) as plantas podem produzir compostos para se protegerem contra ataques. O uso de plantas medicinais no tratamento de doenças de pele, incluindo infecções fúngicas, é uma prática antiga adotada em muitas regiões do mundo (Irobi & Darambola, 1993). Este uso tem sido apoiado pela ciência através do isolamento de compostos com atividade antifúngica a partir de extratos de plantas (Costa et al., 2000; Passos et al., 2002).

2.5.1 Dermatófitos

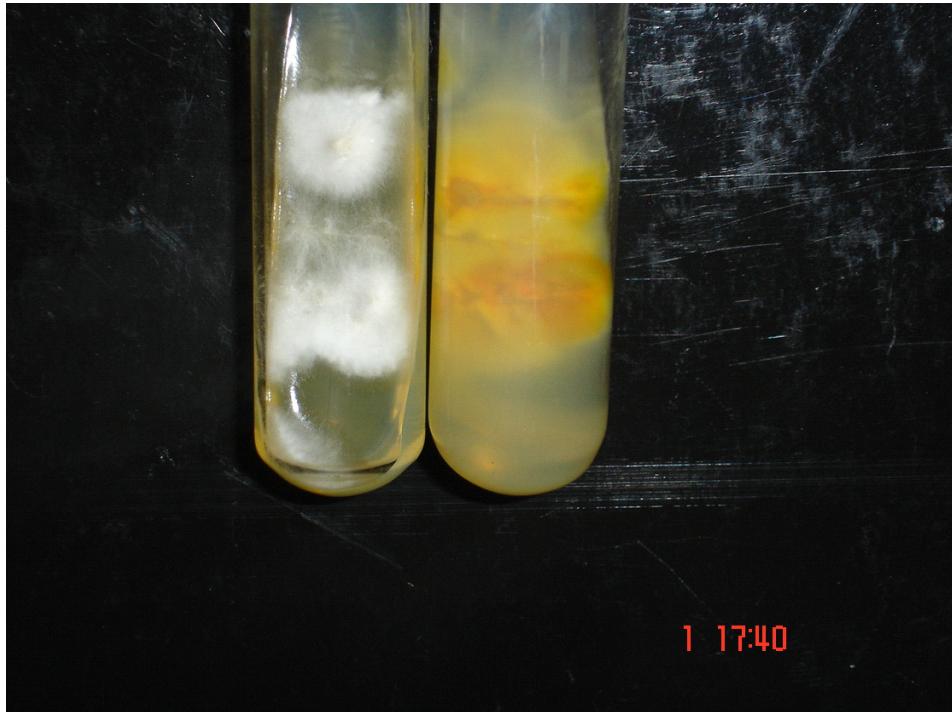
O estudo das dermatofitoses teve início juntamente com a história da Micologia Médica. Em 1839, Robert Remak elucidou a etiologia do *favus*. Quase um século depois, os dermatófitos foram classificados por um dermatologista francês de nome Raymond Jacques Andrien Sabouraud. Hoje, existem muitos fungos pertencentes aos gêneros *Trichophyton*, *Epidermophyton* e *Microsporum*. Entretanto, a denominação dermatófito é utilizada somente para os fungos pertencentes a esses gêneros e que são queratinofílicos e capazes de causar doenças em humanos e animais (Sidrim & Rocha, 2004).

As dermatofitoses são infecções de estruturas queratinizadas, como as unhas, pêlos e estrato córneo da pele, e são as doenças fúngicas mais comuns na clínica veterinária, possuindo grande interesse também pelo seu potencial zoonótico (Garcia et al., 2000; Crespo et al., 2000). São causadas por dermatófitos, que são fungos pertencentes aos gêneros *Microsporum*, *Trichophyton* e *Epidermophyton*, que utilizam a queratina como substrato nutritivo. Estes fungos

são ainda caracterizados por serem filamentosos, hialinos, septados, algumas vezes artroconidiados. Em cães e gatos as principais espécies isoladas pertencem aos gêneros *Microsporum* e *Trichophyton*, sendo o *M. canis* a espécie mais freqüente, possuindo um importante papel como um constante agente de zoonose (Prado et al., 2007).

As espécies de dermatófitos podem ser classificadas, de acordo com a preferência pelo hospedeiro e habitat natural, em antropofílicas, zoofílicas e geofílicas. As espécies antropofílicas (*T. rubrum*, *E. floccosum*, *T. tonsurans*, *T. mentagrophytes* var. *interdigitale*) infectam os seres humanos e, menos comumente, os animais. As espécies zoofílicas (*M. canis*, *T. mentagrophytes* var. *mentagrophytes*, *T. verrucosum*) são usualmente patógenos dos animais, mas são capazes de infectar os humanos. As espécies geofílicas (*M. gypseum*) habitam o solo, sendo uma fonte em potencial de infecção tanto humana quanto animal (Cabañes, 2001; Moriello, 2004; Outerbridge, 2006).

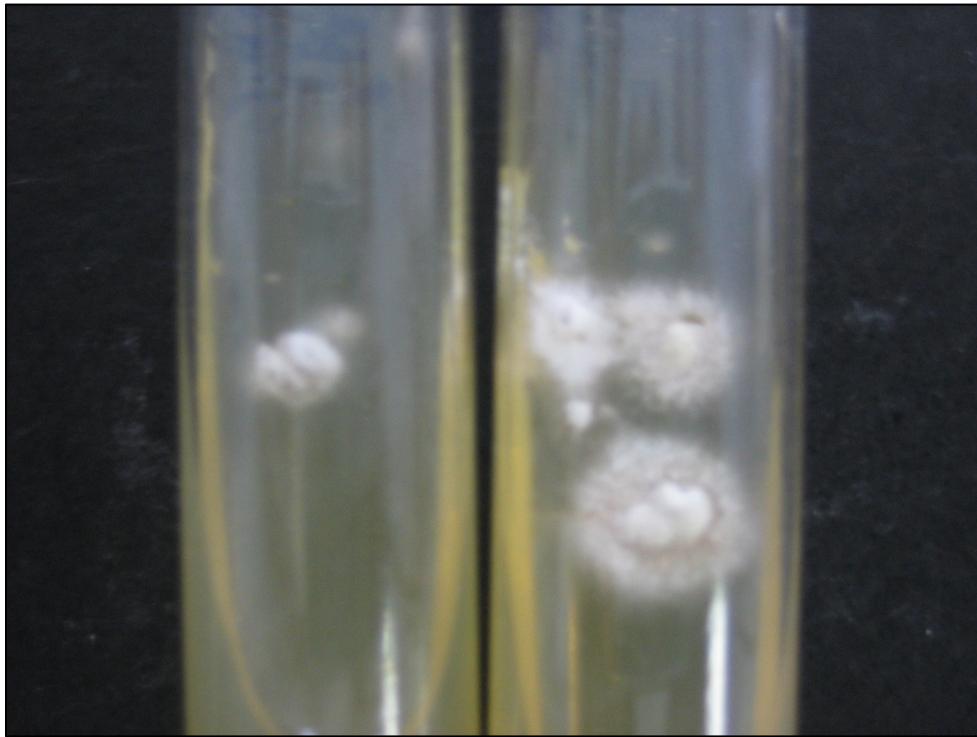
O *M. canis* (Figura 9) é o dermatófito mais comumente isolado em cães e gatos (Brilhante et al., 2003; Cafarchia et al., 2006). Uma vez que os humanos podem se infectar, o *M. canis* se tornou o dermatófito com potencial zoonótico mais freqüentemente encontrado em áreas urbanas (Cafarchia et al., 2006). Porém, os gatos assintomáticos apresentam maior potencial zoonótico do que os cães, uma vez que a incidência de dermatófitos é maior em gatos do que em cães sem sintomatologia clínica (Mancianti et al., 2003; Cafarchia et al., 2006).



(Fonte: CEMM, 2007)

Figura 9: Colônias de *M. canis*, com textura algodonosa e relevo apiculado e radiado.

As espécies do gênero *Trichophyton*, em especial o *T. mentagrophytes* var. *mentagrophytes* (figura 10), também podem causar dermatofitose em animais e no homem, porém sua ocorrência no Brasil é menor em relação às espécies do gênero *Microsporum*. O *T. mentagrophytes* var. *mentagrophytes* é um dermatófito zoofílico, cujos principais hospedeiros são os roedores domésticos ou silvestres. A transmissão para o homem ou para outros animais ocorre ocasionalmente através do contato com roedores infectados ou portadores assintomáticos, ou ainda, através do contato com materiais contaminados presentes no meio ambiente, tais como pêlos ou escamas (Pinter & Stritof, 2004).



(Fonte: CEMM, 2007)

Figura 10: Colônia de *T. mentagrophytes* var. *mentagrophytes* com textura furfurácea.

M. gypseum, *T. terrestre* e *T. ajelloi* são os dermatófitos geofílicos mais comumente isolados de cães e gatos assintomáticos, porém o potencial patogênico destas espécies ainda não está claro (Cafarchia et al., 2006).

2.5.2 O gênero *Candida*:

A história natural das doenças causadas por leveduras é mais bem compreendida à luz da sensibilidade do hospedeiro. O indivíduo normal apresenta mecanismos de defesa inespecíficos e específicos, tais como barreiras anatômicas e fisiológicas, resposta inflamatória e resposta imunológica, que, juntos, representam obstáculo ao estabelecimento da infecção fúngica (Sidrim & Rocha, 2004; Weig & Brown, 2007).

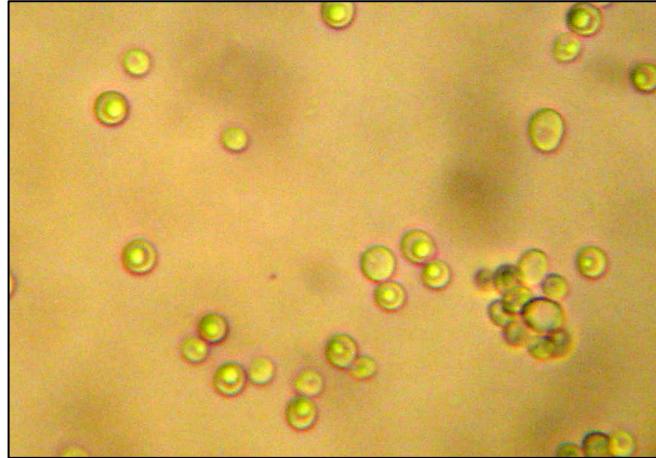
Na Medicina Veterinária, são fatores predisponentes a infecções por leveduras, principalmente do gênero *Candida*: idade, presença de doenças auto-imunes, *Diabetes mellitus*, uso de glicocorticóide, antibioticoterapia, cateterismo venoso e urinário e administração de nutrição parenteral (Heseltine et al., 2003; Moretti et al., 2004; Jin & Lin, 2005). Enfermidades provocadas por *Candida* spp. geralmente acometem apenas animais imunocomprometidos, como observado por Heseltine et al. (2003), que isolaram espécies do gênero apenas em animais portadores de outros processos infecciosos e com sistema imune comprometido. Os sítios anatômicos mais acometidos são: pele, unhas, ouvido, trato urinário e sistema gastrintestinal (Milner et al., 1997; Jin & Lin, 2005).

De acordo com a literatura, a *C. albicans* é a espécie mais comum em casos de candidíases em animais, sendo responsável por diversos quadros clínicos como otite (Duarte et al., 2001; Brito et al., 2007), infecção intestinal (Elad et al., 1998; Ochiai et al., 2000), infecção sistêmica (Heseltine et al., 2003; Brown et al., 2005; Tunca et al., 2006), dermatomicose, entre outros (Raposo et al., 1996; Kozak et al., 2003; Moretti et al., 2004).

O gênero *Candida* é composto por fungos unicelulares e hialinos, que formam colônias de coloração branca, superfície lisa e textura glabrosa úmida (Raposo et al., 1996; Moretti et al., 2004) (Figura 11). Crescem bem dentro de 48 horas, entre temperaturas de 25 e 37°C (Sidrim & Rocha, 2004). Com relação à microscopia, se lâminas forem preparadas diretamente a partir de um fragmento da colônia ou de uma amostra clínica positiva, apenas estruturas unicelulares, denominadas blastoconídeos, que podem estar isoladas ou apresentando brotamento, vão ser visualizadas (Figura 12). Desta forma, torna-se necessário a realização de microcultivo em placa de Petri, para melhor visualização da disposição das estruturas fúngicas típicas de cada espécie, sendo este, muitas vezes, suficiente para identificar o microrganismo (Moretti et al., 2004).



(Fonte: CEMM, 2005)

Figura 11: Colônias de *Candida* spp.

(Fonte: CEMM, 2005)

Figura 12: Blastocônidios de levedura.

2.6 Métodos de estudo da atividade antifúngica *in vitro*

A atividade antifúngica, *in vitro*, é medida a fim de determinar a potência de um antifúngico em solução, a sua concentração em líquido e tecidos corporais e a sensibilidade de um determinado microrganismo a concentrações conhecidas deste antibiótico (Jawetz et al., 1988).

Os testes utilizados na avaliação, *in vitro*, da atividade antifúngica são derivados dos métodos utilizados na avaliação da atividade de antibacterianos. De modo geral, as técnicas disponíveis para essa finalidade nos laboratórios de microbiologia são conhecidas como diluição em caldo, diluição em ágar e difusão em ágar. O princípio desses métodos é expor um inóculo definido do microrganismo a conhecidas concentrações da droga a serem testadas, em condições ótimas de crescimento, e observar se o crescimento fúngico é minimizado ou não. A leitura final dos testes de diluição em meio líquido ou sólido permite identificar a menor concentração da droga que inibe o crescimento do microrganismo problema. No caso da difusão em ágar, o método clássico utiliza disco contendo concentração única da droga a ser avaliada. Nessas condições, a leitura permite, apenas, a análise qualitativa dessa atividade (Sidrim & Rocha, 2004).

A entidade responsável pela normatização de técnicas de laboratório clínico nos Estados Unidos é o *Clinical Laboratory Standards Institute* (CLSI, anteriormente denominado *National Committee for Clinical Laboratory Standards* – NCCLS) que padronizou os testes de sensibilidade de fungos a drogas antimicóticas, definindo variáveis como método e preparação de inóculo, composição e pH do meio a ser utilizado, temperatura e tempo de incubação e determinação dos critérios de leitura do teste (Sidrim & Rocha, 2004).

A macrodiluição em caldo foi o primeiro método padronizado pelo CLSI (1992), porém, por tratar-se de método laborioso e de difícil execução em laboratório de rotina, houve a busca por métodos alternativos. Entre os mais estudados, citam-se a macrodiluição em caldo, com bons resultados, e o Etest. A macrodiluição em placa, realizada segundo parâmetros do CLSI, além da maior facilidade na sua execução, permite a análise de grande número de amostras, com economia de material (Sidrim & Rocha, 2004).

Outros procedimentos, tais como o método de difusão em cavidade ágar, apesar de não ser padronizado pelo CLSI, possibilita análises qualitativas, permitindo-se fazer uma comparação da eficiência da droga testada com outra droga utilizada como referência. Estudos posteriores são necessários para determinação da concentração inibitória mínima (CIM), *in vitro*, para que seja possível correlacionar com resultados *in vivo*.

Nos testes de atividade antimicrobiana de óleos essenciais, a metodologia proposta pelo CLSI não pode ser seguida à risca, devido às propriedades químicas que estes apresentam. Dessa forma, feitas as modificações, esses métodos podem ser usados em algumas situações. Na maioria dos estudos, as zonas de inibição formadas pelos óleos são comparadas com aquelas obtidas pelos antibióticos, no entanto, é importante destacar que esses resultados não devem ser simplesmente comparados, pois as particularidades apresentadas pelos óleos, bem como outras variáveis devem ser levadas em consideração (Nascimento et al., 2007).

As substâncias normalmente testadas pelos métodos propostos pelo NCCLS têm natureza hidrofílica e os testes são padronizados para esta condição. Nos ensaios com óleos essenciais, deve-se considerar que os óleos são voláteis, insolúveis em água, viscosos e complexos. Além disso, podem formar uma suspensão turva que impede a determinação visual da eficácia antimicrobiana do óleo, devido à interferência da dissolução insuficiente dos componentes testados. Sendo assim, a falta de padronização dos testes de susceptibilidade antimicrobiana tem sido um dos empecilhos encontrados para a realização desse tipo de estudo (Nascimento et al., 2007).

Os testes *in vitro* servem como uma indicação inicial da atividade que está sendo pesquisada e, quando utilizados no início de uma triagem de plantas, permitem selecionar as plantas que apresentem melhores resultados, diminuindo gastos, evitando perda de tempo e uso indiscriminado de animais de experimentação (Matan & Matan, 2008; Sharma & Tripathi, 2008).

2.6 Métodos de estudo da atividade antifúngica *in vivo*

Testes *in vivo* são onerosos, demandam tempo e envolvem o uso de animais de laboratório ou da espécie alvo, devendo, por questões éticas e econômicas, ser realizados após um conhecimento prévio da ação da planta que está sendo avaliada. Os testes de eficácia *in vivo* são realizados em animais infectados experimentalmente ou com infecção natural objetivando avaliar a eficácia de um produto natural, e são realizados em animais de laboratório ou na espécie alvo da indicação terapêutica, sendo denominados pré-clínicos ou clínicos, respectivamente. Os resultados dos grupos tratados são comparados percentualmente com os não tratados (Morais & Braz Filho, 2007).

O estudo para validação de uma planta medicinal é aparentemente muito simples, entretanto pode levar a conclusões errôneas quando mal aplicado. A seleção da planta, a forma de administração, as doses, a preparação da planta podem conduzir a falsos resultados. O protocolo de validação deve ser bem pesquisado e analisado, antes da escolha final. Testes *in vitro* devem

preceder os testes *in vivo*, assim como os testes de toxicidade com animais de laboratório devem preceder os testes de eficácia, *in vivo*, com a espécie alvo. O melhor protocolo visa a uma melhor avaliação da atividade medicinal das plantas. Além disso, deve-se respeitar tanto a espécie alvo da indicação terapêutica da planta quanto as espécies de animais de laboratório, para evitar cometer erros éticos sobre a utilização inadequada ou abusiva de animais na experimentação (Morais & Braz Filho, 2007). É ainda de extrema importância salientar que novos modelos de infecção experimental *in vivo*, que se assemelhe à infecção natural e que causem menos sofrimento animal, precisam ser desenvolvidos, já que os modelos utilizados rotineiramente acarretam inúmeros danos ao animal (Dalazen et al., 2005; Lee et al., 2007; Saunte et al., 2008).

3 JUSTIFICATIVA

O aumento da incidência de infecções por fungos nas clínicas veterinárias, juntamente com a resistência que estes agentes têm desenvolvido aos antimicóticos, tem levado a uma busca constante por alternativas terapêuticas eficazes que possam oferecer melhores opções de tratamento aos doentes. No Nordeste do Brasil, várias plantas são utilizadas pelo povo para o tratamento de diversas enfermidades, principalmente em locais mais carentes. Para o seu uso em larga escala, no entanto, estudos que comprovem estas atividades, associados à determinação da toxicidade, fazem-se necessários. Daí, um estudo detalhado, abordando os aspectos químicos, micológicos e toxicológicos dos óleos essenciais do *C. zenhtneri*, *C. nepetaefolius*, *C. argyrophyilloides* e da *L. sidoides*, é importante para a sua validação como prováveis substâncias com atividade antifúngica contra dermatófitos (*M. canis* e *T. mentagrophytes* var. *mentagrophytes*) e leveduras (*C. albicans* e *C. tropicalis*), fungos estes freqüentemente isolados de animais em clínicas veterinárias.

4 HIPÓTESE

Óleos essenciais encontrados na *L. sidoides*, *C. zenhteneri* e *C. nepetaefolius*, bem como, seus constituintes majoritários, apresentam atividade antifúngica contra cepas de *M. canis*, *T. mentagrophytes* var. *mentagrophytes* e *Candida* spp.

5 OBJETIVOS

5.1 Objetivo Geral

Avaliar o potencial antifúngica dos óleos essenciais da *L. sidoides*, *C. zenhteneri*, *C. argyrophyllloides* e *C. nepetaefolius*, bem como de seus principais constituintes contra cepas de *M. canis*, *T. mentagrophytes* var. *mentagrophytes* e *Candida* spp.

5.2 Objetivos Específicos

- 1 Testar a atividade antifúngica dos óleos essenciais da *L. sidoides*, *C. zenhteneri*, *C. argyrophyllloides* e *C. nepetaefolius* pelo método de difusão em ágar e microdiluição em caldo;
- 2 Avaliar a toxicidade aguda e subcrônica dos óleos essenciais das plantas;
- 3 Determinar os constituintes majoritários nas diversas plantas que apresentam atividade antifúngica;
- 4 Sintetizar derivados metilados a partir do eugenol e timol;
- 5 Testar a atividade antifúngica *in vitro* dos constituintes majoritários dos óleos e seus derivados metilados, por difusão em ágar e mirodiluição em caldo;
- 6 Estabelecer um novo modelo de dermatofitose experimental e utilizá-lo para testar a atividade antifúngica *in vivo* do óleo essencial da *L. sidoides*.

CAPÍTULOS I

Chemical Composition, Toxicological Aspects and Antifungal Activity of Essential Oil
from *Lippia sidoides* Cham.

R.O.S. Fontenelle^a, S.M. Morais^{a,b}, E. H. S. Brito^a, M.R. Kerntopf^a, R.S.N. Brilhante^c, R. A. Cordeiro^c, A.R. Tomé^a, M.G.R. Queiroz^d, N.R.F. Nascimento^a, J.J.C. Sidrim^c, M.F.G. Rocha^{a,c*}.

^aVeterinary Faculty, Post-Graduation Program in Veterinary Sciences, State University of Ceará,
Fortaleza, Ceará, Brazil

^bDepartment of Chemistry, State University of Ceará, Fortaleza, Ceará, Brazil

^cDepartment of Pathology and Legal Medicine, Faculty of Medicine, Medical Mycology
Specialized Center, Federal University of Ceará, Fortaleza, Ceará, Brazil

^dDepartment of Clinical Biochemistry, School of Pharmacy, Federal University of Ceará,
Fortaleza, Ceará, Brazil

Corresponding author: Marcos Fábio Gadelha Rocha. Rua Jabaquara, 344. CEP: 60 861-200.
Fortaleza, Ceará, Brazil. Phone: 55 (85) 3295-1736, Fax: 55 (85) 3295-1736. E-mail:
rocha@rapix.com.br

Journal of Antimicrobial Chemotherapy

(Artigo aceito para publicação em março de 2007)

Resumo

Objetivo: O objetivo deste estudo foi testar a atividade antifúngica, *in vitro*, do óleo essencial da *Lippia sidoides* Cham., contra cepas de *Candida* spp. e *Microsporum canis*, avaliar seus efeitos de toxicidade aguda e subcrônica, *in vivo*, e determinar seus constituintes químicos.

Material e métodos: A atividade antifúngica, *in vitro*, foi inicialmente avaliada pela técnica de difusão em ágar; a concentração inibitória mínima (CIM) e a concentração fungicida mínima (CFM) foram determinadas pelo método de microdiluição em caldo. Testes de toxicidade aguda e subcrônica foram realizados em camundongos e ratos, respectivamente. A composição química do óleo essencial foi determinada por cromatografia gasosa acoplada a Espectometria de Massa.

Resultados: O óleo essencial obtido da *L. sidoides* foi efetivo contra todas as cepas testadas pelo método de difusão em ágar. A CIM do óleo essencial da *L. sidoides* foi essencial para as cepas de *M. canis* variou de 4 a 70 mg/L e o CFM variando de 9 a 150 mg/L. A CIM para as cepas de *Candida* spp variou de 620 a 2500 mg/L e CFM variou de 1250 a 5000 mg/L. Os principais constituintes isolados do óleo essencial da *L. sidoides* foram o timol (59.65%), E-cariofileno (10.60%) e *para*-cimeno (9.08%). A administração aguda do óleo essencial acima de 3 g/kg por via oral, em camundongos, foi desprovida de toxicidade. A administração oral por 30 dias do óleo da *L. sidoides* (117.95 mg/kg/day) em ratos, não induziu nenhuma alteração significativa histopatologicamente, hematologicamente ou bioquimicamente.

Conclusão: O óleo essencial da *L. sidoides* constitui uma fonte promissora para pesquisa de novas drogas antifúngicas devido a sua eficácia e a sua baixa toxicidade.

Palavras-chave: *Lippia sidoides*, dematófitos, leveduras, atividade antifúngica.

Abstract

Objectives: The aims of this study were to test the essential oil from *Lippia sidoides* Cham. for antifungal activity, *in vitro*, against *Candida* spp. and *Microsporum canis*, to evaluate its acute and sub-chronic toxicological effects, *in vivo*, and to determine its chemical constituents.

Methods: The antifungal activity, *in vitro*, was initially evaluated by the agar-well diffusion technique, and the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined by the broth microdilution method. The acute and sub-chronic toxicological effects were performed on mice and rats, respectively. The chemical composition of the essential oil was determined by GL-Chromatography coupled to mass spectroscopy.

Results: The essential oil obtained from *L. sidoides* was effective against all tested strains by the agar-well diffusion method. The MICs of *L. sidoides* essential oil for strains of *M. canis* ranged from 4 to 70 mg/L and the MFCs ranged from 9 to 150 mg/L. The MICs for strains of *Candida* spp ranged from 620 to 2500 mg/L and the MFCs ranged from 1250 to 5000 mg/L. The main constituents of *L. sidoides* essential oil were thymol (59.65%), E-caryophyllene (10.60%) and *para*-cymene (9.08%). The acute administration of the essential oil up to 3 g/kg by the oral route to mice was devoid of overt toxicity. The 30-day oral administration of *L. sidoides* oil (117.95 mg/kg/day) to rats did not induce any significant histopathological, haematological or serum biochemical alterations.

Conclusion: The essential oil from *L. sidoides* may be a promising source in the search for new antifungal drugs due to its efficacy and low toxicity.

Key-words: *Lippia sidoides*, dermatophytes, yeasts, antifungal activity.

Introduction

Mycosis constitutes a common health problem, especially in tropical and subtropical developing countries, dermatophytes, *Malassezia* spp. and *Candida* spp. being the most frequent pathogens in humans and animals.¹⁻⁷ In recent years, there has been an increasing search for new antifungal compounds due to the lack of efficacy, side effects and or resistance associated with some of the existing drugs.⁸⁻¹¹ Much attention has been drawn to plant-derived antifungal compounds,¹² based on the knowledge that plants have their own defence system against fungal pathogens.¹³

Natural products obtained from many plants have been attracting scientific interest.⁹⁻¹⁶ More recently, it was demonstrated the antifungal properties of allicin and ajoene isolated from garlic (*Allium sativum*).^{14, 15} In traditional medicine, many essential oils have been claimed to be effective against fungal pathogens, although, most of them are not clinically available. Many authors have reported that essential oils are one of the most promising groups of natural compounds from which a new prototype of antifungal agents may be developed.^{11, 14-21} Therefore, research in this field may lead to the development of effective drugs against pathogenic fungi.^{11, 14}

Widely spread in Brazilian Northeast flora, *Lippia* species are known to be a natural topical antiseptic. Previous studies have reported that the essential oil of *L. sidoides* Cham. shows antimicrobial activity *in vitro* as well as larvicidal effect against *Aedes aegyptii*.^{22, 23}

The aims of this study were to test the essential oil from *L. sidoides* Cham. for *in vitro* antifungal activity against *Candida* spp. and *M. canis*, to evaluate its acute and sub-chronic toxicological effects, *in vivo*, and to determine its chemical constituents.

Materials and Methods

Plant material and essential oil extraction

Plant samples were collected in Horizonte city (3°33'46'' latitude S, 41°05'42'' longitude W), Northeast Brazil. Taxonomic identification was confirmed by experts at the Prisco Bezerra Herbarium (Federal University of Ceará, Brazil), where a voucher sample was deposited with a reference number 25149. *L. sidoides* essential oil was extracted from the leaves by the steam distillation method in a Clevenger apparatus, as described by Craveiro *et al.*²⁴

Gas-Chromatography Mass spectral analysis

The chemical composition of the essential oil was determined by GL-Chromatography coupled to mass spectroscopy performed on a Hewlett – Packard 5971 CG/MS instrument in a polydimethylsiloxano-DB-5 (30 mm x 0.25 m film thickness) fused silica capillary column; the carrier gas was helium (1mL/min). The column temperature ranged from 35 to 180°C; at 4°C/min, them from 180 to 280°C; at 20°C/min; mass spectra was obtained by electronic impact at 70 V. The identification of the constituents was performed by computer-based library search, with retention indices and visual interpretation of the mass spectra.²³

Fungal strains

The strains were obtained from the fungal collection of the Medical Mycology Specialized Center - CEMM (Federal University of Ceará, Brazil), where they were maintained in saline (0.9% NaCl), at 28 °C. At the time of the analysis, an aliquot of each suspension was taken and inoculated onto potato dextrose agar (Difco, Detroit, USA), and then incubated at 28°C for 2-10 days. A total of ten strains of *M. canis*, five strains of *C. albicans* and three strains of *C. tropicalis* were included in this study. Both *M. canis* and *Candida* spp. strains were isolated from dogs and cats. In addition, *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6528) strains were used as a quality control.

Inoculum preparation for antifungal susceptibility tests

For the agar-well diffusion method, based on Tepe *et al.*²⁵ and Gurgel *et al.*¹², stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *M. canis*, respectively, grown on potato dextrose agar (Difco, Detroit, USA), at 28° C. Potato dextrose agar was added to the agar slant and the cultures were gently swabbed to dislodge the conidia. The suspension of conidia with blastoconidia of *Candida* spp. or suspension of hyphal fragments of *M. canis* was transferred to a sterile tube and adjusted by turbidimetry to obtain an inoculum of approximately 10⁶ and 10⁵ cfu /mL for *Candida* spp. and *M. canis*, respectively. The optical densities of the suspensions were spectrophotometrically determined at 530 nm.

For the broth microdilution method, the standardized inoculum ($2.5 - 5 \times 10^3$ cfu/mL) for *Candida* spp. and (5×10^4 cfu/mL) for *M. canis* were also prepared by turbidimetry. Stock inoculum were prepared on day 2 and on day 10 for *Candida* spp. and *M. canis*, respectively, grown on potato dextrose agar at 28°C. Sterile saline solution (0.9%) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *M. canis*²⁶ and *Candida* spp.⁴, respectively. The suspensions of conidia with hyphal fragments of *M. canis* and blastoconidia suspension of *Candida* spp. were transferred to sterile tubes and the volume of both suspensions adjusted to 4 mL with sterile saline solution. The resulting suspension was allowed to settle for 5 min., at 28°C, and its density was read at 530nm and then adjusted to 95% transmittance. The suspensions were diluted to 1:2000 for *Candida* spp and 1:500 for *M. canis*, both with RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with L-glutamine, without sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo.), buffered at pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.), to obtain the inoculum size of approximately $2.5 - 5 \times 10^3$ and 5×10^4 cfu/mL for *Candida* spp. and *M. canis*, respectively.

Agar-well diffusion susceptibility test

The antifungal activity of essential oils from *L. sidoides* was evaluated against *C. albicans* (n = 5), *C. tropicalis* (n = 3) and *M. canis* (n = 10), by the agar-well diffusion method.^{12, 25} Petri dishes with a diameter of 15 cm were prepared with Potato Dextrose Agar (Difco, Detroit, USA). The wells (6 mm in diameter) were then cut from the agar and 0.100 mL of essential oil or drugs was delivered into them. The oil was weighed and dissolved in mineral oil to obtain the test concentrations of 25, 50, 75 and 100 mg/mL. Stock solutions of griseofulvin (1mg/mL; Sigma Chemical Co., St. Louis, USA) and amphotericin B (5 µg/mL; Sigma Chemical Co., USA) were prepared in distilled water and tested as positive controls for *M. canis* and *Candida* spp., respectively. Each fungal suspension was inoculated on to the surface of the agar. After incubation, for 3-5 days for *Candida* spp. and 5-8 days for *M. canis*, at 28-35°C, all dishes were examined for zones of growth inhibition and the diameters of these zones were measured in millimetres. Each experiment was repeated at least twice.

Broth microdilution method

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) for *Candida* spp. were determined by the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute guidelines – CLSI (formerly NCCLS; M27-A2).²⁷ The broth microdilution assay for *M. canis* was performed as previously described,^{26, 28, 29} based on the M38-A document³⁰, in accordance with CLSI.

The essential oil of *L. sidoides* was prepared in 100% mineral oil. Amphotericin B (AMB) (Sigma, Chemical Co., USA) and griseofulvin (Sigma Chemical Co., St. Louis, USA) were prepared in distilled water. For the susceptibility analysis, the essential oil was diluted in mineral oil and tested in a concentration range between 0.002 and 5 mg/mL.

The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each isolate tested. The microplates were incubated at 37 °C and read visually after 2 days for *Candida* spp. and 5 days for *M. canis*. All isolates were run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 80 % inhibition of visible fungal growth. The results were read visually as recommended by CLSI. The MFC was determined by subculturing 100 µL of solution from wells without turbidity, on potato dextrose, at 28°C. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after 2 days for *Candida* spp. and 5 days for *M. canis*.

Animals

Wistar rats (*Rattus norvergicus*; 180-200 g) and Swiss mice (*Mus musculus*; 25-30 g), of both sexes, were housed in temperature-controlled rooms and were given food and water *ad libitum* until used. All the protocols that included animals were approved by the committee for ethics in research of the State University of Ceará, Fortaleza, Ceará, Brazil. The animals were used as recommended by the guide for the care and use of laboratory animals from the National Academy Press (USA; 1996) which fulfils the principles for animal use in Brazil.

Acute and subchronic toxicity

For the acute toxicity analysis, the essential oil was administered to the mice (n = 10 mice per group) orally or intraperitoneally at doses ranging from 100 to 3000 mg/kg. The results

obtained were compared to those for the control animals (3% Tween 80 in saline v/v). The Lethal Dose 50 (LD_{50}) was calculated by the probit method by using SPSS 7.0 for windows. The animals were observed for an additional period of 1 h and the general effects were noted in a table modified from Malone.³¹

In the subchronic toxicity of the essential oil of *L. sidoides*, after 30 days of oral administration to rats, the following parameters were evaluated: haematological, histopathological and serum biochemistry. The rats were separated into two groups ($n = 10/\text{group}$) and treated with *L. sidoides* essential oil (117.95 mg/kg/day) or 3% Tween 80 in saline (v/v) by oral gavages. Blood samples were collected by puncture in the infraorbital plexus at day 0 (one day before starting essential oil or vehicle administration) and then on days 15 and 30. The serum concentration of urea, creatinine, glutamic-oxalacetic (GOT) and glutamic-pyruvic (GPT) transaminases were determined by using commercial kits (Labtest, Lagoa Santa, MG, Brazil). The blood samples collected at day 0 and 30 were used for red cells and leucocytes count and for haemoglobin and hematocrit. The values obtained were compared within and between the groups. Additionally, at the end of the experimental period (30 days), histopathological analysis of heart, lungs, liver, kidneys and spleen were performed by optical microscopy.

Statistical analysis

The antifungal activity evaluated by the agar-well diffusion method was expressed as mean \pm SD of the diameter of the growth inhibition zones (mm). The antifungal activity of the essential oils was analysed by linear correlation for individual analysis and the two-tailed paired Student's t test was used to evaluate differences between the data of essential oils and the controls. The LD_{50} was calculated at 95% confidence intervals, using SPSS 7.0 for Windows. The data obtained from subchronic toxicological studies were expressed as mean \pm 95% confidence intervals and data range. The differences within and between the groups were evaluated by the analysis of variance method followed by the correction of Tukey-Kramer with the significance level set at 5%.

Results

The chemical analysis of the *L. sidoides* is shown in Table 1. The major constituents of the essential oil of *L. sidoides* were thymol (59.65%), E-caryophyllene (10.60%) and *para*-cymene (9.08%).

The essential oil from *L. sidoides* was effective against all tested strains in the agar-well diffusion susceptibility tests (Table 2). The *L. sidoides* oil induced a significant growth inhibition zone ($36.8\text{mm} \pm 12.4$) in the lower concentration (25 mg/mL) against *M. canis* ($n = 10$). In concentrations ≥ 50 mg/mL, this essential oil totally inhibited *M. canis* ($n = 10$) grown in culture. For *Candida* strains ($n = 8$), the maximal inhibition of fungal growth induced by *L. sidoides* oil was $23.3\text{mm} \pm 1.8$, at the higher used dose (100 mg/mL). The positive control, griseofulvin, induced a significant growth inhibition zone ($51.6\text{mm} \pm 6.7$) against *M. canis* ($n = 10$) and amphotericin B induced a significant growth inhibition zone ($10.8\text{mm} \pm 1.5$) against *Candida* spp ($n = 8$).

By way of the broth microdilution method, it was seen that MICs for *M. canis* strains ($n = 6$) ranged from 4 to 70 mg/L and MFCs ranged from 9 to 150 mg/L. The MICs for *Candida* spp. strains ($n = 6$) ranged from 620 to 2500 mg/L and the MFCs ranged from 1250 to 5000 mg/L (Table 3).

The oral or intraperitoneal administration of the essential oil at doses ranging from 100 to 3000 mg/kg did not induce any remarkable alterations in the behaviour pattern of mice. The calculated LD₅₀ for essential oil of *L. sidoides* was 117.95 (110.61-125.29) mg/kg.

The sub-chronic administration of *L. sidoides* essential oil (117.95 mg/kg/day) was devoid of overt toxicity. The body weight, which was not affected by the treatment was 322.9 ± 18.96 g on day 1 and 328.3 ± 22.67 g day 30, compared to 331.1 ± 24.0 g vs. 357.2 ± 21.2 in vehicle treated animals. Moreover, the serum biochemical parameters observed, i.e., creatinine, urea and glutamic-oxalacetic and glutamic-pyruvic transaminases, were not significantly affected (Table 4). The histopathological evaluation of liver, kidneys, lungs, heart and spleen did not reveal any structural alterations in those organs obtained from *L. sidoides* essential oil treated animals or in vehicle treated animals. Similarly, the evaluations of red and white blood cells did not reveal any remarkable sign of hematological toxicity induced by *L. sidoides* essential oil (Table 5).

Discussion

Plant essential oils are a potentially useful source of antimicrobial compounds.^{11, 14} It is often quite difficult to compare the results obtained in different studies, because the compositions of the essential oils can vary greatly depending upon the geographical region, the variety, the age of the plant, the method of drying and the method of extraction of the oil.

In spite of the above-mentioned difficulties, essential oils from medicinal plants are excellent candidates for the development remedies for many infectious diseases, including mycosis, due to the increasing development of antimicrobial resistance as well as the appearance of undesirable effects of some antifungal agents.¹¹

Early reports on *L. sidoides* essential oil revealed its antimicrobial action. Lemos *et al.*²² reported the highest and broadest activity against bacteria and fungi, including yeasts, dermatophytes and non-dermatophyte fungi. The present study shows that the essential oil from *L. sidoides* is quite effective against *M. canis*, the most common species of dermatophytes that cause superficial fungal infection in cats and dogs worldwide.^{2, 26} It induced a significant growth inhibition zone in the lower concentration (25 mg/mL), and in concentrations ≥ 50 mg/mL this essential oil totally inhibited *M. canis* grown in culture. The positive control, griseofulvin induced inhibition zone of $51.6\text{mm} \pm 6.7$ in the agar-well diffusion method.

Concerning *Candida* spp., which are important yeasts involved in human and animal mycosis,^{4, 6, 10} *L. sidoides* essential oil induced significant growth inhibition zones varying from 9.8 ± 0.9 to $23.3\text{mm} \pm 1.8$. Amphotericin B induced inhibition zones of $10.8\text{mm} \pm 1.5$ in the agar-well diffusion method.

Previous research has suggested that several essential oils show important *in vitro* antifungal activity, with varied MIC and MFC values, against dermatophytes, yeasts and other fungi.^{4, 16, 17, 19, 33, 34} In this study the MICs for *M. canis* strains ranged from 4 to 70 mg/L and the MFCs ranged from 9 to 150 mg/L. The MICs for *C. albicans* and *C. tropicalis* ranged from 620 to 2500 mg/L and the MFCs ranged from 1250 to 5000 mg/L. *Candida* spp. and *M. canis* strains used by Brito *et al.*⁴ and Brilhante *et al.*²⁶, respectively, as well as *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6528 were used as controls in MIC determinations and the results were within the recommended limits (*Candida* spp. MIC: 1 mg/L for Amphotericin B and *M. canis* MIC: 1 mg/L for Griseofulvin).

By way of the agar-well diffusion and the broth microdilution methods, this study shows that the essential oil of *L. sidoides* causes fungicidal activity. As there is a good correlation between the MICs, MFCs and the agar-well diffusion values of essential oil of *L. sidoides*, it may be concluded that the antifungal activity of essential oils could be preliminary investigated in the agar-well diffusion test for rapid screening.

The antimicrobial activity of essential oils from *Achillea setacea*,¹⁷ *Pimpinella anisum*,²¹ *Sesuvium portulacastrum*,¹⁸ *Melaleuca alternifolia*,^{11, 19, 20} *Juniperus* spp.,¹⁶ *Allium* spp.,^{14, 15} and *Thymus* spp.^{32, 33} is well-known. The results obtained in the present research were very important to include the *L. sidoides* Cham. in this list of plants with antifungal activity.

Concerning *M. pachydermatis*, which is the most common yeast in dermatitis and otitis externa in dogs,⁵ although it was not the aim of this research, the essential oil from *L. sidoides* Cham. was also tested for antifungal activity against this yeast, *in vitro*, by the agar-well diffusion method. Therefore, our preliminary data evidenced that essential oil from *L. sidoides* was effective in a dose-related way, being, at the lower used dose (25 mg/mL), as efficient (growth inhibition zone: 30.0 mm ± 10.0; n = 10; data not shown) as the positive control itraconazole (29.7 mm ± 9.0; n = 10; data not shown). Therefore, these data reinforce the potential antifungal activity of this essential oil.

To identify the composition of the oil from *L. sidoides* Cham., the oil derived from steam distillation was analysed by gas-chromatography mass spectral. The main component was thymol (59.65%). The main constituents of essential oils, which show important antifungal activity, are phenolic compounds (terpenoids and phenylpropanoids), such as thymol, carvacrol or eugenol, of which antimicrobial activity is well documented.¹⁶ Therefore, the activity of the essential oil from *L. sidoides* against *Candida* spp. *M. canis* and *M. pachydermatis* may partly be explained by the high amounts of thymol, which was previously reported to be effective as antifungal.^{32, 33}

Regarding pharmacokinetics studies with the essential oil from *L. sidoides*, this research was limited in this field. However, due to its high liposolubility, it would appear that the absorption of this essential oil after oral or intraperitoneal routes would not be impaired as can be confirmed by the LD₅₀ experimental protocol (LD₅₀ = 117.95 mg/kg). Corroborating the methodology used for the evaluation of the acute and subchronic toxicity performed in this research, other studies have used a similar strategy for the toxicology study of essential oils.^{34, 35}

In addition, a study using thymol has shown that thymol sulfate and thymol glicuronide can be detected, after one single oral administration, for 24 hours in urine and 41 hours in plasma.³⁶

The use of the essential oils from *L. sidoides* did not induce any significant acute toxicological alterations in the mice. The subchronic daily administration of *L. sidoides* essential oil, per 30 days; p.o, did not induce any remarkable alterations in the biochemical or haematological analyzed parameters nor was there any increase in the weight or structural pattern of the main organs, as revealed by histopathological analysis. Despite additional tests, such as reproductive toxicity analysis, cytotoxic and mutagenesis evaluation must be performed, the present results show that *L. sidoides* essential oil is probably safe for acute use *in vivo*.

In this preclinical phase, the crude essential oil from *L. sidoides* Cham. used by oral or intraperitoneal routes was evaluated to determine whether it induce any intoxication signs (physical, behavioral, biochemical, hematological or histopathological changes), after acute or subchronic experiments. Thus, the results obtained in this stage will certainly be helpful in future clinical studies, where specific tests will be performed to establish the safe profile of this essential oil for clinical use.

Due to its broad spectrum of antifungal effect, *in vitro*, and low toxicity, the essential oil of *L. sidoides* Cham. is a promising source in the search for new antifungal drugs. However, specific pharmacological approaches will be needed in future clinical trials to validate its use as a phytotherapeutic product.

Acknowledgements

The authors thank the financial support of FUNCAP (Ceará State Research Funding) and CNPq (National Counsel for Technological and Scientific Development; Brazil. Proc. CNPq: 478906/2004-8).

References

1. Gueho E, Boekhout T, Ashbee HR *et al.* The role of *Malassezia* species in the ecology of human skin and as pathogens. *Med Mycol* 1998; **36** Suppl 1: 220-29.
2. Brilhante RSN, Cavalcante CSP, Soares-Júnior FA. *et al.* High rate of *Microsporum canis* feline and canine dermatophytoses in Northeast Brazil: epidemiological and diagnostic features. *Mycopathology* 2003; **156**: 303-08.

3. Prado MR, Brito EHS, Girão MD *et al.* Higher incidence of *Malassezia pachydermatis* in the eyes of dogs with corneal ulcer than in healthy dogs. *Vet Microbiol* 2004; **100**: 115-20.
4. Brito EHS, Fontenelle ROS, Brilhante RSN *et al.* Phenotypic characterization and *in vitro* antifungal sensitivity of *Candida* spp. and *M. pachydermatis* strains from dogs. *Vet J* 2006, in press.
5. Girão MD, Prado MR, Brilhante RSN *et al.* *Malassezia pachydermatis* isolated from normal and diseased external ear canals in dogs: A comparative analysis. *Vet J* 2006; **172**: 544-548.
6. Colombo AL, Nucci M, Park BJ *et al.* Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *J Clin Microbiol* 2006; **44**: 2816-23.
7. Nardin ME, Pelegri DG, Manias VG *et al.* Etiological agents of dermatomycoses isolated in a hospital of Santa Fe City, Argentina. *Rev Argent Microbiol* 2006; **38**: 25-7.
8. Barker KS, Rogers PD. Recent insights into the mechanisms of antifungal resistance. *Curr Infect Dis Rep* 2006; **8**: 449-456.
9. Klepser ME. *Candida* resistance and its clinical relevance. *Pharmacotherapy* 2006; **26**: 68S-75S.
10. Aperis G, Myriounis N, Spanakis EK *et al.* Developments in the treatment of candidiasis: more choices and new challenges. *Expert Opin Investig Drugs* 2006; **15**: 1319-36.
11. Mondello F, De Bernardis F, Girolamo A *et al.* In vivo activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic *Candida* species. *BMC Infect Dis* 2006; **6**: 158.
12. Gurgel LA, Sidrim JJC, Martins DT *et al.* *In vitro* antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. *J Ethnopharmacol* 2005; **97**: 409-12.
13. Wojtaszek, P. Oxidative burst an early plant response to pathogen infection. *Biochem J* 1997; **322**: 681-92.
14. Pyun MS, Shin S. Antifungal effects of the volatile oils from *Allium* plants against *Trichophyton* species and synergism of the oils with ketoconazole. *Phytomedicine* 2006; **13**: 394-400.
15. Ledezma E, Apitz-Castro R. Ajoene the main active compound of garlic (*Allium sativum*): a new antifungal agent. *Rev Iberoam Micol* 2006; **23**: 75-80.

16. Cavaleiro C, Pinto E, Gonçalves MJ *et al.* Antifungal activity of *Juniperus* essential oils against dermatophyte, *Aspergillus* and *Candida* strains. *J Appl Microbiol* 2006; **100**: 1333-38.
17. Unlu M., Daferera D, Donmez E *et al.* Compositions and the *in vitro* antimicrobial activities of the essential oils of *Achillea setacea* and *Achillea teretifolia* (Compositae). *J Ethnopharmacol* 2002; **83**: 117-21.
18. Magwa ML, Gundidza M, Gweru N *et al.* (2006). Chemical composition and biological activities of essential oil from the leaves of *Sesuvium portulacastrum*. *J Ethnopharmacol* 2006; **103**: 85-9.
19. Hammer KA, Carson CF, Riley TV. *In vitro* activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. *J Antimicrobial Chemother* 2002; **50**: 195-99.
20. Hammer KA, Carson CF, Riley TV. Antifungal effects of *Melaleuca alternifolia* (tea tree) oil and its components on *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*. *J Antimicrob Chemother* 2004; **53**: 1081-85.
21. Kosalec I, Pepelnjak S, Kustrak D. Antifungal activity of fluid extract and essential oil from anise fruits (*Pimpinella anisum* L., Apiaceae). *Acta Pharm* 2005; **55**: 377-85.
22. Lemos TLG, Matos FJA, Alencar JW *et al.* Antimicrobial activity of essential oils of Brazilian plants. *Phytother Res* 1990; **4**: 82-4.
23. Carvalho, AFU, Melo, VMM, Craveiro, AA *et al.* Larvicidal activity of the essential oil from *Lippia sidoides* Cham. Against *Aedes aegypti* linn. *Mem Inst Oswaldo Cruz* 2003; **98**: 569-71.
24. Craveiro AA, Matos FJA, Alencar JW. A simple and inexpensive steam generator for essential oils extraction. *J Chem Edu* 1976; **53**: 652.
25. Tepe B, Daferera D, Sokmen A *et al.* Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). *Food Chem* 2005; **90**: 333-40.
26. Brilhante RSN, Cordeiro RA, Medrano DJ *et al.* Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can J Microbiol* 2005; **51**: 507-10.
27. National Committee for Clinical Laboratory Standards. 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard, 2nd ed. NCCLS document M27-A2. Clinical and Laboratory Standards Institute, Villanova, Pa, 2002.

28. Jessup CJ, Warner J, Isham I *et al.* Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *J Clin Microbiol* 2000; **38**: 341-44.
29. Fernandez-Torres B, Cabanes FJ, Carrillo-Munoz AJ *et al.* Collaborative evaluation of optimal antifungal susceptibility testing conditions for dermatophytes. *J Clin Microbiol* 2002; **40**: 3999-4003.
30. National Committee for Clinical Laboratory Standards. 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: Approved standard, NCCLS document M38-A. Clinical and Laboratory Standards Institute, Villanova, Pa, 2002.
31. Malone MH, Robichaud RC. (1962). A Hippocratic screening for pure or crude drug material. *Lloydia* 1962; **25**: 320-32.
32. Pina-Vaz C, Gonçalves Rodrigues A, Pinto E *et al.* Antifungal activity of *Thymus* oils and their major compounds. *J Eur Acad Dermatol Venereol* 2004; **18**: 73-8.
33. Pinto E, Pina-Vaz C, Salgueiro L *et al.* Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species. *J Med Microbiol* 2006; **55**: 1367-73.
34. Viana GS, Vale TG, Silva CM *et al.* Anticonvulsant activity of essential oils and active principles from chemotypes of *Lippia alba* (Mill.) N.E. Brown. *Biol Pharm Bull* 2000; **23**: 1314-17.
35. Orafidiya LO, Agbani EO, Iwalewa EO *et al.* Studies on the acute and sub-chronic toxicity of the essential oil of *Ocimum gratissimum* L. leaf. *Phytomedicine* 2004; **11**: 71-6.
36. Kohlert C, Schindler G, Marz RW *et al.* Systemic availability and pharmacokinetics of thymol in humans. *J Clin Pharmacol* 2002; **42**: 731-7.

Table 1 - Chemical composition of *L. sidoides* Cham. essential oil.

*K.I.	Components	Composition (%)
931	α -Thujene	1.48
939	α -Pinene	0.51
991	Myrcene	5.43
1018	α -Terpinene	1.43
1026	<i>para</i>-Cymene	9.08
1031	Limonene	1.01
1050	E- β -Ocimene	0.27
1062	γ -Terpinene	3.83
1098	Linalool	0.28
1171	Umbellulone	0.46
1235	Methyl Thymylether	1.79
1290	Thymol	59.65
1376	α -Copaene	0.66
1419	E-Caryophyllene	10.60
1439	Aromadendrene	0.53
1455	α -Humulene	0.56
1459	Dehidroaromadendrane	0.91
1524	δ -Cadinene	0.35
1581	Caryophyllene Oxide	0.72

*Retention index. The identified constituents are listed in their order of elution from a non-polar column.

Table 2- Antifungal activity of the essential oil from *L. sidoides* Cham. against *M. canis* and *Candida* spp. in agar-well diffusion assay

Strains	Growth inhibition zones (mm)					
	<i>L. sidoides</i> essential oils (mg/mL)				Griseofulvin (mg/mL)	Amphotericin B (μg/mL)
	25	50	75	100	1	5
<i>M. canis</i>						
CEMM 01-3-188	48	TI	TI	TI	55	-
CEMM 01-5-190	35	TI	TI	TI	60	-
CEMM 01-4-104	29	TI	TI	TI	55	-
CEMM 01-3-165	30	TI	TI	TI	60	-
CEMM 01-2-133	20	TI	TI	TI	58	-
CEMM 01-4-097	34	TI	TI	TI	48	-
CEMM 01-3-173	35	TI	TI	TI	47	-
CEMM 01-4-086	35	TI	TI	TI	46	-
CEMM 01-3-004	60	TI	TI	TI	45	-
CEMM 01-4-102	42	TI	TI	TI	42	-
(mean ± SD)	36.8 ± 12.4 ^a	TI	TI	TI	51.6 ± 6.7	-
<i>Candida</i> spp.						
CEMM 01-3-075 (<i>C. albicans</i>)	10	16	24	25	-	14
CEMM 01-3-069 (<i>C. albicans</i>)	10	18	24	24	-	10
CEMM 01-3-077 (<i>C. albicans</i>)	11	17	23	23	-	12
CEMM 01-3-074 (<i>C. albicans</i>)	10	16	22	24	-	11
CEMM 01-3-068 (<i>C. albicans</i>)	10	19	25	25	-	10
CEMM 01-2-063 (<i>C. tropicalis</i>)	10	17	24	24	-	10
CEMM 01-2-078 (<i>C. tropicalis</i>)	08	12	13	20	-	10
CEMM 01-2-081 (<i>C. tropicalis</i>)	09	16	17	21	-	09
(mean ± SD)	9.8 ± 0.9 ^a	16.4 ± 2.1 ^b	21.5 ± 4.2 ^b	23.3 ± 1.8 ^c		10.8 ± 1.5

TI= Total inhibition of fungal growth

Letters means significant differences in the columns at p< 0.05

Each experiment was repeated at least twice

Table 3- Minimum inhibitory and fungicidal concentrations of *L. sidoides* Cham. essential oil against *M. canis* and *Candida* spp. in broth microdilution method

Strains	<i>L. sidoides</i> essential oil	
	MIC (mg/L)	MFC (mg/L)
<i>C. albicans</i>		
CEMM 01-3-075	1250	2500
CEMM 01-3-069	1250	2500
CEMM 01-3-077	620	1250
CEMM 01-3-074	1250	2500
<i>C. tropicalis</i>		
CEMM 01-2-078	2500	5000
CEMM 01-2-063	1250	2500
(Geometric range)	1240	2500
<i>M. canis</i>		
CEMM 01-3-188	10	30
CEMM 01-5-190	30	70
CEMM 01-4-104	70	150
CEMM 01-3-165	9	10
CEMM 01-2-133	9	10
CEMM 01-4-097	4	9
(Geometric range)	13.7	25.6

MIC: Minimum inhibitory concentration

MFC: Minimum fungicidal concentration

CEMM: Specialized Centre of Medical Mycology

Table 4 - Serum biochemical parameters during sub-chronic oral administration of *L. sidoides* Cham. essential oil

Groups	urea (mg/dL)	creatinine (mg/dL)	GOT (U/mL)	GPT (U/mL)
<i>L. sidoides</i>				
<i>Day 0</i>	88.71 ± 19.85 (n=10)	0.87 ± 0.09 (n=10)	100.2 ± 15.89 (n=10)	52.1 ± 9.98 (n=10)
<i>L. sidoides</i>				
<i>15th day</i>	55.96 ± 4.87 (n=09)	0.87 ± 0.16 (n=08)	125.7 ± 10.33 (n=09)	55.54 ± 9.15 (n=09)
<i>L. sidoides</i>				
<i>30th day</i>	46.56 ± 8.65 (n=09)	0.84 ± 0.06 (n=09)	135.3 ± 12.94 (n=09)	41.86 ± 10.52 (n=09)
Vehicle	51.8 ± 18.65	1.04 ± 0.05	138.3 ± 15.89	37.02 ± 13.68
<i>Day 0</i>	(n=10)	(n=10)	(n=10)	(n=10)
Vehicle	64.4 ± 9.53	0.89 ± 0.13	120.3 ± 10.33	66.36 ± 13.77
<i>15th day</i>	(n=10)	(n=10)	(n=10)	(n=10)
Vehicle	48.4 ± 7.68	0.97 ± 0.15	122.2 ± 12.94	50.25 ± 14.25
<i>30th day</i>	(n=10)	(n=10)	(n=10)	(n=10)

Data are expressed as mean ± SD

No statistical differences were noted

Table 5 - Haematological parameters during sub-chronic oral administration of *L. sidoides* Cham. essential oil

Parameters	Day 0		30 th day	
	Mean ± SD	CI (95%)	Mean ± SD	CI (95%)
Red Cells x 10 ⁶ /mm ³	3.6 ± 1.75	3.5 x 10 ⁶ - 3.7 x 10 ⁶	3.5 x 10 ⁶ ± 0.18 x 10 ⁶	3.3 x 10 ⁶ - 3.6 x 10 ⁶
Haemoglobin g/dL	8.9 ± 1.3	8.08 - 9.69	8.4 ± 1.28	7.47 - 9.32
Hematocrit %	28.7 ± 1.2	27.84 - 29.62	28.45 ± 1.17	27.61 - 29.29
Leucocytes x 10 ³ / mm ³	6820 ± 877	6121 - 7519	6590 ± 967.8	5898 - 7282

SD= standard deviation; CI=confidence interval.

No statistical differences were noted

CAPÍTULOS II

ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS OF *CROTON* SPECIES FROM THE BRAZILIAN CAATINGA BIOME

R.O.S. Fontenelle^a, S.M. Moraes^{a,b*}, E. H. S. Brito^a, R.S.N. Brilhante^c, R. A. Cordeiro^c, N.R.F. Nascimento^a, M.R. Kerntopf^d, J.J.C. Sidrim^c, M.F.G. Rocha^{a,c}.

^aFaculty of Veterinary, Postgraduate Program in Veterinary Sciences, State University of Ceará, Fortaleza, Ceará, Brazil

^bDepartment of Chemistry, State University of Ceará, Fortaleza, Ceará, Brazil

^cDepartment of Pathology and Legal Medicine, School of Medicine, Specialized Medical Mycology Center, Federal University of Ceará, Fortaleza, Ceará, Brazil

^dDepartment of Biology, Cariri Region University, Crato, Ceará, Brazil

Corresponding author: Selene Maia de Moraes. Rua Ana Bilhar. No. 601. Apto 400: Meireles. CEP: 60 160-110. Fortaleza, Ceará, Brazil, Phone: 55 (85) 3232-3834. Fax: 55 (85) 3495-8692. E. mail: selene@uece.br

Journal of Applied Microbiology

(Artigo aceito para publicação em março de 2008)

Resumo

Objetivo: O principal objetivo do trabalho foi encontrar novos agentes antifúngicos a partir do óleo essencial de espécies brasileiras de *Croton*.

Métodos e Resultados: As folhas das plantas foram maceradas, os óleos essenciais foram obtidos por arraste em vapor d'água e seus constituintes químicos foram analisados por cromatografia gasosa acoplada a espectometria de massa. Os principais constituintes encontrados no *C. zenhtneri* foram o estragol e o anetol. O metil-eugenol e o biciclogermacreno foram os constituintes majoritários do *C. nepetaefolius* e o espatulenol e o biciclogermacreno foram os principais contituíntes do *C. argyrophyilloides*. A atividade antifúngica, *in vitro*, foi inicialmente avaliada pela técnica de difusão em ágar, a concentração inibitória mínima (CIM) e a concentração fungicida mínima (CFM) foram determinadas pelo método de microdiluição em caldo. Testes de toxicidade aguda foram realizados em camundongos. Os óleos essenciais das espécies de *Croton* demonstraram melhor atividade antifúngica contra as cepas de *M. canis*. Das três plantas analisadas, o *C. argyrophyilloides* apresentou uma melhor atividade para *M. canis* com CIM variando de 9 a 19 $\mu\text{g ml}^{-1}$. A administração aguda dos óleos essenciais até 3 g/kg por via oral, em camundongos, foi desprovida de toxicidade.

Conclusão: Os óleos essenciais foram ativos, *in vitro*, contra o dermatófito *M. canis* e não apresentaram toxicidade aguda *in vivo*.

Significância e Impacto do estudo: Devido a atividade antifúngica e a baixa toxicidade, os óleos essenciais das espécies de *Croton* são fontes promissoras para novos agentes fitoterápicos objetivando o tratamento de dermatofitoses.

Palavras-chave: *Croton* spp., óleo essencial, *M. canis*, *Candida*, veterinária, atividade antifúngica.

Abstract

Aims: To find new antifungal agents among essential oils from Brazilian *Croton* species.

Methods and Results: Plant leaves were steam distilled and the obtained essential oils were analyzed by gas chromatography/mass spectroscopy. The main constituents were estragole and anethole for *C. zehntneri*, methyl-eugenol and bicyclogermacrene for *C. nepetaefolius* and spathulenol and bicyclogermacrene for *C. argyrophyllumoides*. The antifungal activity of essential oils was evaluated against *Candida albicans*, *Candida tropicalis* and *Microsporum canis* by the agar-well diffusion method and the minimum inhibitory concentration (MIC) by the broth microdilution method. Essential oils of *Croton* species demonstrated better activity against *M. canis*. Among the three plants *C. argyrophyllumoides* showed the best results, with MIC ranging from 9 to 19 $\mu\text{g ml}^{-1}$. The acute administration of the essential oil up to 3 g/Kg by the oral route to mice was devoid of overt toxicity.

Conclusions: The studied essential oils are active *in vitro* against the dermatophyte *M. canis* and present relative lack of acute toxicity *in vivo*.

Significance and Impact of the Study: Due to its antifungal activity and low toxicity, the essential oils of studied *Croton species* are promising sources for new phytotherapeutic agents to treat dermatophytosis.

Keywords: *Croton* spp., Essential oil, *Microsporum canis*, *Candida* spp., Veterinary, antifungal activity.

Introduction

During the past several years there has been an increasing incidence of fungal infections due to growth in immunocompromised people, such as organ transplant, cancer and HIV/AIDS patients. This fact, coupled with the resistance to antibiotics and the toxicity during prolonged treatment with many antifungal drugs (Giordani *et al.*, 2001), has prompted an extensive search for newer drugs to treat mycosis (Fostel and Lartey, 2000).

The conventional treatment of fungal diseases is limited in comparison with antibiotic therapy for bacterial infection. Part of the reason is that fungi are eukaryote organisms, thus making it difficult to develop a drug that is selectively toxic to the fungal cell and not to the host (Harris, 2002). In addition to the small number of drugs available for mycosis treatment, the emerging resistance has been encouraging the search for alternatives that are more efficient, cheaper and less toxic than traditional therapies and natural products (Cavaleiro *et al.*, 2006).

The use of plant extracts and phytochemicals with known antifungal properties can be of great significance in therapeutic treatments. In recent years, a number of studies have been conducted in different countries to prove such efficiency (Prasad *et al.*, 2004; Pyun and Shin, 2006; Ledezma and Apitz-Castro, 2006; Unland and Higgins, 2006; Rasooli *et al.*, 2006; Matasyoh *et al.*, 2007; Fontenelle *et al.*, 2007). Antimicrobial properties of plants are due to compounds synthesized in their secondary metabolism (Nascimento *et al.*, 2000).

Many plants from Brazilian biomes have been used as natural medicines by local population for treatment of many diseases, including mycosis (Souza *et al.*, 2002; Duarte *et al.*, 2005; Cruz *et al.*, 2007) and several plants have shown antimicrobial properties (Bertini *et al.*, 2005; Botelho *et al.*, 2007). Considering that the *Caatinga* scrublands is a biome with extreme

diversity of medicinal plants, more phytochemical and pharmacological research is needed to establish the potential use of these plants as alternative treatments (Almeida et al., 2006).

Widespread in the flora of northeastern Brazilian, mainly in the *Caatinga* region, *Croton* species are used for several purposes in folk medicine. Some pharmacological activities of *Croton* essential oils have been validated, such as antispasmodic, antimicrobial and antihypertensive activity for *Croton nepetaefolius* (De Albuquerque et al., 1974; Lahlou et al., 2000; Magalhães et al., 2004). In addition, the antinociceptive and antimicrobial effects of *Croton zehntneri* have been demonstrated (Lemos et al., 1990; Oliveira et al., 2001). Morais et al. (2006) have reported that the essential oils of *C. zehntneri*, *C. nepetaefolius*, *C. argyrophyllumoides* and *C. sonderianus* showed *in vitro* larvicidal effect against *Aedes aegypti*. However, the antifungal properties of the essential oil from above-cited *Croton* species remain unknown.

The aims of this study were to determine the chemical constitution of the essential oils from *C. nepetaefolius*, *C. argyrophyllumoides* and *C. zehntneri* species, to test their essential oils *in vitro* antifungal activity against *Candida* spp. and *M. canis*, and to evaluate their acute toxicological effects *in vivo*.

Materials and Methods

Plant material and extraction of essential oils

Plant samples were collected in Viçosa city, Ceará State ($3^{\circ}33'46''$ latitude S, $41^{\circ}05'42''$ longitude W), situated in Northeastern Brazil. The taxonomic identification was confirmed by botanists of the Prisco Bezerra Herbarium (Federal University of Ceará, Brazil), where voucher specimens were deposited with reference numbers 32448, 32444 and 32446 for *C. nepetaefolius*, *C. argyrophyllumoides* and *C. zehntneri* respectively. The *Croton* species' essential oils were

extracted from leaves by the steam distillation method in a Clevenger type apparatus, as described by Craveiro et al. (1976).

Gas-chromatography / mass spectral (GC-MS) analysis

The chemical analysis of the essential oils constituents was performed on a Shimadzu QP-2010 instrument employing the following conditions: column: DB-5ms (Agilent, part No. 122-5532) coated fused silica capillary column (30 m x 0.25mm x 0.25 μ m); carrier gas: He (1 mL/min, in constant linear velocity mode); injector temperature was 250 °C, in split mode (1:100), and the detector temperature was 250 °C. The column temperature programming was 35 to 180 °C at 4 °C/min then 180 to 280 °C at 17 °C/min, and at 280 °C for 10 min; mass spectra: electron impact 70 eV. The injected sample volume was 1 L. Compounds were identified by their GC retention times relative to known compounds and by comparison of their mass spectra with those present in the computer data bank (National Institute for Standard Technology – NIST – 147,198 compounds) and published spectra (Adams, 2001; Stenhammar et al., 1974).

Fungal strains

A total of ten strains of *M. canis*, five strains of *C. albicans* and three strains of *C. tropicalis* were included in this study. Both *M. canis* and *Candida* spp. strains were isolated from symptomatic dogs and cats. The strains were stored in the fungal collection of the Specialized Medical Mycology Center – CEMM (Federal University of Ceará, Brazil), where they were maintained in saline (0.9% NaCl), at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated into potato dextrose agar (Difco, Detroit, USA), and then incubated at 28 °C for 2-10 days.

Inoculum preparation for antifungal susceptibility tests

For the agar-well diffusion method, based on Gurgel et al. (2005) and Fontenelle et al. (2007), stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *M. canis*, respectively, grown on potato dextrose agar (Difco, Detroit, USA) at 28° C. Potato dextrose agar was added to the agar slant and the cultures were gently swabbed to dislodge the conidia. The suspensions with blastoconidia of *Candida* spp. or suspension of hyphal fragments of *M. canis* were transferred to a sterile tube and adjusted by turbidimetry to obtain inocula of approximately 10^6 cfu/mL blastoconidia of *Candida* spp. and 10^5 cfu/mL hyphal fragments or conidia of *M. canis*. The optical densities of the suspensions were spectrophotometrically determined at 530 nm and then adjusted to 95% transmittance.

For the broth microdilution method, standardized inocula ($2.5 - 5 \times 10^3$ cfu/mL for *Candida* spp. and 5×10^4 cfu/mL for *M. canis*) were also prepared by turbidimetry. Stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *M. canis* cultures, respectively, grown on potato dextrose agar at 28°C. Sterile normal saline solution (0.9%; 3 mL) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat for *M. canis* (Brilhante et al., 2005) and the blastoconidia from *Candida* spp. (Brito et al., 2007). The suspensions of conidia with hyphal fragments of *M. canis* and blastoconidia suspension of *Candida* spp. were transferred to sterile tubes, and the volume of both suspensions adjusted to 4 ml with sterile saline solution. The resulting suspensions were allowed to settle for 5 min at 28°C, and their density was read at 530nm and then adjusted to 95% transmittance. The suspensions were diluted to 1:2000 for *Candida* spp. and 1:500 for *M. canis*, both with RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with L-glutamine, without sodium bicarbonate (Sigma

Chemical Co., St. Louis, Mo.), buffered to pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.), to obtain the inoculum size of approximately $2.5 - 5 \times 10^3$ for *Candida* spp. and 5×10^4 cfu/ml for *M. canis*.

Agar-well diffusion susceptibility test

The antifungal activity of essential oils from *Croton* species were evaluated against *C. albicans* (n=5), *C. tropicalis* (n=3) and *M. canis* (n=10), by the agar-well diffusion method according to Gurgel et al. (2005) and Fontenelle et al. (2007). Petri dishes with 15 cm diameter were prepared with potato dextrose agar (Difco, Detroit, USA). The wells (6 mm in diameter) were then cut from the agar and 0.100 mL of essential oil was delivered into them. The oil was weighed and dissolved in mineral oil to obtain the test concentrations of 25.000, 50.000, 75.000 and 100.000 $\mu\text{g mL}^{-1}$. Stock solutions of griseofulvin (1000 $\mu\text{g mL}^{-1}$; Sigma Chemical Co., St. Louis, USA) and amphotericin B (5 $\mu\text{g mL}^{-1}$; Sigma Chemical Co., USA) were prepared in distilled water and tested as positive controls for *M. canis* and *Candida* spp., respectively. Each fungal suspension was inoculated on the surface of the agar. After incubation, for 3-5 days for *Candida* spp. and 5-8 days for *M. canis*, at 28 °C, all dishes were examined for zones of growth inhibition and the diameters of these zones were measured in millimeters. Each experiment was repeated at least twice.

Broth microdilution method

The minimum inhibitory concentration (MIC) for *Candida* spp. was determined by the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute – CLSI (formerly NCCLS; M27-A2), (NCCLS, 2002). The broth microdilution assay for *M. canis* was performed as described by Jessup et al. (2000), Fernandez-Torres et al. (2002) and Brilhante

et al. (2005), based on the M38-A document (CLSI; formerly NCCLS, 2002). The Minimum fungicidal concentration (MFC) for both *Candida* spp. and *M. canis* were determined according Fontenelle et al. (2007). In addition, *C. parapsilosis* (ATCC 22 019) and *C. krusei* (ATCC 6528) strains were used as quality controls for broth microdilution method.

The essential oils of *Croton* species were prepared in 100% mineral oil. Amphotericin B (AMB) (Sigma, Chemical Co., USA) and griseofulvine (Sigma Chemical Co., St. Louis, USA) were prepared in distilled water. For the susceptibility analysis, the essential oils were tested in concentrations ranging from 4 to 5000 $\mu\text{g ml}^{-1}$.

The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each essential oil tested. The microplates were incubated at 37°C and read visually after 2 days for *Candida* spp. and 5 days for *M. canis*. The assays for all essential oils were run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 100% inhibition of visible fungal growth. The results were read visually as recommended by CLSI. The MFC was determined by subculturing 100 μL of solution from wells without turbidity, on potato dextrose, at 28 °C. *The MFCs were determined* as the lowest concentration resulting in no growth on the subculture after 2 days for *Candida* spp. and 5 days for *M. canis*.

Animals

Swiss mice (*Mus musculus*; 25-30 g), of both sexes, were housed in temperature-controlled rooms and given water and food *ad libitum* until used. All the protocols that included animals were approved by the research ethics committee of the State University of Ceará, Fortaleza, Ceará, Brazil. The animals were used as recommended by the guide for the care and use of

laboratory animals from the National Academy Press (USA; 1996) which is in line with the principles for animal use in Brazil.

Acute toxicity

For the acute toxicity analysis, the essential oil was administered to the mice ($n = 10$ mice per group) orally or intraperitoneally at doses ranging from 100 to 3000 mg/kg. The results obtained were compared with those for the control animals (3% Tween 80 in saline v/v). The lethal dose 50 (LD_{50}) was calculated by the probit method by using SPSS 7.0 for Windows. The animals were observed for an additional period of 1 h and the general effects were noted in a modified table from a previous work (Malone and Robichaud, 1962).

Statistical analysis

The antifungal activity evaluated by the agar-well diffusion method was expressed as mean \pm SD of the diameter of the growth inhibition zones (mm). The antifungal activity of the essential oils was analyzed by linear correlation for individual analysis and by the one-way ANOVA followed by Tukey test to evaluate differences between the effect of different doses of each essential oil. The LD_{50} was calculated at 95% confidence intervals, using SPSS 7.0 for Windows.

Results

The chemical analyses demonstrated that *C. nepetaefolius* has methyl-eugenol (15.7%) and bicyclogermacrene (14.1%) as main constituents, while the main constituents of *C. argyrophyllumoides* are spathulenol (20.3%) and bicyclogermacrene (11.7%), and those of *C. zehntneri* are estragole (72.9%) and anethole (14.3%) (Table 1).

Through the agar-diffusion method, this study shows that essential oils from *C. nepetaefolius* and *C. argyrophyilloides* were effective only against *M. canis* strains ($n = 10$). Nevertheless, the essential oil from *C. zehntneri* was effective against both *M. canis* and *Candida* species (Tables 2 and 3). Briefly, *C. nepetaefolius* essential oil was effective against *M. canis* at concentrations of 25000, 50000, 75000 and 100000 $\mu\text{g ml}^{-1}$, but no statistically significant difference was found among the three last concentrations. *C. argyrophyilloides* also presented a relevant antifungal activity against *M. canis*, showing a tendency to a dose-dependent effect, and the best activity was obtained using 100000 $\mu\text{g ml}^{-1}$ (growth inhibition zone: 31.2 mm \pm 9.9). *C. zehntneri* inhibited *M. canis* growth at all concentrations and the best effect (22 mm \pm 7.3) was obtained with the highest concentration (Table 2). The positive control, Griseofulvin, induced significant growth inhibition zones (51.6mm \pm 12.4). On the other hand, the negative control, mineral oil, was devoid of antifungal effect against all strains of *M. canis* ($n = 10$) and *Candida* spp ($n = 8$).

The essential oils obtained from *C. nepetaefolius* and *C. argyrophyilloides* were ineffective against *Candida* spp. strains ($n=8$) in the agar-well diffusion susceptibility tests, even in higher concentration (100000 $\mu\text{g mL}^{-1}$) (Table 3). On the other hand, *C. zehntneri* essential oil was effective against these yeasts. The maximal inhibition of fungal growth was obtained with 100000 $\mu\text{g mL}^{-1}$ (12.6mm \pm 1.1), but there were no differences among the tested concentrations. The positive control, amphotericin B, had a significant effect (10.7mm \pm 1.5) (Table 3).

Through the broth microdilution method, it was seen that the MIC of *C. zehntneri* essential oil against *M. canis* strains ($n = 6$) ranged from 620 to 1250 $\mu\text{g ml}^{-1}$ and the MFC ranged from 1250 to 2500 $\mu\text{g ml}^{-1}$. The MIC of *C. zehntneri* essential oil for *Candida* spp. strains ($n = 6$) was $\geq 2500 \mu\text{g ml}^{-1}$ and the MFC was $\geq 5000 \mu\text{g mL}^{-1}$. *C. argyrophyilloides* and *C. nepetaefolius*

essential oils were effective only against *M. canis* strains ($n = 6$), with MIC ranging from 9 to 19 $\mu\text{g ml}^{-1}$ and MFC from 39 to 78 $\mu\text{g ml}^{-1}$ for *C. argyrophyllumoides* essential oil and MIC > 5000 $\mu\text{g ml}^{-1}$ for *C. nepetaefolius* essential oil (Table 4).

Concerning acute toxicity analysis, the oral administration of the essential oils at doses ranging from 100 to 3000 mg/Kg induced no remarkable alterations in the behavior pattern of the mice, such as: trembles, convulsions, dyspnea and ataxia. After the intraperitoneal administration, the calculated LD₅₀ for *C. nepetaefolius* and *C. argyrophyllumoides* were 163.8 mg/Kg (155.3-172.3) and 168.6 mg/Kg (160.5-176.7), respectively. *C. zehntneri* was devoid of any overt toxicity after intraperitoneal administration. None of the essential oils tested presented remarkable signs of toxicity after oral administration up to 3 g/Kg.

Discussion

The data from this study provide evidence that the essential oils from *C. nepetaefolius*, *C. argyrophyllumoides* and *C. zehntneri* could be an alternative natural source to treat dermatophytosis. However, a specific study of the safety and *in vivo* efficacy of them and clinical trials are still required to evaluate the practical relevance of the *in vitro* results. The acute toxicity analysis of these *Croton* essential oils revealed that the oral administration at doses ranging from 100 to 3000 mg/Kg induced no remarkable alterations in the mice behavior pattern. *C. zehntneri* was devoid of any overt toxicity after intraperitoneal administration. Although additional tests must be performed, the results show that the essential oils of these *Croton* species have no acute toxicity. These preliminary toxicity data will be helpful in specific studies to establish the safe profile of these essential oils.

Among natural products, essential oils are one of the most promising groups from which a new prototype of antifungal agents can be developed (Pyun & Shin, 2006). The ease of use and relative lack of toxicity of many essential oils leads to numerous possible formulations of topical application for dermatomycoses (Harris, 2002). Corroborating these opinions, different authors have evidenced that plant essential oils are effective against some pathogenic fungi responsible for these diseases (Harris, 2002; Duarte et al., 2005; Pyun and Shin, 2006; Fontenelle et al., 2007; Tullio et al., 2007). In particular, we have demonstrated that essential oil of *Lippia sidoides* Cham. is devoid of overt *in vivo* toxicity and has *in vitro* activity against *M. canis*, *Candida* species and *M. pachydermatis* (Fontenelle et al., 2007), and now we have also shown that essential oils from *C. nepetaefolius*, *C. argyrophylloides* and *C. zehntneri* posses *in vitro* antifungal properties with no acute toxicity.

Several previous studies have demonstrated the activity of essential oils against dermatophytes and *Candida* spp. (Tepe et al., 2005; Cavaleiro et al., 2006; Magwa et al., 2006; Matasyoh et al., 2007; Fontenelle et al., 2007). However, there is no specific study of the antifungal activity of essential oils from *C. zehntneri*, *C. nepetaefolius* and *C. argyrophylloides*. In the present study using agar-well diffusion and broth microdilution methods, we have demonstrated that *C. zehntneri* essential oils have *in vitro* antifungal activity against both *M. canis* and *Candida* spp. However, *C. nepetaefolius* and *C. argyrophylloides* were efficient only for *M. canis*. *C. nepetaefolius* essential oil was effective against *M. canis* at concentrations of 25000, 50000, 75000 and 100000 µg ml⁻¹, but no statistically significant difference was found among the three last concentrations. There is no difference among the higher doses, because the interval lies in the saturation area of the dose-response curve. So, we have differences only for the extremities.

Among these three plants *C. argyrophyilloides* showed the best results against the dermatophyte, with MIC ranging from 9 to 19 $\mu\text{g ml}^{-1}$ and MFC from 39 to 78 $\mu\text{g ml}^{-1}$. The results obtained here are very important to establish the essential oils of these *Croton* species, particularly from *C. argyrophyilloides*, as promising plants from which a new prototype of phytotherapeutic agent against dermatophyte can be developed.

The antifungal activity of the essential oil of *C. zehntneri* may be attributed to its major constituent estragole (methyl-chavicol) and/or anethole, which have shown antifungal properties against *Aspergillus parasiticus* (Karapinar, 1990; Singh et al., 2006). The major constituents of *C. nepetaefolius* were methyl-eugenol (15.7%) and bicyclogermacrene (14.1%). Methyl-eugenol was isolated and identified as the antifungal constituent of the oil from *Artemisia dracunculus* L. var. *dracunculus* (Meepagala et al., 2002). *C. argyrophyilloides* presented as main constituents spathulenol (20.3%) and bicyclogermacrene (11.7%), which were identified as antifungal constituents of the essential oil of *Hyptis suaveolens* (L.) Poit. from Tanzania, and confirming the hypothesis that these constituents have the capacity for inhibition the tested fungal strains since they showed activity against other fungal species (Malele et al., 2003).

We suppose that there is a correlation between the antifungal activity of the studied essential oils and their main components. Corroborating this hypothesis, previous studies have demonstrated that essential oils in which spathulenol and caryophyllene oxide are the main compounds have inhibitory activity on filamentous fungi species (Farag et al., 2004; Wenqiang et al., 2006). Although the antifungal activity of bicyclogermacrene from essential oils is unknown, the antimicrobial potential of terpenoid compounds has already been described (Cavin et al., 2006; Cavaleiro et al., 2006). The anti-*Candida* activity of *C. zehntneri* essential oil might be related to its main compound, methyl chavicol. The antifungal potential of methyl chavicol alone

(Shin and Kang, 2003) or in combination with ketoconazole (Shin and Pyu, 2004), has been previously described, particularly against *Candida* species. Additionally, the effect of anethole, a compound with a well-known antifungal potential (Fujita et al., 2007; Kordali et al., 2005) might contribute to this anti-*Candida* effect.

In brief, due to their antifungal activity and its low toxicity, *Croton* species essential oils are promising sources of new phytotherapeutic agents to treat mycoses, especially dermatophytosis. However, *in vivo* studies must be performed to confirm this efficacy *in vitro*.

Acknowledgements

The authors acknowledge the financial support of FUNCAP (Ceará State Foundation for Scientifical and Technological Development) and CNPq (National Council for Technological and Scientifical Development; Brazil).

References

1. Adams, R.P. (2001) *Identification of essential oil components by Gas Chromatography Quadrupole Mass Spectroscopy*, Allured Publishing Corporation, USA.
2. Almeida, C.F.C.B.R., Amorim, E.L.C., Albuquerque, U.P. and Maia, M.B. (2006) Medicinal plants popularly used in the Xingó region – a semi-arid location in Northeastern Brazil. *J Ethnobiol Ethnomed* **15**, 1-7.
3. Bertini, L.M., Pereira, A.F., Oliveira, C.L.L., Menezes, E. A., Morais, S.M., Cunha, F.A. and Cavalcante, E.S.B. (2005) Perfil de sensibilidade de bactérias frente a óleos

essenciais de algumas plantas do Nordeste do Brasil. *Infarma* **17**, 80-83.

4. Botelho, M.A., Nogueira, N.A.P, Bastos, G.M., Fonseca, S.G.C., Lemos, T.L.G., Matos, F.J.A., Montenegro, D., Heukelbach, J., Rao, V.S. and Brito, G.A.C. (2007) Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res* **40**, 349-356.
5. Brilhante, R.S.N., Cordeiro, R.A., Medrano DJ, Monteiro, A.J., Sidrim, J.J.C. and Rocha, M.F.G. (2005) Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can J Microbiol* **51**, 507-510.
6. Brito, E.H.S., Fontenelle, R.O.S., Brilhante, R.S.N., Cordeiro, R.A., Soares Junior, F.A., Sidrim, J.J.C. and Rocha, M.F.G. (2007) Phenotypic characterization and *in vitro* antifungal sensitivity of *Candida* spp. and *M. pachydermatis* strains from dogs. *Vet J*, **174**, 147-153.
7. Cavaleiro, C., Pinto, E., Gonçalves, M.J. and Salgueiro, L. (2006) Antifungal activity of *Juniperus* essential oils against dermatophyte, *Aspergillus* and *Candida* strains. *J Appl Microbiol* **100**, 1333-1338.
8. Cavin, A.L., Hay, A.E., Marston, A., Stoeckli-Evans, H., Scopelliti, R., Diallo, D. and Hostettmann, K. (2006). Bioactive diterpenes from the fruits of *Detarium microcarpum*. *J Nat Prod* **69**, 768-773.
9. Craveiro, A.A., Matos F.J.A. and Alencar, J.W. (1976) A simple and inexpensive steam generator for essential oils extraction. *J Chem Edu* **53**, 652.

10. Cruz, M.C.S., Santos, P.O., Barbosa, A.M. Jr., Melo, D.L.F.M., Alviano, C.S., Antoniolli, A.R., Alviano, D.S. and Trindade, R.C. (2007) Antifungal activity of Brazilian medicinal plants involved in popular treatment of mycoses. *J Ethnopharmacol* **111**, 409-412.
11. De Albuquerque, M.M., Lyra, F.D., Mello, J.F., Lima, O.G., Monache, F.D. Diu, E. and Laurinete, C.M.B. (1974) Antimicrobial substances of superior plants. 44 Isolation of diterpene acid from *Croton* aff. *argyrophyllumoides* Muell Arg. (Euphorbiaceae). *Rev Inst Antibiot* **14**, 83-89.
16. Duarte, M.C.T., Figueira, G.M., Sartoratto, A., Rehder, VLG. and Delarmelina, C. (2005) Anti-*Candida* activity of Brazilian medicinal plants. *J Ethnopharmacol* **97**, 305-311.
17. Farag, R.S., Shalaby, A.S., El-Baroty, G.A., Ibrahim, N.A., Ali, M.A. and Hassan, E.M. (2004) Chemical and biological evaluation of the essential oils of different *Melaleuca* species. *Phytother Res.* **18**, 30-35.
18. Fernandez-Torres, B., Cabanes, F.J., Carrillo-Munoz, A.J., Esteban, A., Inza, I., Abarca, L. and Guarro, J. (2002) Collaborative evaluation of optimal antifungal susceptibility testing conditions for dermatophytes. *J Clin Microbiol* **40**, 3999-4003.
19. Fontenelle, R.O.S., Morais, S.M., Brito, E.H.S., Kerntopf, M.R., Brilhante, R.S.N., Cordeiro, R.A., Tomé, A.R., Queiroz, M.G.R., Nascimento, N.R.F., Sidrim, J.J.C. and Rocha, M.F.G. (2007) Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. *J Antimicrob Chem* **59**, 934-940.
20. Fostel, J. and Lartey, P. (2000) Emerging novel antifungal agents. *Drug Discov Today* **5**, 25-32.

21. Fujita, K., Fujita, T. and Kubo, I. (2007) Anethole, a potential antimicrobial synergist, converts a fungistatic dodecanol to a fungicidal agent. *Phytother Res* **21**, 47-51.
22. Giordani, R., Trebaux, J., Masi, M. and Regli, P. (2001) Enhanced antifungal activity of ketoconazole by *Euphorbia characias* latex against *Candida albicans*. *J Ethnopharmacol* **78**, 1-5.
23. Gurgel, L.A., Sidrim, J.J.C, Martins, D.T., Cechinel-Filho, V. and Rao, V.S. (2005) *In vitro* antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. *J Ethnopharmacol* **97**, 409-412.
24. Harris, R. (2002) Progress with superficial mycoses using essential oils. *Intern J Aromath* **12**, 83-91.
25. Jessup, C.J., Warner, J., Isham, I., Hasan, I. and Ghannoum, M.A. (2000) Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *J Clin Microbiol* **38**, 341-344.
26. Karapinar, M. (1990) Inhibitory effects of anethole and eugenol on the growth and toxin production of *Aspergillus parasiticus*. *Food Microbiol* **10**, 193-199.

27. Kordali, S., Kotan, R., Mavi, A., Cakir, A., Ala, A. and Yildirim, A. (2005) Determination of the chemical composition and antioxidant activity of the essential oil of *Artemisia dracunculus* and of the antifungal and antibacterial activities of Turkish *Artemisia absinthium*, *A. dracunculus*, *Artemisia santonicum*, and *Artemisia spicigera* essential oils. *J Agric Food Chem* **53**, 9452-9458.
28. Lahlou, S., Leal-Cardoso, J.H. and Magalhães, P.J. (2000) Essential oil of *Croton nepetaefolius* decreases blood pressure through an action upon vascular smooth muscle: studies in DOCA-salt hypertensive rats. *Planta Med* **66**, 138-143.
29. Ledezma, E. and Apitz-Castro, R. (2006) Ajoene the main active compound of garlic (*Allium sativum*): a new antifungal agent. *Rev Iberoam Micol* **23**, 75-80.
30. Lemos, T.L.G., Matos, F.J.A., Alencar, J.W., Craveiro, A.A., Clark, A.M. and McChesney, J.D. (1990) Antimicrobial Activity of Essential Oils of Brazilian Plants. *Phytother Res* **4**, 82-83.
31. Magalhães, P.J., Lahlou, S. and Leal-Cardoso, J.H. (2004) Antispasmodic effects of the essential oil of *Croton nepetaefolius* on guinea-pig ileum: a myogenic activity. Fundam. *Clin Pharmacol* **18**, 539-546.
32. Magwa, M.L., Gundidza, M., Gweru, N. and Humphrey, G. (2006) Chemical composition and biological activities of essential oil from the leaves of *Sesuvium portulacastrum*. *J Ethnopharmacol* **103**, 85-89.
33. Malele, R.S., Mutayabarwa, C.K., Mwangi, J.W., Thoithi, G.N., Lopez, A.G., Lucini, E.I. and Zygallo, J.A. (2003) Essential oil of *Hyptis suaveolens* (L.) Point. From Tanzania: Composition and antifungal activity. *J Essent O Res* **3**, 1-4.
34. Malone, M.H. and Robichaud, R.C. (1962) A Hippocratic screening for pure or crude drug material. *Lloydia* **25**, 320-332.

35. Matasyoh, J.C., Kiplimo, J.J., Karubiu, N.M. and Hailstorks, T.P. (2007) Chemical composition and antimicrobial activity of essential oil of *Tarchonanthus camphoratus*. *Food Chem* **101**, 1183-1187.
36. Meepagala, K.M., Sturtz, G. and Wedge, D.E. (2002) Antifungal constituents of the essential oil fraction of *Artemisia dracunculus L. Var. dracunculus*. *J Agric Food Chem* **50**, 6989-6992.
37. Morais, S.M., Cavalcanti, E.S.B., Bertini, L.M., Oliveira, C.L.L., Rodrigues, J.R.B., and Cardoso, J.H.L. (2006) Larvicidal activity of essential oils from Brazilian *Croton* species against *Aedes aegypti* L. *J Am Mosq Control Assoc* **22**, 161-164.
38. Nascimento, G.G.F., Locatelli, J., Freitas, P.C. and Silva, G.L. (2000) Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. *Braz J Microbiol* **31**, 247-256.
39. NCCLS M27A (2002) *Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard M27A*. National Committee Clinical Laboratory Standards, Wayne, PA; USA: vol. 27. 9.
40. NCCLS M38A (2002) *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: Approved standard M38A*. National Committee Clinical Laboratory Standards, Wayne, PA; USA: vol. 22. 16.
41. Oliveira, A.C., Leal-Cardoso, J.H., Santos, C.F., Morais, S.M. and Coelho de Souza, A.N. (2001) Antinociceptive effects of the essential oil of *Croton zehntneri* in mice. *Braz J M Biol Res* **34**, 1471-1474.
42. Prasad, N.R., Anandi, C., Balasubramanian, S. and Pugalendi, K.V. (2004) Antidermatophytic activity of extracts from *Psoralea corylifolia* (Fabaceae) correlated with the presence of a flavonoid compound. *J Ethnopharmacol* **91**, 21-24.

43. Pyun, M.S. and Shin, S. (2006) Antifungal effects of the volatile oils from *Allium* plants against *Trichophyton* species and synergism of the oils with ketoconazole. *Phytomedicine* **13**, 394-400.
44. Rasooli, I., Rezaei, M.B. and Allameh, A. (2006) Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlock*. *Food con* **17**, 359-364.
45. Shin, S. and Kang, C.A. (2003) Antifungal activity of the essential oil of *Agastache rugosa* Kuntze and its synergism with ketoconazole. *Lett Appl Microbiol* **36**, 111-115.
46. Shin, S. and Pyun, M. S. (2004) Anti-*Candida* effects of estragole in combination with ketoconazole or amphotericin B. *Phytother Res* **18**, 827-830.
47. Singh, G., Maurya, S., Lampasona, M.P. and Catalan, C. (2006) Chemical constituents, antifungal and antioxidative potential of *Foeniculum vulgare* volatile oil and its acetone extract. *Food control* **17**, 745-752.
48. Souza, L.K.H., Oliveira, C.M.A, Ferri, P.H., Santos, S.C., Júnior, J.G.O., Miranda, A.T.B., Lião, L.M. and Silva, M.RR. (2002) Antifungal properties of Brazilian Cerrado plants. *Braz J Microbiol* **33**, 247-249.
49. Stenhagen, S., Abrahamson, E. and MacLafferty, F.W. (1974) Registry of Mass Spectral Data. J. Wiley & Sons, New York.
50. Tepe, B., Daferera, D, Sokmen, A., Sokmen, M. and Polissiou, M. (2005) Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). *Food Chem* **90**, 333-340.

51. Tullio, V., Nostro, A., Mandras N., Dugo, P., Banche, G., Cannatelli, M.A., Cuffini, A.M., Alonzo, V. and Carlone, N.A. (2007). Antifungal activity of essential oils against filamentous fungi determined by broth microdilution and vapor contact methods. *J Appl Microbiol* **102**, 1544-1550.
52. Unland, F.C. and Higgins, R. (2006) Evaluation of the susceptibility of *Aeromonas salmonicida* to oxytetracycline and tetracycline using antimicrobial disk diffusion and dilution susceptibility tests. *Aquaculture* **257**, 111-117.
53. Wenqiang, G., Shufen, L., Ruixiang, Y. and Yanfeng, H. (2006) Comparison of composition and antifungal activity of *Artemisia argyi* Lévl. et Vant inflorescence essential oil extracted by hydrodistillation and supercritical carbon dioxide. *Nat Prod Res* **20**, 992-998.

Table 1 - Chemical composition of the essential oils of *Croton* species

K.I.*	components	composition (%)**		
		C. a.	C. n.	C. z.
939	α -Pinene	5.6	-	-
976	Sabinene	4.7	-	-
991	Myrcene	-	-	0.4
1033	1,8-Cineole	11.2	8.0	0.6
1050	E- β -Ocimene	-	-	0.4
1189	α -Terpineol	-	3.5	-
1196	Methyl chavicol***	-	-	72.9
1285	Anethole	-	-	14.3
1298	Ortho-Vanillin	-	5.0	-
1339	β -Elemene	-	-	0.2
1391	β -Elemene	6.2	3.7	0.3
1404	Methyl-Eugenol	-	15.7	-
1413	E- α -Bergamotene	-	4.6	-
1419	E-Caryophyllene	5.7	11.4	1.6
1435	E- α -Bergamotene	-	9.1	0.2
1439	Aromadendrene	-	3.4	-
1455	α -Humulene	-	5.9	0.2
1461	Alloaromadendreno	-	-	0.3
1480	Germacrene-D	-	-	0.9
1485	β -Selinene	3.2	-	-
1500	Bicyclogermacrene	11.7	14.1	5.1
1505	Cupareno	-	-	0.2
1506	β -Bisabolene	-	3.0	0.2
1524	δ -Cadinene	-	-	0.2
1578	Spathulenol	20.3	4.3	0.6
1581	Caryophyllene Oxide	-	4.4	-
1593	Veridiflorol	-	-	0.3

* Retention index. The identified constituents are listed in their order of elution from a non-polar column.

** % peak area of the compounds in GC-FID chromatograms.

*** Methyl chavicol: Estragole

C. a.: *Croton argyrophyllumoides*

C. n.: *Croton nepetaefolius*

C. z.: *Croton zenhtneri*

-: Components was not detected

Table 2- Antifungal activity of the essential oils of *Croton* species against *M. canis* in the agar-well diffusion assay

Essential oils/drug	Growth inhibition zones (mean ± SD): <i>Microsporum canis</i>				
	Essential oils and drug concentrations ($\mu\text{g mL}^{-1}$)				
	25000	50000	75000	100000	1000
<i>C. nepetaefolius</i>	10.2±1.7 ^a	18.6±4.6 ^b	18.7±4.2 ^b	19.8±7.2 ^b	-
<i>C. argyrophyilloides</i>	14.8±4.0 ^a	21.1±4.2 ^{a,b}	26.2±5.5 ^b	31.2±9.9 ^c	-
<i>C. zehntneri</i>	11.8 ±3.0 ^a	16.8±5.8 ^{a,b}	18.7±4.4 ^b	22±7.3 ^b	-
Griseofulvin	-	-	-	-	51.6 ± 6.7

Small letters mean significant differences in the rows at p< 0.05.

Each experiment was performed in duplicate.

(n=10)

Table 3 - Antifungal activity of the essential oils of *Croton* species against *Candida* spp in the agar-well diffusion assay

essential oils/drug	growth inhibition zones (mean ± SD): <i>Candida</i> spp				
	essential oil and drug concentrations ($\mu\text{g ml}^{-1}$)				
	25000	50000	75000	100000	5
<i>C. nepetaefolius</i>	NI	NI	NI	NI	-
<i>C. argyrophyilloides</i>	NI	NI	NI	NI	-
<i>C. zehntneri</i>	7.4 ± 1.1 ^a	9.0 ± 1.4 ^a	10.0 ± 1.5 ^a	12.6 ± 1.1 ^a	-
Amphotericin B	-	-	-	-	10.75 ± 1.5

NI= No inhibition of fungal growth.

Small letters mean significant differences in the rows at p< 0.05.

Each experiment was done in duplicate.

(n=8)

Table 4- Minimum inhibitory and fungicidal concentrations of essential oils of *Croton* species against *M. canis* and *Candida* spp.

Strains	Essential oil <i>C. nepetaefolius</i>		Essential oil <i>C. argyrophyllumoides</i>		Essential oil <i>C. zenhtneri</i>	
	MIC*	MFC*	MIC	MFC	MIC	MFC
<i>C. albicans</i>						
CEMM 01-3-075	NI	NI	NI	NI	>5000	-
CEMM 01-3-069	NI	NI	NI	NI	>5000	-
CEMM 01-3-077	NI	NI	NI	NI	>5000	-
CEMM 01-3-074	NI	NI	NI	NI	>5000	-
<i>C. tropicalis</i>						
CEMM 01-2-078	NI	NI	NI	NI	2500	5000
CEMM 01-2-063	NI	NI	NI	NI	2500	5000
(Geometric range)	-	-	-	-	2500	5000
<i>M. canis</i>						
CEMM 01-3-188	>5000	-	9	39	620	1250
CEMM 01-5-190	>5000	-	9	39	620	1250
CEMM 01-4-104	>5000	-	9	39	1250	2500
CEMM 01-5-189	>5000	-	19	78	620	1250
CEMM 01-4-097	>5000	-	19	78	1250	2500
CEMM 01-3-165	>5000	-	19	78	1250	2500
(Geometric range)	-	-	13.57	55.15	880.34	1767.77

*MIC: Minimum inhibitory concentration expressed in $\mu\text{g ml}^{-1}$

*MFC: Minimum fungicidal concentration $\mu\text{g ml}^{-1}$

CEMM: Specialized Medical Mycology Center.

NI=No inhibition.

Each experiment was repeated at least twice.

Broth microdilution method

CAPÍTULOS III

Antifungal Alkylphenols against *Candida* spp. and *Microsporum canis*

R.O.S. Fontenelle^a, S.M. Morais^{a,b*}, E. H. S. Brito^a, R.S.N. Brilhante^c, R. A. Cordeiro^c, Y.C. Lima^b, N.V.Gramosa^d, J.J.C. Sidrim^c, M.F.G. Rocha^{a,c}.

^aVeterinary Faculty, Post-Graduation Program in Veterinary Sciences, State University of Ceará, Fortaleza, Ceará, Brazil

^bDepartment of Chemistry, State University of Ceará, Fortaleza, Ceará, Brazil

^cDepartment of Pathology and Legal Medicine, Faculty of Medicine, Medical Mycology Specialized Center, Federal University of Ceará, Fortaleza, Ceará, Brazil

^dDepartment of Organic and Inorganic Chemistry, Federal University of Ceará, Fortaleza, Ceará, Brazil

Corresponding author: Selene Maia de Morais. Rua Ana Bilhar. No. 601. Apto 400: Meireles. CEP: 60 160-110. Fortaleza, Ceará, Brazil, Phone: 55 (85) 3232-3834. Fax: 55 (85) 3495-8692. E. mail: selene@uece.br

Journal of Antimicrobial Chemotherapy

(Artigo submetido)

Resumo

Objetivo: O principal objetivo do trabalho foi testar alquilfenóis (timol, eugenol, estragol e anetol) e seus derivados metilados (metil-timol e metil-eugenol) para avaliar o potencial antifúngico *in vitro*, contra cepas de *Candida* spp. e *Microsporum canis* de origem animal.

Material e métodos: A atividade antifúngica, *in vitro*, foi inicialmente avaliada pela técnica de difusão em ágar, a concentração inibitória mínima (CIM) e a concentração fungicida mínima (CFM) foram determinadas pelo método de microdiluição em caldo. Os devidos metilados foram obtidos com DMSO em condições alcalinas. As estruturas foram confirmada com base na espectrometria de massa.

Resultados: Todos os compostos testados tiveram uma boa atividade antifúngica, como podemos observar: timol (CIM contra *Candida* spp = 0.039 mg/mL e CIM contra *M. canis* = 0.0048-0.0097 mg/mL), eugenol (CIM = 0.15-0.62 mg/mL para *Candida* spp e CIM para *M. canis* = 0.039 mg/mL), anetol e estragol (CIM = 0.62-1.25 mg/mL para *Candida* spp e *M. canis* CIM = 0.078-0.15 mg/mL e 0.019-0.039 mg/mL, respectivamente). Por outro lado, a metilação diminui o efeito dos compostos analisados (metil-eugenol CIM = 0.31-0.62 mg/mL para *Candida* spp e CIM para *M. canis* = 0.078-0.15 mg/mL e metil-timol MIC=0.62-1.25 mg/mL para *Candida* spp e CIM para *M. canis*=0.078-0.15 mg/mL).

Conclusão: Os alquilfenóis comumente encontrados em óleos essenciais extraídos de plantas foram ativos, *in vitro*, contra *Candida* spp. e *M. canis*, cepas de origem animal. Desta forma, futuros estudos prescissam ser realizados para o uso destas substâncias como compostos antimicrobianos.

Palavras-chave: alquilfenóis, dermatófitos, leveduras, atividade antifúngica, antimicrobianos.

Abstract

Objectives: The aims of this study were to test alkylphenols (thymol, eugenol, estragole and anethole) and methyl derivatives (methyl-thymol and methyl-eugenol) for antifungal activity, *in vitro*, against animal strains of *Candida* spp. and *Microsporum canis*.

Methods: The *in vitro* antifungal activity was initially evaluated by the agar-well diffusion technique, and the MIC and minimum fungicidal concentration (MFC) were determined by the broth microdilution method. Derivatives were synthesized via methylation of the phenolic group with dimethyl sulfate in alkaline conditions. The structures were confirmed on the basis of mass and related spectral evidence.

Results: All tested compounds have good antifungal activity, as follows: thymol (MIC against *Candida* spp = 0.039 mg/mL and MIC against *M. canis* = 0.0048-0.0097 mg/mL), eugenol (MIC = 0.15-0.62 mg/mL for *Candida* spp and MIC for *M. canis* = 0.039 mg/mL), anethole and estragole (MIC = 0.62-1.25 mg/mL for *Candida* spp and *M. canis* MIC = 0.078-0.15 mg/mL and 0.019-0.039 mg/mL, respectively). However, the methylation decreases the effect of parent compounds (methyl-eugenol MIC = 0.31-0.62 mg/mL for *Candida* spp and MIC for *M. canis* = 0.078-0.15 mg/mL and methyl-thymol MIC=0.62-1.25 mg/mL for *Candida* spp and MIC for *M. canis*=0.078-0.15 mg/mL).

Conclusions: Alkylphenols common in plants have *in vitro* antifungal activity against the *Candida* spp. and *M. canis* strains obtained from dogs. However, further studies will be required to evaluate the usefulness of these substances as veterinary antimicrobials.

Keywords: alkylphenols, dermatophytes, yeasts, antifungal activity, veterinary antimicrobials

Introduction

Fungal infections, especially those caused by dermatophytes and *Candida* spp., are very common in humans and animals. They are assuming greater significance both in developed and developing countries, particularly due to the advent of immunosuppressive drugs and diseases.¹ In addition to these factors, several studies have reported resistance of fungal strains to the limited arsenal of known drugs.^{2,3} To overcome these drawbacks, a search for new and effective products to treat fungal infection is needed.

The antifungal effect of constituents from many aromatic plants has been described in several studies.⁴⁻⁷ Over 30,000 different components have been isolated from these plants and have been suggested to have potent antifungal activity.⁸ In this sense, eugenol (the main component of clove oil) and thymol (the major component of *Lippia sidoides* or *Thymus vulgaris*) are two of the most important representatives. Their antibacterial properties are well known.⁸⁻⁹ Carvacrol and eugenol were evaluated for their therapeutic efficacy in the treatment of experimental oral candidiasis induced by *Candida albicans* in immunosuppressed rats and were considered to be strong antifungal agents.⁴ The ethers anethole and estragole are also found frequently in several essential oils and many authors have demonstrated their antimicrobial activity. For example, antifungal activity of anethole against *Aspergillus parasiticus* strains.¹¹

Previous studies by our research team of the essential oils of species of *Crotons* and *Lippia sidoides*, plants native to the Caatinga (scrublands) biome, have shown their antifungal activity against strains of *Candida* spp. and *Microsporum canis*.^{6,12} These same studies identified and quantified the main constituents of these essential oils, by gas chromatography along with mass spectrometry. The main constituent found in the essential oil of *Lippia sidoides* is thymol (59.65%), while in the essential oil of *Croton nepetaefolius* it is methyl-eugenol (15.73%). The

essential oil of *Croton zenhtneri* contains two main constituents, estragole (72.9%) and anethole (14.3%).

Based on these studies, we carried out the present work to evaluate the antifungal activity of the main compounds found in these oils and derivatives against *Candida* spp. and *M. canis* strains isolated from symptomatic dogs.

Materials and Methods

Antifungal agents

Estragole, anethole and eugenol were purchased from Sigma Chemical Co. (St. Louis, MO, USA), other reagents from VETEC, Nuclear and Dinâmica (São Paulo, Brazil). TLC analyses were performed on a 3-10 cm aluminum sheet precoated with silica gel 60-254 (Merck) (Solvent used: petroleum ether, chloroform and methanol). SiO₂, 200–400 mesh (Merck), was used for column chromatography. Mass spectra were obtained on a 5791A Hewlett Packard instrument coupled with a Hewlett Packard 5890A serie II gas-chromatograph in the following analytical conditions: DB-5 capillary column (30 m, 0.25 mm, 0.25 m film thickness), helium as carrier gas.

Methylation of eugenol and thymol

The methyl derivatives were synthesized by the following method Furniss *et al.*¹³ Eugenol or thymol (0.05 mols) was suspended in a NaOH cold solution of (13.3g, 0.33mols) in water (81.25 mL) with vigorous stirring. Dimethyl sulfate (14.4 g, 0.12 mols) was added in one portion, and the mixture was shaken vigorously for 20 minutes, while the temperature was maintained below 35° C by external cooling. A second portion of dimethyl sulfate (14.4 g, 0.12 mols) was added

and the temperature was allowed to rise to 40-45°C. At the end of the reaction, the reactional mixture was transferred to a separator funnel. The organic layer, containing the methyl-derivatives, was washed with distilled water and dried with anhydrous sodium sulfate. The solvent was then evaporated to furnish the corresponding methyl derivatives. These compounds were purified by column chromatography and analyzed by gas chromatography along with mass spectrometry comparing mass spectra of compounds with literature data.¹⁴

Fungal strains

The strains were obtained from the fungous collection of the Specialized Medical Mycology Center – CEMM (Federal University of Ceará, Brazil), where they were maintained in saline (0.9% NaCl), at 28 ° C. *At the time of the analysis, an aliquot of each suspension was taken and inoculated into potato dextrose agar (Difco, Detroit, USA), and then incubated at 28° C for 2-10 days.* A total of six strains of *M. canis*, four strains of *C. albicans* and two strains of *C. tropicalis* were included in this study. Both *M. canis* and *Candida* spp. strains were isolated from symptomatic dogs. In addition, *C. parapsilosis* (ATCC 22 019) and *C. krusei* (ATCC 6528) strains were used as a quality controls.

Inoculum preparation for antifungal susceptibility tests

For the agar-well diffusion method, based on Gurgel *et al.*¹⁵ and Fontenelle *et al.*⁶ stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *M. canis*, respectively, grown on potato dextrose agar (Difco, Detroit, USA), at 28° C. Potato dextrose ágar was added to the agar slant and the cultures were gently swabbed to dislodge the conidia. The suspensions with blastoconidia of *Candida* spp. or suspension of hyphal fragments of *M. canis* were transferred to

a sterile tube and adjusted by turbidimetry to obtain an inoculum of approximately 10^6 and 10^5 cfu/ml for *Candida* spp. and *M. canis*, respectively. The optical densities of the suspensions were spectrophotometrically determined at 530 nm and then adjusted to 95% transmittance.

For the broth microdilution method, the standardized inocula for *Candida* spp. ($2.5 - 5 \times 10^3$ cfu/mL) and *M. Canis* (5×10^4 cfu/mL) were also prepared by turbidimetry. Stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *M. canis* cultures, respectively, grown on potato dextrose agar at 28° C. Sterile normal saline solution (0.9%; 3 ml) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat for *M. canis*,¹⁵ and the blastoconidia from *Candida* spp.³ The suspensions of conidia with hyphal fragments of *M. canis* and the blastoconidia suspension of *Candida* spp. were transferred to sterile tubes, and the volume of both suspensions were adjusted to 4 ml with sterile saline solution. The resulting suspension was allowed to settle for 5 min at 28° C, and its density was read at 530nm and then adjusted to 95% transmittance. The suspensions were diluted to 1:2000 for *Candida* spp. and 1:500 for *M. canis*, both with RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with L-glutamine, without sodium bicarbonate (Sigma Chemical Co., USA), and buffered at pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., USA) to obtain inoculum sizes of approximately $2.5 - 5 \times 10^3$ for *Candida* spp. and 5×10^4 cfu/ml for *M. canis*.

Agar-well diffusion susceptibility test

The screening of the antifungal activity of the alkylphenols and derivatives was evaluated against *Candida* spp (n=2) and *M. canis* (n=2) strains, by the agar-well diffusion method according to Gurgel *et al.*¹⁴ and Fontenelle *et al.*⁶ Petri dishes 15 cm in diameter were prepared

with potato dextrose agar (Difco, Detroit, USA). Wells (6 mm in diameter) were then cut from the agar and 0.100 ml of the compounds was placed in them. The constituents were weighed and dissolved in DMSO to obtain a test concentration of 10 mg/mL. Stock solutions of griseofulvin ($1000 \mu\text{g ml}^{-1}$; Sigma Chemical Co., St. Louis, USA) and amphotericin B ($5 \mu\text{g ml}^{-1}$; Sigma Chemical Co., USA) were prepared in distilled water and tested as positive controls for *M. canis* and *Candida* spp., respectively. Each fungal suspension was inoculated on the surface of the agar. After incubation at 28°C , for 3-5 days for *Candida* spp. and 5-8 days for *M. canis*, all the dishes were examined for growth-inhibition zones and the diameters of these zones were measured in millimeters. Each experiment was repeated at least twice.

Broth microdilution method

The minimum inhibitory concentration (MIC) for *Candida* spp. was determined by the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute – CLSI (formerly NCCLS; M27-A2).¹⁷ The broth microdilution assay for *M. canis* was performed as described by Jessup *et al.*,¹⁸ Fernandez-Torres *et al.*,¹⁹ and Brilhante *et al.*,¹⁶ based on the M38-A document.²⁰ The minimum fungicidal concentrations (MFC) for both *Candida* spp. and *M. canis* were found according Fontenelle *et al.*⁶

Arylpropanoids and derivatives, Amphotericin B (AMB) (Sigma, Chemical Co., USA) and griseofulvin (Sigma Chemical Co., USA), were mixed with DMSO to improve solubility in distilled water. For the susceptibility analysis, the compounds were tested in concentrations ranging from 4 to $5000 \mu\text{g ml}^{-1}$.

The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each isolate tested. The microplates were incubated at 37°C and

read visually after two days for *Candida* spp. and five days for *M. canis*. All isolates were run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 100 % inhibition of visible fungal growth. The results were read visually as recommended by CLSI. The MFC was determined by subculturing 100 µL of solution from wells without turbidity on potato dextrose agar at 28° C. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after two days for *Candida* spp. and five days for *M. canis*.

Results

The antifungal activity of the chemical compounds was initially evaluated by the agar diffusion method using strains of *M. canis* and *Candida* spp. The inhibition zones for *Candida* spp. were 15.5 mm for thymol, 17.5 mm for eugenol and 9.5 mm for methyl-eugenol. Regarding the strains of *M. canis*, all the tested compounds totally inhibited growth in culture. The positive control, amphotericin B, had a significant effect (10.5 mm) against *Candida* spp., and griseofulvin induced a significant growth inhibition zone (51.5 mm) against *M. canis* (Table 1).

Through the broth microdilution method, the thymol effect against *Candida* spp. (n=06) showed a MIC of 0.039 mg/mL and MFC 0.078mg/mL. The microdilution susceptibility test showed a MIC for eugenol ranging from 0.15 to 0.62 mg/mL and MFC 0.31 to 1.25 mg/mL. The Methyl-eugenol MIC ranged from 0.31 to 0.62 and the MFC ranged 0.62 to 1.25 mg/mL. Anethole, estragole and methyl-thymol presented a MIC varying from 0.62 to 1.25, while the MFC was 1.25 mg/mL for anethole and ranged from 1.25 to 2.5 mg/mL for estragole and methyl-thymol. The MIC of amphotericin B varied from 0.25 to 2.0 mg/mL (Table 2).

Concerning the strains *M. canis* (n=6), for thymol the MIC ranged from 0.0048 to 0.0097 mg/mL and the MFC ranged from 0.0097 to 0.019 mg/mL. For estragole the MIC varied from

0.019 to 0.039 mg/mL and the MFC from 0.078 to 0.039. The MIC for anethole and methyl-thymol ranged from 0.078 to 0.15mg/mL and the MFC from 0.15 to 0.31. Eugenol presented MICs of 0.039 mg/mL for all strains of *M. canis* and MFCs of 0.078 mg/mL. For methyl-eugenol the MIC varied from 0.07 to 0.15 mg/mL and the MFC from 0.15 to 0.31 mg/mL (Table 2).

Discussion

Essential oils have been used throughout human history to treat various ailments, including fungal infections.^{4, 21-24} Some constituents previously studied have demonstrated antifungal activity, such as thymol against strains of *Candida albicans*,⁹ anethole against *Aspergillus parasiticus*, *Saccharomyces cerevisiae* and *Candida albicans*^{11,25} and estragole against *Candida* species.²⁶ In this work, these constituents were compared with their methylated derivatives methyl-thymol and methyl-eugenol, utilizing microorganisms from symptomatic dogs.

The agar well-diffusion method has been used for screening of antifungal susceptibility testing. This method is simple, convenient and has been used for antifungal susceptibility testing of essential oils and chemical compounds.^{6,10,27,28} However, the zone diameter from this method is affected by the fungal concentration and the degree of drug diffusion into the agar, that's why this method has been used only for screening of compounds which present antifungal activity, including plant derivatives. We adopted agar well-diffusion and broth dilution method for yeast and filamentous fungi. Broth dilution method appears to be the one most commonly used for antifungal susceptibility testing of tea tree oil.²⁸ In the present study, we have demonstrated that all compounds have *in vitro* antifungal activity against both *M. canis* and *Candida* spp.

In the present study, the MIC values for thymol against *Candida* spp. were 0.078-0.31 mg/mL and at a higher concentration thymol had fungicidal activities. Corroborating these data, Botelho *et al.*⁹ also showed that *C. albicans* of human origin was inhibited by thymol.

Methyl-thymol also showed antifungal activity for the *Candida* spp. and *M. canis* strains, although it presented higher MICs and MFCs than thymol, which had antifungal activity against *Candida* spp. strains (MICs=1.25-2.5 mg/mL) and against *M. canis* strains (MICs varying from 0.15 to 0.31 mg/mL). Therefore this thymol derivative showed lesser MIC values, demonstrating that methylation of thymol decreases the antifungal activity.

Methyl-eugenol presented MICs ranging from 0.62 to 1.25 mg/mL against *Candida* spp. strains and from 0.15 to 0.31 mg/mL against *M. canis* strains. In another study, methyl-eugenol was isolated and identified in essential oils of *Croton nepetaefolius* and *Artemisia dracunculus* L. var. *dracunculus*, presenting antifungal activity.³⁰

Eugenol presented MICs ranging from 0.31 to 1.25 mg/mL against *Candida* spp. strains, and MIC of 0.07 mg/mL against *M. canis* strains. These MIC values are lower than those found by Silva et al. (2005), who reported MIC of 0.25 mg/mL for the five strains of *M. canis* tested. These authors also reported antifungal activity, through the agar dilution technique against strains *M. gypsum* (MIC=0.22 mg/mL), *T. rubrum* and *T. mentagrophytes* (MICs=0.125 mg/mL). Gayoso *et al.*³¹ showed that eugenol is a strong antifungal agent against *C. albicans*, *C. tropicalis*, *C. krusei*, *T. rubrum*, *T. mentagrophytes* and *G. candidum*. According to their results, eugenol may be a potential leading compound for the development of antifungal drugs.

Yen and Chang,³² evaluated the synergistic effects of eugenol in combination with cinnamaldehyde, a major constituent of cinnamomum essential oils, whose antifungal activity was already known.³³ The authors observed a significant synergy in this combination against

Laetiporus sulphureus, and both methyl-eugenol and eugenol presented MFCs from 1.25 to 2.5 mg/mL for *Candida* spp., but against *M. canis* methyl-eugenol showed lower activity (MFCs from 0.31 to 0.62 mg/mL) than eugenol (MFCs = 0.15 mg/mL). Therefore, methylation of eugenol appears to decrease the antifungal activity.

Regarding the mechanism by which thymol and eugenol act, Chami et al.¹⁰ studied the surface alteration induced by these compounds on yeast and concluded that the antifungal activity of eugenol and thymol involve alterations of both the membrane and cell wall of yeast.

Estragole and anethole both showed MICs ranging from 1.25 to 2.5 mg/mL against *Candida* spp. strains and from 0.07 to 0.038 mg/mL and 0.31 to 0.15 mg/mL, respectively, against strains of *M. canis*. These results corroborate those of Fujita et al.²⁵ who observed antifungal activity of anethole against *Saccharomyces cerevisiae* and *Candida albicans*. The antifungal potential of estragole alone,³⁴ or in combination with ketoconazole²⁵ has been previously described, particularly against *Candida* species. Additionally, the effect of anethole, a compound with a well-known antifungal potential might contribute to this anti-*Candida* effect.^{25,35}

Our results suggest that thymol, methyl-thymol, eugenol, methyl-eugenol, estragole and anethole have antifungal activity against the *Candida* spp. and *M. canis* obtained from dog. However, further studies will be required to evaluate the usefulness of these substances as veterinary antimicrobials.

Acknowledgements

The authors thank the financial support of FUNCAP (Ceará State Research Funding) and CNPq (National Counsel for Technological and Scientific Development; Brazil, Proc. CNPq: 478906/2004-8; Proc. CNPq: 304811/2006-8).

Transparency declarations

FUNCAP and CNPq did not play any role in the design, execution, analysis or reporting of the present research. The authors Raquel O S Fontenelle, Erika Helena S Brito, Raimunda S N Brilhante, Rossana A Cordeiro, José Júlio C Sidrim and Marcos Fábio G Rocha were responsible for antifungal tests. Selene M Moraes, Ynayara C Lima and Nilce V. G. P. S. Brasil performed chemistry synthesis and spectroscopic analysis.

References

1. Singh S, Beena PM. Comparative study of different microscopic techniques and culture media for the isolation of dermatophytes. *Ind J of Med Microbiol* 2003; 21: 21-4.
2. Giordani R, Trebaux J, Masi M, Regli, P. Enhanced antifungal activity of ketoconazole by *Euphorbia characias* latex against *Candida albicans*. *J Ethnopharmacol* 2001; 78: 1-5.
3. Brito EHS, Fontenelle ROS, Brilhante RSN, Cordeiro RA, Soares Junior FA, Sidrim JJC, Rocha MFG. Phenotypic characterization and *in vitro* antifungal sensitivity of *Candida* spp. and *M. pachydermatis* strains from dogs. *Vet J* 2007; 174: 147-53.
4. Pyun MS, Shin S. Antifungal effects of the volatile oils from *Allium* plants against *Trichophyton* species and synergism of the oils with ketoconazole. *Phytomedicine* 2006; 13: 394-400.
5. Unland FC, Higgins R. Evaluation of the susceptibility of *Aeromonas salmonicida* to oxytetracycline and tetracycline using antimicrobial disk diffusion and dilution susceptibility tests. *Aquaculture* 2006; 257: 111-7.
6. Fontenelle ROS, Moraes SM, Brito EHS, Kerntopf MR, Brilhante RSN, Cordeiro RA, Tomé AR, Queiroz MGR, Nascimento NRF, Sidrim JJC, Rocha MFG. Chemical composition,

toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. *J Antimicrob Chem* 2007; 59: 934-940.

7. Matasyoh JC, Kiplimo JJ, Karubiu NM, Hailstorks TP. Chemical composition and antimicrobial activity of essential oil of *Tarchonanthus camphoratus*. *Food Chem* 2007; 101: 1183-7.
8. Vázquez BI, Fente C, Franco CM, Vázquez MJ, Cepeda A. Inhibitory effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. *Food Microbiol* 2001; 67: 157-63.
9. Botelho MA, Nogueira NAP, Bastos GM, Fonseca SGC, Lemos TLG, Matos FJA, Montenegro D, Heukelbach J, Rao VS, Brito GAC. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res* 2007; 40: 349-56.
10. Chami N, Chami F, Bennis S, Trouillas J, Remmal A. Antifungal Treatment With Carvacrol and Eugenol of Oral Candidiasis in Immunosuppressed Rats. *Brazilian J Infect Dis* 2004; 8(3): 217-226.
11. Karapinar M. Inhibitory effects of anethole and eugenol on the growth and toxin production of *Aspergillus parasiticus*. *Food Microbiol* 1990; 10: 193-9.
12. Fontenelle ROS, Morais SM, Brito EHS, Brilhante RSN, Cordeiro RA, Nascimento NRF, Kerntopf MR, Sidrim JJC, Rocha MFG. Antifungal activity of essential oils of *Croton* species from the Brazilian *Caatinga* biome. *J Appl Microbiol* 2008; 104: 1383-90.
13. Furniss, B. S.; Hannaford, A. J.; Smith, P. W. G.; Tatchell, A. R. *Vogel's Textbook of Practical Organic Chemistry*, Longman Scientific & Technical, London, 1989.
14. Adams, RP. *Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy*. Allured Publishing Corporation, Illinois, USA, 2005.

15. Gurgel LA, Sidrim JJC, Martins DT *et al.* *In vitro* antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. *J Ethnopharmacol* 2005; 97: 409-12.
16. Brilhante RSN, Cordeiro RA, Medrano DJ *et al.* Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can J Microbiol* 2005; 51: 507-10.
17. National Committee for Clinical Laboratory Standards. 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard, 2nd ed. NCCLS document M27-A2. Clinical and Laboratory Standards Institute, Villanova, Pa, 2002.
18. Jessup CJ, Warner J, Isham I *et al.* Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *J Clin Microbiol* 2000; 38: 341-44.
19. Fernandez-Torres B, Cabanes FJ, Carrillo-Munoz AJ *et al.* Collaborative evaluation of optimal antifungal susceptibility testing conditions for dermatophytes. *J Clin Microbiol* 2002; 40: 3999-4003.
20. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: Approved standard, NCCLS document M38-A. Clinical and Laboratory Standards Institute, Villanova, Pa, 2002.
21. Pina-Vaz C, Gonçalves Rodrigues A, Pinto E. Antifungal activity of *Thymus* oils and their major compounds. *J Eur Acad Dermatol Venereol* 2004; 18: 73-8.
22. Hammer KA, Carson CF, Riley TV. Antifungal effects of *Melaleuca alternifolia* (tea tree) oil and its components on *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*. *J Antimicrob Chemother* 2004; 53: 1081-85.
23. Kosalec I, Pepelnjak S, Kustrak D. Antifungal activity of fluid extract and essential oil from anise fruits (*Pimpinella anisum* L., Apiaceae). *Acta Pharm* 2005; 55: 377-85.

24. Cavaleiro C, Pinto E, Gonçalves MJ *et al.* Antifungal activity of *Juniperus* essential oils against dermatophyte, *Aspergillus* and *Candida* strains. *J Appl Microbiol* 2006; 100: 1333-38.
25. Fujita K, Fujita T, Kubo I. Anethole, a potential antimicrobial synergist, converts a fungistatic dodecanol to a fungicidal agent. *Phytother Res* 2007; 21: 47-51.
26. Shin S, Pyun MS. (2004) Anti-*Candida* effects of estragole in combination with ketoconazole or amphotericin B. *Phytother Res* 2002; 18: 827-30.
27. Silva MRR, Oliveira Jr JG, Fernandes OFL, Passos XS, Costa, CR, Souza LKH, Lemos JA, Paula JR. Antifungal activity of *Ocimum gratissimum* towards dermatophytes. *Mycoses* 2005; 48: 172-5.
28. Lee S, Han J, Lee G, Park M, Choi I, Jeung E. Antifungal Effect of Eugenol and Nerolidol against *Microsporum gypseum* in a Guinea Pig Model. *Biol Pharm Bull* 2007; 30: 184-8.
29. Hammer KA, Carson CF, Riley TV. *In vitro* activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. *J Antimicrobial Chemother* 2002; 50: 195-99.
30. Meepagala KM, Sturtz G, Wedge DE. Antifungal constituents of the essential oil fraction of *Artemisia dracunculus L. Var. dracunculus*. *J Agric Food Chem* 2002; 50: 6989-92.
31. Gayoso CW, Lima EO, Oliveira VT, Pereira FO, Souza EL, Lima IO, Navarro DF. Sensitivity of fungi isolated from onychomycosis to *Eugenia cariophyllata* essential oil and eugenol. *Fitoterapia* 2005; 76: 247-9.
32. Yen TB, Chang ST. Synergistic effects of cinnamaldehyde in combination with eugenol against wood decay fungi. *Biores Technol* 2006; 106: 1-8.
33. Wang SY, Chen PF, Chang ST. Antifungal activities of essential oils and their constituents from indigenous cinnamon (*Cinnamomum osmophloeum*) leaves against wood decay fungi. *Biores Technol* 2005; 96: 813-8.

34. Shin S, Kang CA. Antifungal activity of the essential oil of *Agastache rugosa* Kuntze and its synergism with ketoconazole. *Lett Appl Microbiol* 2003; 36: 111-5.
35. Kordali S, Kotan R, Mavi A, Cakir A, Ala A, Yildirim, A. Determination of the chemical composition and antioxidant activity of the essential oil of *Artemisia dracunculus* and of the antifungal and antibacterial activities of Turkish *Artemisia absinthium*, *A. dracunculus*, *Artemisia santonicum*, and *Artemisia spicigera* essential oils. *J Agric Food Chem* 2005; 53: 9452-8.

Table 1. Antifungal activity of compounds against *M. canis* and *Candida* spp.

Strains	Growth inhibition zones (mm)							
	Constituents (10 mg/mL)							
	THY	M-THY	EUG	M-EUG	ANE	EST	GRI (1mg/mL)	AMP (5mg/mL)
<i>M. canis</i>								
CEMM 01-3-188	35	22	26	20	16	23	55	-
CEMM 01-5-190	40	20	30	20	20	20	47	-
<i>Candida</i> spp.								
CEMM 01-3-075	18	18	12	10	11	11	-	14
CEMM 01-3-069	17	13	8	7	9	8	-	10

THY: thymol; M-THY: methyl-thymol; EUG: eugenol; M-EUG: methyl-eugenol; ANE: anethole; EST: estragole; GRI: gryseofulvin; AMP: amphotericin B

CEMM: Specialized Centre of Medical Mycology

TI= total inhibition

Each experiment was repeated at least twice

Table 2 MIC and MFC of compounds against *M. Canis* and *Candida* spp. in the broth microdilution method

Strains		MIC and MFC (mg/mL)													
		Constituents (10 mg/mL)													
		THY		M-THY		EUG		M-EUG		ANE		EST		AMP	GRI
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	(μ g/mL)	(μ g/mL)
<i>C. albicans</i>	(n=4)	0.039 (4) ^a	0.078 (4)	1.25 (4)	2.5 (4)	0.62 (3)	1.25 (3)	0.62 (3)	1.25 (3)	0.62 (4)	1.25 (4)	1.25 (3)	2.5 (3)	0.5 (2)	-
						0.15 (1)	0.31 (1)	0.31 (1)	0.62 (1)			0.62 (1)	1.25 (1)	0.25 (2)	
<i>C. tropicalis</i>	(n=2)	0.039 (2)	0.078 (2)	1.25 (1)	2.5 (1)	0.31 (2)	0.62 (2)	0.31 (2)	0.62 (2)	0.62 (2)	1.25 (2)	0.62 (2)	1.25 (2)	1.0 (1)	-
						0.62 (1)	1.25 (1)							0.5 (1)	
<i>C. krusei</i>	(n=1)	0.15	0.31	1.25	2.5	0.62	1.25	0.62	1.25	0.62	1.25	0.62	1.25	0.5	-
ATCC 22019															
<i>C. parapsiloses</i>	(n=1)	0.15	0.31	1.25	2.5	0.62	1.25	0.62	1.25	0.62	1.25	0.62	1.25	2.0	-
ATCC 6528															
<i>M. canis</i>	(n=6)	0.0097 (3)	0.019 (3)	0.15 (4)	0.31 (4)	0.039 (6)	0.078 (6)	0.15 (2)	0.31 (2)	0.15 (3)	0.31 (3)	0.039 (4)	0.078 (4)	-	0.25 (2)
		0.0048 (3)	0.0097 (3)	0.078 (2)	0.15 (2)			0.078 (4)	0.15 (4)	0.078 (3)	0.15 (3)	0.019 (2)	0.039 (2)		0.125 (3)
														0.006 (1)	

^a Represents the number of strains of a species for the MIC and MFC indicated

THY: thymol; M-THY: methyl-thymol; EUG: Eugenol; M-EUG: methyl-eugenol; ANE: anethole; EST: estragole
Each experiment was repeated at least twice

CAPÍTULOS IV

Short Communication

Experimental dermatophytosis on the outer ear of guinea pigs: a model that mimics natural infection

R.O.S. Fontenelle^a, S.M. Morais^{a,b*}, E. H. S. Brito^a, R.S.N. Brilhante^d, N. R. F. Nascimento^a, R. A. Cordeiro^d, J.T. Valença Júnior^c, J..J.C. Sidrim^d, M.F.G. Rocha^{a,d}

^aVeterinary Faculty, Post-Graduation Program in Veterinary Sciences, State University of Ceará, Fortaleza, Ceará, Brazil

^bDepartment of Chemistry, State University of Ceará, Fortaleza, Ceará, Brazil

^cDepartment of Pathology and Legal Medicine, Faculty of Medicine, Federal University of Ceará, Fortaleza, Ceará, Brazil

^dDepartment of Pathology and Legal Medicine, Faculty of Medicine, Medical Mycology Specialized Center, Federal University of Ceará, Fortaleza, Ceará, Brazil

Corresponding author: Marcos Fábio Gadelha Rocha. Rua Suécia. No. 171: Vila Betânia. CEP: 60 740-810. Fortaleza, Ceará, Brazil, Phone: 55 (85) 3232-3834. Fax: 55 (85) 3495-8692. E. mail: rocha@rapix.com.br

Microbes and Infection

(Artigo em fase de elaboração)

Resumo

No presente estudo, um novo modelo de dermatofitose experimental foi testado. O objetivo foi encontrar um modelo de infecção mais próximo do natural e que acarretasse menos danos para o animal. A infecção experimental foi induzida pela aplicação de 0,5 mL da suspensão de fragmentos de hifas de *T. mentagrophytes* var. *mentagrophytes* no pavilhão auditivo de cobaias de ambos os sexos. Os primeiros sinais da infecção foram observados no sétimo dia após a inoculação em todos os animais. O curso clínico da infecção foi monitorado por análises clínicas, micológicas e histológicas. As culturas micológicas foram positivas em 100% dos animais expostos ao fungo e os principais sinais clínicos foram alopecia e descamação. Os exames histopatológicos da biópsia de pele observaram a presença de um moderado infiltrado inflamatório, rico em linfócito, neutrófilo e macrófagos, e hifas de *T. mentagrophytes* var. *mentagrophytes* foi detectado em cortes histológicos obtidos com PAS. O experimento demonstrou que o método é efetivo, causando menos estresse e riscos de morte ao animal. Este método ainda se mostrou simples, prático e próximo à infecção natural.

Abstract

In the present study a new model of experimental dermatophytosis was tested. The aim was to achieve maximum similarity to a natural infection through a procedure that is easy to carry out and also reduces the animals' suffering. The experimental infection was induced by a single inoculation of 0.5 mL of a suspension with hyphal fragments of *T. mentagrophytes* var. *mentagrophytes* on the outer ear of guinea pigs of both sexes. The first signs of infection were observe on the seventh day after inoculation in all animals. The clinical course was monitored by clinical, mycological and histological analyses. The mycological cultures were positive for 100% of the animals exposed to the fungus and the main clinical signs observed were alopecia and scaling. The histopathological evaluation of the skin biopsies revealed the presence a moderate inflammatory infiltrate, rich in lymphocytes, neutrophils and macrophages, and *T. mentagrophytes* var. *mentagrophytes* hyphae were detected in histological sections stained with periodic acid-Schiff. The experiment demonstrated that this method, besides being effective, causes less stress, harm and risk of death to the animals. It was relatively fast and practical for development of the infection and the clinical aspects the lesions here were similar to natural lesions.

1. Introduction

Experimental infection is a valuable method of studying the pathogenesis of fungal infections, to evaluate the prophylactic efficacy of antifungal therapeutic drugs and to study the immunology of dermatophytosis (Cavalcanti et al., 2002; Saunte et al., 2008; Vermout et al., 2004). Dermatophytes are a group of highly specialized fungi that infect keratinized tissues, such as the hair, nail and stratum corneum of humans and other animals. The dermatophytes include three genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. The species that most commonly infect animals are *Microsporum canis*, *Trichopyton mentagrophytes* and *Microsporum gypseum* [2-4]. The use of guinea pigs as animal models for dermatophytosis is based on the predisposition of this species to skin fungal infections with clinical features comparable to those seen in humans (Saunte et al., 2008).

When establishing an animal model it is crucial to find a method that ensures a high infection rate. Various methods have previously been used to render the skin more susceptible to infection, such as scarification, abrasion with a scalpel and inoculate fixed to the flank by bandaging for approximately 24hs [8]. In these studies, the experimental infections are commonly induced in the posterior dorsal region, cheek pouch, hind thigh and other skin areas [1,5-7], causing suffering and stress to the animals.

The idea for the present study began with our observation of an outbreak of dermatopytosis caused by *T. mentagrophytes* var. *mentagrophytes* in a colony of guinea pigs, where the main site affected was the outer ear. Since our work entails *in vitro* and *in vivo* evaluation of the antifungal activity of natural products, we felt that induced infection at this site could serve as a new model that more nearly mimics natural infection and is easy to carry out while at the same time reducing the animals' suffering.

2. Materials and Methods

2.1. Animals

Twelve guinea pigs, of both sexes, were used in the study. The animals, weighing approximately 600g, were kept in controlled rooms (temperature: $23\pm2^\circ\text{ C}$, relative humidity: $50\pm10\%$, frequent ventilation and 12h light cycle). All protocols that included animals were approved by the research ethics committee of State University of Ceará, Fortaleza, Brazil. The animals were used as recommended by the guide for the care and use of laboratory animals from the National Academy Press (USA; 1996), which is in line with the principles for animal use in Brazil.

2.2. Fungal strain

In the present study, *T. mentagrophytes* var. *mentagrophytes* strain (CEMM 1-4-085) was obtained from the fungal collection of the Specialized Medical Mycology Center – CEMM (Federal University of Ceará, Brazil), where it was maintained in saline (0.9% NaCl), at 28° C . At the time of the analysis, an aliquot of each suspension was taken and inoculated in potato dextrose agar (Difco, Detroit, USA), and then incubated at 28° C for six days. The identification of *T. mentagrophytes* was based on phenotypic features, such as a description of the macro and micromorphology. Skin perforation and vitamin requirement tests were considered, as well as the production of the enzyme urease [Brilhante et al., 2003].

2.3. Inoculation

The stock inocula were prepared on day 10, grown on potato dextrose agar (Difco, Detroit, USA), at 28° C . The suspensions with hyphal fragments of *T. mentagrophytes* var. *mentagrophytes* were transferred to a sterile tube and adjusted by turbidimetry to obtain an

inoculum of approximately 10^6 cfu/ml. The optical densities of the suspensions were spectrophotometrically determined at 530 nm. The suspension (0.5 ml) was gently inoculated on the ear flap with a swab, a single time. The guinea pigs were divided into three groups of four animals each: two groups treated on both the right and left ears and a negative control that was not inoculated with the fungal suspension. One of the groups infected with *T. mentagrophytes* var. *mentagrophytes* was then treated with cetoconazol. The fungal infection in each animal was confirmed by hair cultures and clinical evaluation of infected skin lesions.

2.4. Clinical evaluation

Clinical assessment of the guinea pigs was done according to a modification of the method published by Lee et al. (2007). The lesions were clinically followed-up daily, starting on day 0, until resolution was observed. Clinical evaluation of the inoculated animals was performed using a modified lesion score from 0 to 4, as follows: score 0, no visible lesion; score 1, moderate scaling; score 2, hair rarefaction and heavier scaling; score 3, hair loss and crust formation; score 4, alopecia and crusts.

2.5. Mycologic and histopathologic evaluation

Mycological evaluation. Epidermal flakes were scraped from the animals and hairs were collected manually at intervals of seven days after inoculation. The epidermal samples and hairs were seeded in tubes containing potato dextrose agar (Difco, Detroit, USA) and Sabouraud dextrose agar 2% (SGA; Difco, Detroit, USA), with chloramphenicol, and maintained in an incubator at 28° C.

Histopathological evaluation. The guinea pigs experimentally inoculated with *T. mentagrophytes* var. *mentagrophytes* were submitted to local anesthesia and skin fragments of their mycotic ears were collected with scissors and tweezers. These samples

were fixed in 10% buffered formalin, embedded in paraffin, cut into 5-m-thick sections, and stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). All the samples were immediately taken to the Histology Laboratory of the Department of Pathology and Forensic Medicine of Federal University of Ceará, where they were analyzed. The amount of fungal elements (hyphae and conidia), and degree of hyperkeratosis, acanthosis (epidermal hyperplasia), and spongiosis were evaluated semi-quantitatively by one pathologist in a blind setup. A positive histological examination was defined as detection of fungi.

3. Results

The experimental infection of guinea pigs with *T. mentagrophytes* resulted in lesions in all animals that were exposed to the fungus. The first signs of infection were observed on the 7th day after inoculation in all the infected animals and were manifested in the form of moderate scaling, corresponding to lesion score 1. These alterations became more evident around the 14th day, with the development of hair rarefaction and squamosis, score 2 (Fig. 1a). The lesions progressively increased in diameter, with total hair loss and crust formation, score 3, between the 18th and 21st days (Fig. 1b). Between the 22nd and 25th days, the inoculation site showed areas of alopecia and crusts, score 4 (Fig. 1c). *T. mentagrophytes* were re-isolated from hairs and epidermal flakes of all inoculated animals from day 7, 14, 21, 28 and 33 (Fig. 1d). The mycological cultures were positive in 100% of the inoculated animals.

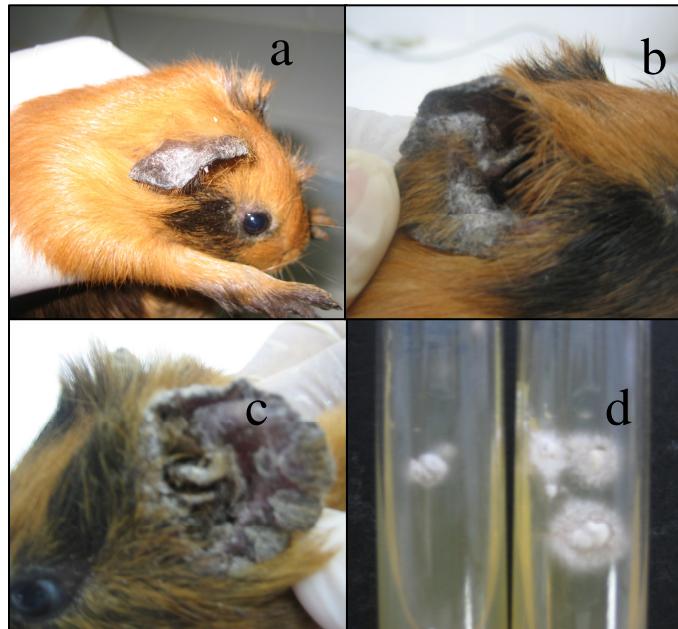


Fig. 1. Experimental lesion produced by *T. mentagrophytes* var. *mentagrophytes* in guinea pigs after 14 days of evolution (a), after 21 days (b), after 28 days (c) and colonies of *T. mentagrophytes* isolates from the guinea pigs.

The positive cases were related to the first week of infection. After the start of treatment with cetoconazol, on the 14th day, the mycological tests were negative. The group treated with cetoconazol had reduced lesion scores compared to the positive control during the entire experimental period.

In the histological sections stained with hematoxylin-eosin, the animals infected with *T. mentagrophytes* var. *mentagrophytes* showed moderate inflammatory infiltrate rich in lymphocytes, neutrophils and macrophages on the skin diffusely. We observed foci of granulation tissue with neovascularization, extravasation of red blood cells and collections of neutrophils (micro abscesses). The inflammatory infiltrate extended to the dermo-epidermal junction. There were also foci of parakeratosis with accumulations of

neutrophils (Fig. 5). *T. mentagrophytes* hyphae were detected in the stratum corneum in histological sections stained with PAS (Fig. 2).

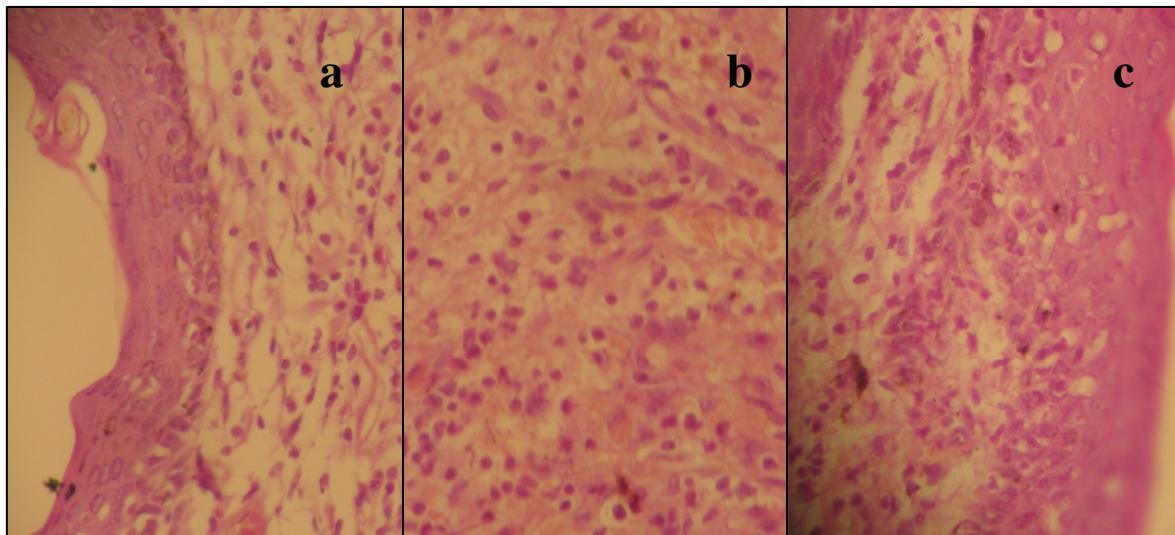


Fig. 2. Histological section of the skin of a guinea pig inoculated with *T. mentagrophytes* after 30 days of evolution. Histological sections showed moderate infiltrate rich in lymphocytes, neutrophils and macrophages, diffusely on the skin. Staining: hemosylin-eosin. Magnification: 200x

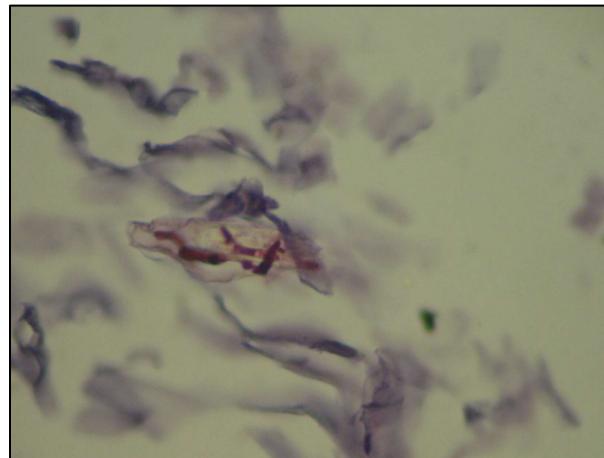


Fig. 3. Histological section of the skin of a guinea pig inoculated with *T. mentagrophytes* after 30 days of evolution. Presence of hyphal fragments in the stratum corneum, visible after staining with PAS. Magnification: 400x

4. Discussion

In this study a new model of experimental dermatophytosis was developed and showed itself to be practical and fast, without the need to use abrasions, bandages and general anesthesia, thus causing less harm and suffering to the animals. Such procedures have been described by various authors, such as Cavalcante et al. (2002). In that study, the hair in the posterior dorsal region of the animals was removed and a skin area was submitted to scarification with a scalpel. After the inoculation of the suspension (of *Microsporum canis*), the site was covered with polyethylene film and kept in place with an elastic bandage for 24h.

In comparison with the technique described by Cavalcanti et al. (2002) and other authors [8,9], the experimental model presented in this study, using the outer ear of guinea pigs without subjecting the animals' skin to any type of aggression or invasive procedures, showed significant results, both in relation to the clinical profile and time for the infection to appear.

The first signs of infection in this work were observed on the 7th day after inoculation in 100% of the animals, manifested in the form of scaling. These findings corroborate those described by Vermout et al. (2004), who observed signs, although slight, typical of dermatophytosis on the 7th day after inoculation in guinea pigs in evaluating immunogenicity and protective efficacy of a *Microsporum canis* metalloprotease subunit vaccine. Regarding the time for manifestation of the infection, our findings are similar to those described by Cavalcanti et al. (2002), where the first signs were detected around the 5th day after inoculation in 87.5% of the animals, through the presence of mild edema, erythema and mild shedding.

The literature, although extensive due to the many techniques of experimental infection described [1-4], does not report any study where the outer ear was the inoculation site, or techniques that do not require aggression and are less invasive for establishment of the infection. The method described here appears to cause less stress, harm and risk of death to the animals, and is quick and practical for developing experimental dermatophytosis.

The present results obtained by experimental induction of *T. mentagrophytes* var. *mentagrophytes* led us to conclude that the new model of experimental dermatophytosis in guinea pigs produces lesions similar to natural ones, is relatively simple to perform and is less traumatic for the animals.

Acknowledgements

The authors thank the financial support of FUNCAP (Ceará State Research Funding) and CNPq (National Counsel for Technological and Scientific Development; Brazil, Proc. CNPq: 478906/2004-8).

References

- [1] J.N. Cavalcanti, J.L. Guerra, W. Gambale, B. Corrêa, C.R. Paula, Histopathologic and mycologic aspects of experimental infection of guinea pigs with *Microsporum canis*, *Braz. J. vet. Res. anim. Sci.* 39 (2002) 238-243.
- [2] R.S.N. Brilhante, R.A. Cordeiro, D.J. Medrano, A.J. Monteiro, J.J.C. Sidrim, M.F.G. Rocha, Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can J Microbiol* 51 (2005) 507-510.
- [3] J.J.C. Sidrim, M.F.G. Rocha, *Micologia Médica à Luz de Autores Contemporâneos*. 2004.
- [4] R.S.N. Brilhante, C.S.P. Cavalcante, F.A. Soares-Júnior, R.A. Cordeiro, J.J.C. Sidrim, M.F.G. Rocha, High rate of *Microsporum canis* feline and canine dermatophytoses in Northeast Brazil: epidemiological and diagnostic features. *Mycopathology* 156 (2003) 303-308.
- [5] M.S.P. Arruda, S. Gilioli, F.R. Vilani-Moreno, Experimental Dermatophytosis in Hamsters inoculated with *Trichophyton mentagrophytes* in the cheek pouch. *Rev. Inst. Med. trop. S. Paulo* 43 (2001) 29-32.
- [6] P. Dalazen, A. Molon, M.W. Biavatti, M.R.O. Kreuger, Effects of the topical application of the extract of *Vernonia scorpioides* on excisional wounds in mice. *Braz. J. Pharmacog.* 15 (2005) 82-87.

- [7] A. Apisariyakul, N. Vanittanakom, D. Buddhasukh, Antifungal activity of turmeric oil extracted from *Curcuma longa* (Zingiberaceae). J. Ethnopharmacology 49 (1995) 163-169.
- [8] S. Lee, J. Han, G. Lee, M. Park, I. Choi, K. Na, E. Jeung. Antifungal Effect of Eugenol and Nerolidol against *Microsporum gypseum* in a Guinea Pig Model. Biol. Pharm. Bull 30 (2007) 184-188.
- [9] J. Guillot, L. Latié, M. Deville, L.Halos, R. Chermette, Evaluation of the dermatophyte test médium RapidVet-D. Vet. Dermatology 12 (2001) 123-127.
- [10] S.M. Vermout, F.D. Brouta, F.F. Descamps, B.J. Losson, B.R. Mignon. Evaluation of immunogenicity and protective efficacy of a *Microsporum canis* metalloprotease subunit vaccine in guinea pigs. Immunology and Medical Microbiology 40 (2004) 75-80.

CAPÍTULOS V

Antifungal effect of essential oil of *Lippia sidoides* against *Trychophyton mentagrophytes* var. *mentagrophytes* in guinea pigs

R.O.S. Fontenelle^a, S.M. Morais^{a,b*}, E. H. S. Brito^a, R.S.N. Brilhante^d, N. R. F. Nascimento^a, R. A. Cordeiro^d, S.G.C. Fonseca^e, J.T. Valença Júnior^c, P.R.N. Raquel^a, J..J.C. Sidrim^d, M.F.G. Rocha^{a,d}

^aVeterinary Faculty, Post-Graduation Program in Veterinary Sciences, State University of Ceará, Fortaleza, Ceará, Brazil

^bDepartment of Chemistry, State University of Ceará, Fortaleza, Ceará, Brazil

^cDepartment of Pathology and Legal Medicine, Faculty of Medicine, Federal University of Ceará, Fortaleza, Ceará, Brazil

^dDepartment of Pathology and Legal Medicine, Faculty of Medicine, Medical Mycology Specialized Center, Federal University of Ceará, Fortaleza, Ceará, Brazil

^eDepartment of Pharmacia, Faculty of Medicine, Federal University of Ceará, Fortaleza, Ceará, Brazil

Corresponding author: Marcos Fábio Gadelha Rocha. Rua Suécia. No. 171: Vila Betânia. CEP: 60 740-810. Fortaleza, Ceará, Brazil, Phone: 55 (85) 3232-3834. Fax: 55 (85) 3495-8692. E. mail: raquelbios@yahoo.com.br

Resumo

No presente estudo objetivou-se avaliar a atividade antifúngica do óleo essencial da *Lippia sidoides* em cobaias infectados com *T. mentagrophytes* var. *mentagrophytes*. A análise da atividade antifúngica, *in vitro*, determinando a concentração inibitória mínima (CIM) foi realizada através do método de microdiluição em caldo. O curso clínico da infecção e a avaliação da atividade antifúngica, *in vivo*, foram monitorados através de análises clínicas, micológicas e histológicas. A pomada com o óleo essencial da *L. sidoides* e base de carbopol foi manipulada na concentração de 1% e foi topicalmente aplicada na pele do pavilhão auditivo dos animais, infectados com *T. mentagrophytes* var. *mentagrophytes*, diariamente por 3 semanas. O óleo essencial da *L. sidoides* apresentou atividade antifúngica, *in vitro*, com o CIM de 0.019 mg/mL. *In vivo* o óleo essencial foi clinicamente efetivo com a diminuição das lesões depois da segunda semana de aplicação. Também foi observada negatividade nas culturas de pelo e escama de pele dos animais tratados com cetoconazol e *L. sidoides*. Os exames histopatológicos revelaram que o grupo tratado com o óleo essencial *L. sidoides* apresentou menor grau de hiperqueratose e infiltração das células inflamatórias que o controle positivo. Em conclusão os resultados sugerem que o óleo essencial da *L. sidoides* podem ser um achado promissor como nova droga antifúngica.

Abstract

This study investigates the antifungal activity of the essential oil of *Lippia sidoides* in guinea pigs infected by *T. mentagrophytes* var. *mentagrophytes*. Skin lesion scoring, hair culture and histopathological examination of skin tissues were performed and the minimum inhibitory concentration (MIC) was calculated to evaluate the antifungal effect of the essential oil of *Lippia sidoides*. The MIC was 0.019 mg/mL. The essential oil was a 1% concentration with a base of carbopol and was applied topically daily for three weeks to the skin lesion on the outside ear flap infected with *T. mentagrophytes* var. *mentagrophytes*. The essential oil of *L. sidoides* was clinically effective in improving the lesion during the third week of application, as determined by skin lesion scoring. The hair culture test was also negative and the histopathological examination revealed that treated groups had a lower degree of hyperkeratosis and inflammatory cell infiltration than the positive control. Taken together, these results suggest that the essential oil of *L. sidoides* may be a promising source in the search for new antifungal drugs.

1. Introduction

Dermatophytes are a group of highly specialized fungi which, through a long evolutionary process, have become able to invade, colonize and maintain themselves in keratinized tissues. These organisms are divided into three genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. (Brilhante et al., 2005; Sidrim & Rocha., 2004). In Brazil, the most frequently diagnosed agents in the clinical dermatology of dogs and cats are *Microsporum canis*, *Trichopyton mentagrophytes* and *Microsporum gypseum* (Brilhante et al., 2005).

Widespread in northeast Brazil, *Lippia* species are known for their natural topical antiseptic properties. Previous studies have reported that the essential oil of *Lippia sidoides* Cham. shows antimicrobial activity *in vitro* as well as larvicidal effect against *Aedes aegyptii* (Carvalho et al., 2003; Botelho et al., 2007). Another study by Fontenelle et al. (2007) found *in vitro* antifungal activity of the essential oil da *Lippia sidoides* against different strains of *Microsporum canis* and *Candida* spp. In the present study, the antifungal activity of the essential oil of *Lippia sidoides* was evaluated *in vitro* and *in vivo* against *T. mentagrophytes* var. *mentagrophytes*.

2. Materials and Methods

2.1. Plant material and essential oil extraction

The plant samples were collected in the city of Horizonte, Ceará ($3^{\circ}33'46''$ S latitude, $41^{\circ}05'42''$ W longitude), in northeast Brazil. Taxonomic identification was confirmed by experts at the Prisco Bezerra Herbarium (Federal University of Ceará), where a voucher sample was deposited with reference number 25149. The *L. sidoides* essential oil was extracted from the leaves by steam distillation in a Clevenger apparatus, as described by Craveiro et al. (1976).

2.2. Experimental Animals

Twenty guinea pigs of both sexes, weighing approximately 600g, were housed in controlled rooms (temperature: $23\pm2^{\circ}$ C, relative humidity: $50\pm10\%$, frequent ventilation and 12-hour light cycle). All protocols that included animals were approved by the research ethics committee of the State University of Ceará, Fortaleza, Brazil. The animals were used as recommended by the guide for the care and use of laboratory animals from

the National Academy Press (USA; 1996), which is in line with the principles for animal use in Brazil.

2.3. Fungal strain

The *T. mentagrophytes* var. *mentagrophytes* strain (CEMM 1-4-085) was obtained from the fungal collection of the Specialized Medical Mycology Center – CEMM (Federal University of Ceará), where it was maintained in saline (0.9% NaCl) at 28° C. At the time of the analysis, an aliquot of each suspension was taken and inoculated in potato dextrose agar (Difco, Detroit, USA), and then incubated at 28 ° C for six days. The identification of *T. mentagrophytes* var. *mentagrophytes* was based on phenotypic features, such as a description of the macro and micromorphology. Skin perforation and vitamin requirement tests were considered, as well as the production of the enzyme urease (Brilhante et al., 2003).

2.4. Determination of the Minimum Inhibitory Concentration (MIC)

Inoculum preparation for antifungal susceptibility tests

For the broth microdilution method, the standardized inocula (5×10^4 cfu/mL) for *T. mentagrophytes* var. *mentagrophytes* were prepared by turbidimetry. Stock inocula were prepared on day 10 for *T. mentagrophytes* var. *mentagrophytes* grown on potato dextrose agar at 28° C. Sterile saline solution (0.9%) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *T. mentagrophytes* var. *mentagrophytes*. The suspensions of conidia with hyphal fragments of *T. mentagrophytes* var. *mentagrophytes* were transferred to sterile tubes and the volume of both suspensions was adjusted to 4 mL with sterile saline solution. The resulting suspension was allowed to settle for five minutes at 28° C, and its density was read at 530nm and then adjusted to 95% transmittance. The suspensions were diluted to 1:500 for *T. mentagrophytes* var. *mentagrophytes*, with both RPMI 1640 medium (Roswell Park Memorial Institute – 1640) and L-glutamine, buffered to pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.), to obtain an inoculum size of approximately 5×10^4 cfu/mL for *T. mentagrophytes* var. *mentagrophytes*.

Broth microdilution method

The broth microdilution assay for *T. mentagrophytes* var. *mentagrophytes* was performed as previously described by Jessup et al., (2000), Fernandez-Torres et al. (2002) and Brilhante et al. (2005), based on the M38-A document (CLSI; formerly NCCLS M38A 2002).

For the susceptibility analysis, the essential oil was diluted in mineral oil and tested at a concentration range between 0.002 and 5 mg/mL. The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each isolate tested. The microplates were incubated at 37° C and read visually after five days for *T. mentagrophytes* var. *mentagrophytes*. The isolate was run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 80% inhibition of visible fungal growth. The results were read visually as recommended by CLSI.

2.5. Animal Infection

The animal model for infection was chosen based on Fontenelle (2008). The stock inocula of *T. mentagrophytes* var. *mentagrophytes* were prepared on day 10, grown on potato dextrose agar (Difco, Detroit, Mi.), at 28° C. The suspensions with hyphal fragments were transferred to a sterile tube and adjusted by turbidimetry to obtain an inoculum of approximately 10⁶ cfu/ml. The optical densities of the suspensions were spectrophotometrically determined at 530 nm. The suspension (0.5 ml) was gently inoculated on the ear flap with a swab, a single time. The guinea pigs were divided into three groups of four animals each: two groups treated on both the right and left ears and a negative control that was not inoculated with the fungal suspension. One of the groups infected with *T. mentagrophytes* var. *mentagrophytes* was then treated with ketoconazol. The fungal infection in each animal was confirmed by hair cultures and clinical evaluation of infected skin lesions.

2.6. Drug Application

The topical drugs were mixed in carbopol as a vehicle. The concentrations of the *L. sidoides* essential oil and ketoconazol were 1% and 2%, respectively. The drugs were applied topically to the infected animals as ointments, with 0.2 g of carbopol as a positive control, daily for three weeks from 14 days post-inoculation.

2.7. Evaluation of Efficacy

Drug efficacy was evaluated every seven days following treatment, through clinical lesion scoring, hair culture and histopathological examination of skin tissues. Clinical assessment of the guinea pigs was done according to a modification of the method published by Lee et al. (2007). The lesions were clinically followed daily, starting on day 0, until resolution was observed. The clinical evaluation of the inoculated animals was performed using a modified lesion score from 0 to 4, as follows: score 0, no visible lesion; score 1, moderate scaling; score 2, hair rarefaction and heavier scaling; score 3, hair loss and crust formation; score 4, alopecia and crusts.

2.7. Mycological and histopathological evaluation

Mycological evaluation. Epidermal flakes were scraped from the animals and hairs were collected manually at intervals of seven days after inoculation. The epidermal samples and hairs were seeded in tubes containing potato dextrose agar (Difco, Detroit, Mi.) and Sabouraud dextrose agar 2% (SGA; Difco, Detroit, Mi.), with chloramphenicol, and maintained in an incubator at 28° C.

Histopathological evaluation. The guinea pigs experimentally inoculated with *T. mentagrophytes* var. *mentagrophytes* were submitted to local anesthesia and skin fragments of their mycotic ears were collected with scissors and tweezers. These samples were fixed in 10% buffered formalin, embedded in paraffin, cut into 5- μm -thick sections, and stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). All the samples were immediately taken to the Histology Laboratory of the Department of Pathology and Forensic Medicine of Federal University of Ceará, where they were analyzed. The amount of fungal elements (hyphae and conidia), and degree of hyperkeratosis, acanthosis (epidermal hyperplasia), and spongiosis were evaluated semi-quantitatively by a single pathologist in a blind setup. A positive histological examination was defined as detection of fungi.

3. Results

MIC. The broth microdilution method showed that the MIC of the essential oil of *L. sidoides* for *T. mentagrophytes* var. *mentagrophytes* was 0.019 mg/mL.

Skin Lesion Scoring. The first signs of infection were observed on the seventh day after inoculation in all the infected animals and were manifested in the form of slight scaling, corresponding to lesion score 1. These alterations became more evident and well-defined around the fifteenth day (the first day of drug treatment), with hair loss and crust

formation, corresponding to lesion score 3. In contrast, the lesion scores of the positive control were maintained until eight days after treatment, while the scores of the treated groups continuously decreased following topical treatment. The groups treated with the essential oil of *L. sidoides* and cetoconazol had lower lesion scores than the positive control group.

Hair Culture. On day 10, all hairs of the inoculation areas were positive on culture medium. The hair cultures of the groups treated with *L. sidoides* essential oil and cetoconazol gradually declined with time. The hairs of the cetoconazol-treated group did not produce a fungal colony after day eight days of treatment.

4. Discussion

Various essential oils have long been used in popular medicine for many applications, among them to produce topical antiseptics (Nascimento et al., 2007). This has prompted a great deal of scientific interest seeking to confirm the antimicrobial activity of essential oils (Almeida et al., 2006; Arruda et al., 2006; Lee et al., 2007; Fontenelle et al., 2007; Fontenelle et al., 2008; Sharma and Tripathi, 2008; Matan and Matan, 2008; Martos et al., 2008).

In this effort, many methods of *in vitro* and *in vivo* investigation have been developed producing reliable results that can be reproduced and validated. However, this task has been hampered by the peculiarities of many essential oils, such as volatility, insolubility in water and complexity, all of which significantly interfere in the results. For this reason, tests of microbial susceptibility must take into account the technique used, the culture medium, microorganisms and essential oil studied. In this study, we evaluated the antifungal activities the essential oil of *L. sidoides* *in vitro* and *in vivo* against a strain of *T. mentagrophytes* var. *mentagrophytes* by the broth microdilution and experimental dermatophytosis methods, respectively.

Earlier reports on *L. sidoides* essential oil revealed its antimicrobial action. Lemos et al. (1990) reported the highest and broadest activity against bacteria and fungi, including yeasts, dermatophytes and non-dermatophyte fungi. Fontenelle et al. (2007) showed that the essential oil of *L. sidoides* is very effective against *M. canis*, superficial fungal infection in cats and dogs worldwide (Brilhante et al., 2003; Brilhante et al., 2005), and against *Candida* spp., which are important yeasts involved in human and animal mycosis (Aperis et al., 2006; Colombo et al., 2006; Brito et al., 2007). In the present study, the

essential oil of *L. sidoides* was active against a strain of *T. mentagrophytes* var. *mentagrophytes*, a dermatophyte that most often infects guinea pigs.

By the *in vitro* and *in vivo* evaluation, this study shows that the essential oil of *L. sidoides* has fungicidal activity. There was a good correlation between the *in vitro* and *in vivo* activity, showing the broad antifungal activity of this oil.

In previous studies the oils, extracts and constituents of plants have been tested *in vivo* and showed antifungal activity (Apisariyaul et al., 1995; Dalazen et al., 2005; Lee et al., 2007). Experimental infections are a valuable instrument for the study of the pathogenesis of infectious fungi, to evaluate the prophylactic efficacy of therapeutic antifungal drugs. In this study, treatment of the outer ear flap of infected guinea pigs with an ointment containing the essential oil of *L. sidoides* reduced the infection after three weeks, corroborating the report of Dalazen et al. (2005), who evaluated the activity of an ethanol extract of *Vernonia scorpioides* against the microorganisms in infected ulcers and found improvement 20 days after infection.

The chemical composition of this oil was described previously by Fontenelle et al. (2007). The main component is thymol (59.65%). This constituent is described in several articles as an antifungal agent (Pina-Vaz et al., 2004; Pinto et al., 2006). Bennis et al. (2004) studied the action mechanism of this constituent and observed surface alteration induced by thymol on yeast, concluding that its antifungal activity involves alterations of both the yeast membrane and cell wall.

The acute and sub-chronic toxicity of this oil was also described by Fontenelle et al. (2007). Due to its broad spectrum of antifungal effect, both *in vitro*, and *in vivo*, allied with low toxicity, the essential oil of *L. sidoides* is a promising source in the search for new antifungal drugs.

Acknowledgements

The authors thank the financial support of FUNCAP (Ceará State Research Funding) and CNPq (National Counsel for Technological and Scientific Development; Brazil, Proc. CNPq: 478906/2004-8).

References

- [1] Aperis G, Myriounis N, Spanakis EK *et al.* Developments in the treatment of candidiasis: more choices and new challenges. *Expert Opin Investig Drugs* 2006; **15**: 1319
- [2] A. Apisariyakul, N. Vanittanakom, D. Buddhasukh, Antifungal activity of turmeric oil extracted from *Curcuma longa* (Zingiberaceae). *J. Ethnopharmacology* 49 (1995) 163-169.
- [3] C. F. C. Almeida, E. L. C. Amorin, U. P. Albuquerque and M. B. Maia. Medical plants popularly used in the Xingó region – a semi-arid location in Northeastern Brazil. **Journal Ethnobiol Etnomed**, v. 15, p. 1-7, 2006.
- [4] Botelho, M.A., Nogueira, N.A.P, Bastos, G.M., Fonseca, S.G.C., Lemos, T.L.G., Matos, F.J.A., Montenegro, D., Heukelbach, J., Rao, V.S. and Brito, G.A.C. (2007) Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res* 40, 349
- [5] R.S.N. Brilhante, R.A. Cordeiro, D.J. Medrano, A.J. Monteiro, J.J.C. Sidrim, M.F.G. Rocha, Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. **Can J Microbiol** 51 (2005) 507-510.
- [6] Brito, E.H.S., Fontenelle, R.O.S., Brilhante, R.S.N., Cordeiro, R.A., Soares Junior, F.A., Sidrim, J.J.C. and Rocha, M.F.G. (2007) Phenotypic characterization and *in vitro* antifungal sensitivity of *Candida* spp. and *M. pachydermatis* strains from dogs. *Vet J*, **174**, 147-153.

- [7] Carvalho, AFU, Melo, VMM, Craveiro, AA *et al.* Larvicidal activity of the essential oil from *Lippia sidoides* Cham. Against *Aedes aegypti* linn. *Mem Inst Oswaldo Cruz* 2003; **98**: 569-71.
- [8] Colombo AL, Nucci M, Park BJ *et al.* Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *J Clin Microbiol* 2006; **44**: 2816
- [9] Craveiro, A.A., Matos F.J.A. and Alencar, J.W. (1976) A simple and inexpensive steam generator for essential oils extraction. *J Chem Edu* **53**, 652.
- [10] Fernandez-Torres B, Cabanes FJ, Carrillo-Munoz AJ *et al.* Collaborative evaluation of optimal antifungal susceptibility testing conditions for dermatophytes. *J Clin Microbiol* 2002; **40**: 3999-4003.
- [11] Fontenelle ROS, Morais SM, Brito EHS, Kerntopf MR, Brilhante RSN, Cordeiro RA, Tomé AR, Queiroz MGR, Nascimento NRF, Sidrim JJC, Rocha MFG. Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. *J Antimicrob Chem* 2007; **59**: 934
- [12] Fontenelle ROS, Morais SM, Brito EHS, Brilhante RSN, Cordeiro RA, Nascimento NRF, Kerntopf MR, Sidrim JJC, Rocha MFG. Antifungal activity of essential oils of *Croton* species from the Brazilian *Caatinga* biome. *J Appl Microbiol* 2008; **104**: 1383
- [13] Jessup, C.J., Warner, J., Isham, I., Hasan, I. and Ghannoum, M.A. (2000) Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *J Clin Microbiol* 38, 341
- [14] J.J.C. Sidrim, M.F.G. Rocha, Micologia Médica à Luz de Autores Contemporâneos. 2004.
- [15] R.S.N. Brilhante, C.S.P. Cavalcante, F.A. Soares-Júnior, R.A. Cordeiro, J.J.C. Sidrim, M.F.G. Rocha, High rate of *Microsporum canis* feline and canine dermatophytoses in Northeast Brazil: epidemiological and diagnostic features. *Mycopathology* 156 (2003) 303-308.
- [16] M.S.P. Arruda, S. Gilioli, F.R. Vilani-Moreno, Experimental Dermatophytosis

- in Hamsters inoculated with *Trichophyton mentagrophytes* in the cheek pouch. Rev. Inst. Med. trop. S. Paulo 43 (2001) 29-32.
- [17] P. Dalazen, A. Molon, M.W. Biavatti, M.R.O. Kreuger, Effects of the topical application of the extract of *Vernonia scorpioides* on excisional wounds in mice. **Braz. J. Pharmacog.** 15 (2005) 82-87.
- [18] S. Lee, J. Han, G. Lee, M. Park, I. Choi, K. Na, E. Jeung. Antifungal Effect of Eugenol and Nerolidol against Microsporum gypseum in a Guinea Pig Model. **Biol. Pharm. Bull** 30 (2007) 184-188.
- [19] Lemos TLG, Matos FJA, Alencar JW *et al*. Antimicrobial activity of essential oils of Brazilian plants. *Phytother Res* 1990; **4**: 82
- [20] Martos, M. V.; Navajas, Y. R.; Lopez, J. F.; Lavarez, J. P. Antifungal activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) essential oils. **Food Control**, v. 19, p. 1130-1138, 2008.
- [21] Matan, N. & Matan, N. Antifungal activities of anise oil, lime oil, and tangerine oil against molds on rubberwood (*Hevea brasiliensis*). **International Biodeterioration & Biodegradation**, v. 62, p. 75-78, 2008.
- [22] C. Pina-Vaz, A. Gonçalves Rodrigues, E. Pinto *et al*. Antifungal activity of *Thymus* oils and their major compounds. **J Eur Acad Dermatol Venereol** 2004; **18**: 73
- [22] Pinto E, Pina-Vaz C, Salgueiro L *et al*. Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species. *J Med Microbiol* 2006; **55**: 1367-73.
- [23] Sharma, N. & Tripathi, A. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. *Microbiological Research*, v. 163, p. 337

9 CONCLUSÕES GERAIS

- Os óleos essenciais das três espécies de *Croton* apresentaram atividade, *in vitro*, contra cepas de *M. canis*. Entretanto, apenas o *Croton zenhteneri* apresentou atividade contra cepas de *Candida* spp.;
- O óleo essencial da *Lippia sidoides* apresentou atividade, *in vitro*, contra cepas de *M. canis*, *T. mentagrophytes* e *Candida* spp.;
- A administração, por longo prazo, de OELs é relativamente segura e, provavelmente, desprovida de toxicidade significativa;
- Os constituintes majoritários dos óleos essenciais apresentaram atividade antifúngica, *in vitro*, contra cepas de *M. canis* e *Candida* spp.;
- O óleo essencial da *Lippia sidoides* apresentou atividade *in vivo*, contra *T. mentagrophytes*.

10 PERSPECTIVAS

- A partir deste estudo sobre a atividade antifúngica dos óleos essenciais da *Lippia sidoides*, *Croton zenhteneri*, *Croton argyrophyilloides* e *Croton nepetaefolius*, bem como de seus principais constituintes contra cepas de *Microsporum canis*, *Trichophyton mentagrophytes* e *Candida* spp. e dos resultados dos testes de sensibilidade e toxicidade, observou-se que essas plantas constituem fontes viáveis para uma análise farmacológica mais específica para o tratamento de diversas enfermidades causadas por fungos, o que possibilita a realização de testes com grupos alvo, visando à otimização de novas drogas com atividade antifúngica.

11 REFERÊNCIAS GERAIS

- ABDELGALEIL, S. A. M.; ABBASSY, M. A.; BELAL, A.S.; RASOUL, M. A. A. A. Bioactivity of two major constituents isolated from the essential oil of *Artemia judaica* L. **Bioresource Technology**, v. 99, p. 5947-5950, 2008.
- ALMEIDA, C. F. C. B. R.; AMORIM, E. L. C.; ALBUQUERQUE, U. P. and MAIA, M. B. Medical plants popularly used in the Xingó region – a semi-arid location in Northeastern Brazil. **Journal Ethnobiol Etnomed**, v. 15, p. 1-7, 2006.
- AMARAL, J. F. DO. **Atividade antiinflamatória, antinociceptiva e gastroprotetora do óleo essencial do Croton sonderianus Muell. Arg.** Dissertação (Mestrado em Farmacologia). Universidade Federal do Ceará, 2004.
- BAJPAI, V. K.; SHUKLA, S.; KANG, S. C. Chemical composition and antifungal activity of essential oil and various extract of Silene armeria L. **Bioresource Technology**, doi: 10.1016/j.biortech, 2008.
- BARRACA, S. A. **Manejo e produção de plantas medicinais e aromáticas.** Relatório do estágio supervisionado em Produção Vegetal II. Universidade de São Paulo, 1999.
- BARRETO, J. E. F. **Efeito gastroprotetor do óleo essencial do Croton zenhtneri Pax et Hoffm.** Dissertação (Mestrado em Ciências Fisiológicas). Universidade Estadual do Ceará, 2005.

BARROS, S. B. M; DAVINO, S. C. Em: OGA, S. **Fundamentos de Toxicologia**, p. 61-70. Editora Atheneu, São Paulo, 1996.

BERTINI, L.M., PEREIRA, A.F., OLIVEIRA, C.L.L., MENEZES, E. A., MORAIS, S.M., CUNHA, F.A. and CAVALCANTE, E.S.B. Perfil de sensibilidade de bactérias frente a óleos essenciais de algumas plantas do Nordeste do Brasil. **Infarma**, v. 17, 80-83, 2005.

BOTELHO, M. A.; BASTOS, G. M.; FONSECA, S. G. C.; MATOS, F. J. A.; MONTENEGRO, D.; RAO, V. S. and BRITO, G. A. C. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. **Braz J. Med. Biol. Res.** v. 40, P. 349-356, 2007.

BOURNE, K. Z.; BOURNE, N.; REISING, S. F.; STANBERRY, L. R. Plant products as topical microbicide candidates: assessment of *in vitro* and *in vivo* activity against herpes simples virus type 2. **Antiviral Research**, v. 42, p.219-236, 1999.

BRILHANTE, R. S. N.; CAVALCANTE, C. S. P.; SOARES-JÚNIOR, F. A.; CORDEIRO, R. A.; SIDRIM, J. J. C.; ROCHA, M. F. G. High rate of *Microsporum canis* feline and canine dermatophytoses in Northeast Brazil: epidemiological and diagnostic features. **Mycopathology**, v. 156, p. 303-308, 2003.

BRITO, E.H.S.; FONTENELLE, R.O.S.; BRILHANTE, R.S.N.; CORDEIRO, R.A.; SOARES JÚNIOR, F.A.; SIDRIM, J.J.C.; ROCHA, M.F.G. (2007) Phenotypic characterization and *in vitro* antifungal sensitivity of *Candida* spp. and *M. pachydermatis* strains from dogs. **Vet J**, v.174, p. 147-153, 2007.

BROWN, M. R; THOMPSON, C. A.; MOHAMED, F. M. Systemic candidiasis in an apparently immunocompetent dog. **Journal of Veterinary Diagnostic Investigation**, v. 17, p. 272-276, 2005.

BRUNETON, J. **Pharmacognosy Phytochemistry Medicinal**. In: BRUNETON, J. Terpenoids and steroids essential oils. 2 ed., Inglaterra: Lavoisier Publishing. p. 405-426, 1995.

CABAÑES, F.J. Dermatofotosis animales. Recientes avances. **Rev. Iberoam Micology**. v. 17, p. 8-12, 2000.

CAFARCHIA, C.; ROMITO, D.; CAPELLI, G.; GUILLOT, J.; OTRANTO, D. Isolation of *Microsporum canis* from the hair coat of pet dogs and cats belonging to owners diagnosed with *M.canis* tinea corporis. **Europea Society of Veterinary Dermatology**, v. 17, p. 327-331, 2006.

CALIXTO, J. B.; BEIRITH, A.; FERREIRA, J. Naturally occurring antinociceptive substances from plants. **Rev. Phytotherapy**, v. 14, p. 401-418, 2000.

CANUTO, K. M. **Efeito atinociceptivo e antiedematógeno do óleo essencial do Croton argyrophylloides Muell. Arg.** Dissertação (Mestrado em Ciências Fisiológicas). Universidade Estadual do Ceará, 2005.

CATUNDA JÚNIOR, F. E. A. **Estudo químico dos óleos essenciais de espécies do gênero Croton**. Monografia (Lic. Plena em Química). Universidade Estadual do Ceará, 2003.

CHANG, H. T.; CHENG, Y. H.; WU, C. L.; CHANG, S. T.; CHANG, T. T. and SU, Y. C. Antifungal activity of essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* Florin leaf against plant pathogenic fungi. **Bioresource Technology**, v. 99, p. 6266-6270, 2008.

COELHO-DE-SOUZA, A. N.; CRIDDLE, D. N. e LEAL-CARDOSO, J. H. Selective modulatory effect of the essential oil of *Croton zehntneri* on isolated smooth preparations of the guinea-pig. **Phytotherapy Research**, v. 12, p. 189-194, 1997.

COSTA, T. R.; FERNANDES, O. F. L.; SANTOS, S. C.; OLIVEIRA, C. M. A.; LIÃO, L. M.; FERRI, P. H.; PAULA, J. R. P.; FERREIRA, H. D.; SALES, H.N.; SILVA, M. R. R. Antifungal activity of volatile constituents of *Eugenia dysenterica* leaf oil. **Journal of Ethnopharmacology**, v. 72, p. 111-117, 2000.

COSTA, S. M. **Contribuição ao conhecimento químico de plantas do Nordeste do Brasil *Lippia sidoides* Cham.** Tese (doutorado em Química Orgânica). Universidade Federal do Ceará, 2001.

COTÉ, L. & OLENSON, J. J. Antagonism of anethole of adrenhal extract on glycogen formation in adrenalectomized rats. **Fed. Proc.** V. 10, p. 175-176, 1951.

COUNCIL OF EUROPE. **Opinion of the scientific committee of food on estragol (1-allyl-4-methoxybenzene).** Strasbourg: Committee of Experts on Flavouring Substances. Adopted on 26 September, 2001.

CRAVEIRO, A. A.; ALENCAR, J. W.; MATOS, F. J. A. Estudo de óleos essenciais de plantas do Nordeste brasileiro. **Ciência Cultural**, v. 28, p. 180, 1976.

CRAVEIRO, A. C.; FERNANDES, A. G.; ANDRADE, C. H. S.; MATOS, F. J. A.; ALENCAR, J. W.; MACHADO, M. I. L. **Óleos Essenciais de plantas do Nordeste.** Fortaleza, CE: Edições UFC, 1981.

CRUZ, G. M. P. **Efeito do eugenol e do metil-eugenol sobre o potencial composto do nervo ciático do rato.** Dissertação de Mestrado. Universidade Estadual do Ceará, 2001.

CRUZ, M. C.S.; SANTOS, P. O.; BARBOSA, A. M. JR.; D. L. F. M.; ALVIANO, C. S.; ANTONIOLLI, A. R.; ALVIANO, D. S. and TRINDADE, R. C. Antifungal activity of Brazilian medicinal plants involved in popular treatment of mycoses. **Journal Ethnopharmacol**, v.111, p. 409-412, 2007.

CRESPO, M. J.; ABARCA, M. L.; CABANES, F. J. Evaluation of different Preservation and Store Methods for *Malassezia* spp. **Journal Clinic Microbiology**, v. 38 (10), p. 3872-5, 2000.

DALLMEIER, K.; CARLINI, E. A. Aesthetic, hypothermic, myorelaxant and antivonvulsant effects of synthetic eugenol derivates. **Pharmacology**, v. 22, p. 113-127, 1981.

DEBA, F.; XUAN, T. D.; YASUDA, M.; TAWATA, S. Chemical composition and antioxidant, antibacterial and antifungal acvties of the essential oils from *Bidens pilosa* Linn. Var. *Radiata*, v. 19, p. 346-352, 2008.

DE VINCENZI, M.; SILANO, M.; MAIALETTI, F.; SCAZZOCCHIO, B. Constituents of aromatic plants: II. **Fitoterapia**, Milão, Itália, v. 71, p. 725-729, 2000.

DIKBAS, N.; KOTAN, R.; DADASOGLU, F.; SAHIN, F. Control of *Aspergillus flavus* with essential oil and methanol extract of *Satureja hortensis*. v. 124, p. 179-182, 2008.

DUARTE, E. R.; RESENDE, J. C.; ROSA, C. A.; HAMDAN, J. S. Prevalence of yeasts and mycelia fungi in bovine parasitic otitis in the State of Minas Gerais, Brazil. **Journal of Veterinary Medicine Series B – Infectious Diseases and Veterinary Public Health**. v.48, p. 631-635, 2001.

DUARTE, M. C. T., FIGUEIRA, G. M., SANTORATTO, A.; REHDER, V. L. G. and DELARMELINA, C. Anti-*Candida* activity of Brazilian medicinal plants. **Journal Ethopharmacology**, v. 97, p. 305-311, 2005.

EATON, D. L.; KLAASSEN, C. D. **Principles of toxicology**. Em: Casarett and Doull's toxicology: the basic science of poisons. 5^a ed. McGraw-hill, New York, 1995.

ELAD, D.; BRENNER, J.; MARKOVICS, A.; YAKOBSON, B.; SHLOMOVITZ, S.; BASAN, J. Yeasts in the gastrointestinal tract of reweaned calves and possible involvement of *Candida glabrata* in neonatal calf diarrhea. **Mycopathologia**, v. 141, p. 7-14, 1998.

ERBS, G.; JENSEN, T. T.; SILIPO, A.; GRANT, W.; DOW, J. M.; MOLINARO, A.; PARRILI, M.; NEWMAN, M. A. An antagonist of lipid A action in mammals has complex effects on lipid A induction of defence responses in the model plant *Arabidopsis thaliana*. **Microbes and Infection**, v. 10, p. 571-574, 2008.

ESCOBAR, R. G. Eugenol: propriedades farmacológicas y toxicológicas. Ventajas y desvantajas de su uso. **Review Cubana Estomatology**, v.39, 2002.

FENNER, R. As plantas utilizadas na medicina popular brasileira com potencial atividade antifúngica. **Revista Brasileira de Ciências Farmacêuticas, Brazilian Journal of Pharmaceutical Sciences.** v. 42, p. 369-394, 2006.

FERNANDES, A. G.; ALENCAR, J. W.; MATOS, F. J. A. Canelas silvestres nordestinas: aspectos botânicos, químicos e farmacológicos. **Ciencia Cultural,** v. 32, p. 26-33, 1971.

FONTENELLE, R. O. S.; MORAIS, S. M.; BRITO, E. H. S.; KERNTOPF, M. R.; BRILHANTE, R. S. N.; CORDEIRO, R. A.; TOMÉ, A. R.; QUEIROZ, M. G. R.; NASCIMENTO, N. R. F.; SIDRIM, J. J. C.; ROCHA, M. F. G. Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. **J Antimicrob Chem.** v.59, p. 934-940, 2007.

FRANCHOME, P.; PENOEL, D. D. L'aromatherapie exactement – **Encyclopedie de l'utilisation therapeutique des huiles essentielles.** Limoges: Roger Jolois, 1995.

GIORDANI, R.; HADEF, Y.; KALOUSTIAN, J. Compositions and antifungal activities of essential oils of some Algerian aromatic plants. **Fitoterapia,** v. 79, p. 199-203, 2008.

GARCIA, M. E.; BLANCO, J. L. Principales enfermedades fúngicas que afectan a los animales domésticos. **Rev. Iberoam Micology,** v. 17, p. S2-S7, 2000.

GUERRERO, M. F. Quercetin 3, 7 – dimethyl ether: a vasorelaxante flavonoid isolated from *Croton schiedeanus* Schlecht. **Journal Pharm. Pharmacol.,** v. 54, p. 1373-1378, 2002.

GUPTA, A. K.; KOHLI, Y.; FAERGEMANN, J.; SUMMERBELL, R. C. *In vitro* susceptibility of the seven *Malassezia* species to ketoconazole, voriconazole, itraconazole and terbinafine. **British Journal of Dermatology**, v. 142, p. 758-765, 2000.

GURGEL, L. A.; SIDRIM, J. J. C.; MARTINS, D. T.; CECHINEL-FILHO, V. S. *In vitro* antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. **Journal of Ethnopharmacology**, v. 97, 409-412, 2005.

HESELTINE, J. C.; PANCIERA, D. L.; SAUNDERS, G. K. Systemic candidiasis in a dog. **Journal of the American Veterinary Medical Association**, v. 223, p. 821-824, 2003.

HIBBET, D. S.; BINDER, M.; BISCHOFF, J. F.; BLACKWELL, M.; CANNON, F.; ERIKSSON, O. E.; HUHNDORF, S. et al. A higher-level phylogenetic classification of the fungi. **Mycological Research**, v. 111, p. 509-547, 2007.

IROBI, O. M. & DARAMBOLA, S. O. Antifungal activities of crude extract of *Mitracarpus villosus* (Rubiaceae). **Journal of Ethnopharmacology**, v. 40, p. 137-140, 1993.

JAWETZ, E.; MELNICK, L. J.; ADELBERG, E. A. **Microbiología Médica**, Ed. Guanabara, 15 ed, p. 568, 1988.

JIN, Y. & LIN, D. Fungal urinary tract infections in the dog and cat: a retrospective study (2001-2004). **Journal of the American Animal Hospital Association**, v. 41, p. 373-381, 2005.

KORDALI, S.; CAKIR, A.; OZER, H.; CAKMAKCI, R.; KESDEK, M.; METE, E. Antifungal, phytotoxic and insecticidal properties of essential oil isolated from *Turkish Origanum acutidens* and its three components, carvacrol, thymol and ρ -cymene. **Bioresource Technology**, doi: 10.1016/j.biortech.2008.04.048, 2008.

KOTAN, R.; KORDALI, S.; CAKIR, A.; KESDEK, M.; KAYA, Y.; KILIC, H. Antimicrobial and insecticidal activities of essential oil isolated from Turkish *Salvia hydrangea* DC. Ex Benth. **Biochemical Systematics and Ecology**, v. 36, p. 360-368, 2008.

KOZAK, M.; BILEK, J.; BELADICOVA, V.; BELADIKOVA, K.; BARANOVA, Z.; BUGARSKY, A. Study of the dermatophytes in dogs and the risk of human infection. **Bratisl. Lek. Listy**, v. 19, p. 211-217, 2003.

LAHLOU, S.; LEAL-CARDOSO, J. H.; MAGALHÃES, P. J. Essential oil of *Croton nepetaefolius* decreases blood pressure through an action upon vascular smooth muscle: studies in DOCA-salt hypertensive rats. **Planta Med.** v.66, p. 138-143, 2000.

LAVABRE, M. **Aromaterapia**: a cura pelos óleos essenciais. 2 ed. Rio de Janeiro: Record, 1993.

LE BOURHIS, B. & SOENEN, A. M. Recherches sur lactino psychotrope de quelques substancias aromatiques utilices alimentation. **Food Cosmet. Toxicol.**, v. 11, p. 1-9, 1973.

LEE, S. J.; HAN, J.; LEE, G S.; PARK, M.; CHOI, I. G.; NA, K. J.; JEUNG, E. B. Antifungal effect of eugenol and nerolidol against *Microsporum gypseum* in a Guinea Pig Model. **Biol. Pharm. Bull.**, v. 30, p. 184-188, 2007.

LIMA, C. C. **Efeito miorelaxante do metileugenol em músculos liso de roedores.** Máster Thesis, Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, Fortaleza, Ceará, CE, Brazil, 1998.

LIMA, C. C.; CRIDDLE, D. N.; COELHO-DE-SOUZA, A. N.; MONTE, F. J. Q.; JAFFAR, M.; LEAL-CARDOSO, J. H. Relaxant and antispasmodic action of methyleugenol on guinea-pig isolated. **Planta Medica**, v. 66, p. 408-411, 2000.

LOPES JÚNIOR, J. E. G. **Avaliação da atividade citotóxica, antimicrobiana, larvicida e antiinflamatória de óleos essenciais do *Croton zehntneri* PAX ET HOFFM., Anetol e análogos.** Fortaleza. (Mestrado em Ciências Fisiológicas). Centro de Ciências da Saúde, Universidade Estadual do Ceará, 2005.

MACIEL, A. S.; VIANA, J. A. Dermatofitose em cães e gatos: uma revisão. **Clínica Veterinária**, v. 57, p. 74-82, 2005.

MAGALHÃES, P. J.; LAHLOU, S.; LEAL-CARDOSO, J. H. Antispasmodic effects of the essential oil of *Croton nepetaefolius* on guinea-pig ileum: a myogenic activity. **Fundam. Clin Pharmacol**, v.18, p. 539-546, 2004.

MAGALHÃES, P. J. C.; CRIDDLE, D. N.; RAQUEL, A. T.; MELO, E. M.; MOTA, T. L.; LEAL-CARDOSO, J. H. Intestinal myorelaxant and antispasmodic of the essential oil of *Croton nepetaefolius*, and constituents cineole, methyl-eugenol and therpineol. **Phytotherapy Research**, v, 12, p. 172-173, 1998.

MANCIANTI, F.; NARDONI, S.; CORAZZA.; DACHILLE, P.; PONTICELLI, C.; Environmental detection of *Microsporum canis* arthrospores in the households of

infected cats and dogs. **Journal of Feline Medicine and Surgery**, v. 5, p. 323-328, 2003.

MAGWA, M. L.; GUNDIDZA, M.; GWERU, N.; HUMPHREY, G. Chemical composition and biological activities of essential oil from the leaves of *Sesuvium portulacastrum*. **Journal Ethnopharmacology**, v. 103, p. 5-89, 2006.

MARCUS, C. & LICHTENSTEIN, P. Biologically active components of anise: Toxicity and interactions with insecticides in insects. **Journal Agric. Food Chem.** v. 27, p. 1217-1223, 1979.

MARKOWITZ, K.; MOYNIHAN, M.; LIU, M.; KIM, S. Biologic properties of eugenol and zinc oxide-eugenol. **Oral Surg Oral Med Oral Pathol**, v. 73, p. 729-737, 1992.

MARTOS, M. V.; NAVAJAS, Y. R.; LOPEZ, J. F.; LAVAREZ, J. P. Antifungal activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) essential oils. **Food Control**, v. 19, p. 1130-1138, 2008.

MATAN, N. & MATAN, N. Antifungal activities of anise oil, lime oil, and tangerine oil against molds on rubberwood (*Hevea brasiliensis*). **International Biodegradation & Biodegradation**, v. 62, p. 75-78, 2008.

MATOS, J. M. D.; MATOS, M. E. O. **Farmacognosia**: curso teórico – prático. Fortaleza: edições UFC, 1989.

MATOS, F. J. A. **Plantas Medicinais** – Guia de seleção e emprego de plantas usadas em fitoterapia no Nordeste do Brasil. 2 ed. Fortaleza: Imprensa Universitária – UFC, 2000.

MENDES-GIANNINI, M. J. S.; MELHEM, M. S. C. **Infecções fúngicas**. In: FERREIRA, A. W.; ÁVILA, S. L. M. – Diagnóstico laboratorial. Rio de Janeiro: Guanabara-Koogan, 1996. 216-275.

MENDONÇA, M. C. S. **Efeito do ácido indolbutírico no enraizamento de estacas de alecrim-pimenta (*Lippia sidoides* Cham.)**. Dissertação (Mestrado em Agronomia), Universidade Federal do Ceará, 1997.

MILNER, R. J.; PICARD, J.; TUSTIN, R. Chronic episodic diarrhea associated with apparent intestinal colonization by the yeasts *Saccharomyces cerevisiae* and *Candida famata* in a German shepherd dog. **Journal of the South African Veterinary Association**, v. 68, p. 147-149, 197.

MOTSEI, M. L.; LINDSEY, K. L.; VAN STANDEN, J.; JAGER, A. K. Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. **Journal of Ethnopharmacology**, v. 86, p. 235-241, 2003.

MORAIS, S. M.; BRAZ-FILHO. **Produtos Naturais estudos químicos e biológicos**. Fortaleza: EdUECE, 2007.

MORAIS, S. M.; CAVALCANTI, E. S. B.; BERTINI, L. M.; OLIVEIRA, C. L. L.; RODRIGUES, J. R. B.; CARDOSO, J. H. L. Larvicidal activity of essential oils from Brazilian *Croton* species against *Aedes aegypti* L. **J Am Mosq Control Assoc.**, v. 22, p. 161-164, 2006.

MORETTI, A., POSTERARO, B., BONCIO, L., MECHELLI, L., GASPERIS, E., AGNETTI, F., RASPA, M. Diffuse cutaneous candidiasis in a dog. Diagnosis by PCR-REA. **Revista Iberoamericana de Micologia**, v. 21, p. 139-142, 2004.

MORIELLO, K. A. Treatment of dermatophytosis in dogs and cats: review of published studies. **Veterinary Dermatology**, v. 15, p. 99-107, 2004.

MUELLER, R. S.; BETTENAY, S. V.; SHIPSTONE, M. Cutaneous candidiasis in a dog caused by *Candida guilliermondii*. **Veterinary Record**, v. 150, p. 728-730, 2002.

NAGABABU, E.; LAKSHMAIAH, N. Inhibition of microsomal lipid peroxidation and monooxygenase activities by eugenol. **Free Radical Research**, v. 20, p. 235-266, 1994.

NARDI, G. M. Anti-inflammatory and antioxidant effects of Croton celtidifolius bark. **Phytomedicine**, v. 10, p. 176-184, 2003.

NASCIMENTO, P. F. C.; NASCIMENTO, A. C.; RODRIGUES, C. S.; ANTONIOLLI, A. R.; SANTOS, P. O.; BARBOSA JÚNIOR, A. M.; TRINDADE, R. C. Atividade antimicrobiana dos óleos essenciais: uma abordagem multifatorial dos métodos. **Revista Brasileira de Farmacognosia**, v. 17, p. 108-113, 2007.

OCHIAI, K.; VALENTINE, B. A.; ALTSCHUL, M. Intestinal candidiasis in a dog. **Veterinary Record**, v. 146, p.228-229, 2000.

OLIVEIRA, A. C. Antinociceptive effects of the essential oil of *Croton zenhtneri* in mice. **Braz. J. Med. Biol. Res.**, v. 34, p. 1471-1474, 2001.

OLIVEIRA, H. D. Mecanismo de ação do óleo essencial do *Croton zehntneri* Pax et Hoffm. em músculo detrusor de rato. Dissertação (Mestrado em Ciências Fisiológicas). Universidade Estadual do Ceará. 2005.

OUTERBRIDGE, C. A. Mycologic disorders of the skin. **Clinical Techniques in Small Animal Practice**, v. 21, p. 128-134, 2006.

OZAWA, H.; OKABAYASHI, K.; KANO, R.; WATARI, T.; WATANABE, S.; HASEGAWA, A. Rapid identification of *Candida tropicalis* from canine cystitis. **Mycopathologia**, v. 160, p. 159-162, 2005.

PANTHONG, A.; KANJANAPOTHI, D.; TAESOTIKUL, T.; PHANKUMMOON, A.; PANTHONG, K.; REUTRAKUL, V. Anti-inflammatory activity of methanolic extracts from *Ventilago harmandiana* Pierre. **Journal of Ethnopharmacology**, v. 92, p. 237-242, 2004.

PASSOS, X. S.; SANTOS, S. C.; FERRI, P. H.; FERNANDES, O. F. L.; PAULA, T. F.; GARCIA, A. C. F.; SILVA, M. R. R. Atividade antifúngica de *Caryocar brasiliensis* (Caryocaraceae) sobre *Cryptococcus neoformans*. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 35, p.623-627, 2002.

PRADO, M. R. Isolamento de *Microsporum canis*, *Malassezia* spp. e *Candida tropicalis* em cães: um destaque para teste de sensibilidade de *Malassezia pachydermatis* in vitro. Tese (Doutorado em Ciências Veterinárias). Universidade Estadual do Ceará. 2005.

RAPOSO, B. R.; NOBRE, M. O.; FERNANDES, C. G.; PORTO, M. Candidíase cutânea em um canino. **Revista da Faculdade de Zootecnia, Veterinária e Agronomia**, 2-3, 11-14, 1996.

RABELO, Michelle. **Avaliação do efeito antiedematógeno e antinociceptivo do óleo essencial de *Ocimum gratissimum* (OEOG) Labiateae**. Fortaleza, 116p. Dissertação (Mestrado em Ciências Fisiológicas) – Centro de Ciências da Saúde, Universidade Estadual do Ceará, 2003.

RAKOTONIRAINY, M. S. & LAVEDRINE, B. Screening for antifungal activity of essential oils and related compounds to control the biocontamination in libraries and archives storage areas. **International Biodeterioration & Biodegradation**, v. 55, p. 141-147, 2005.

RATES, S. M. K. Plants as source of drugs. **Toxicon**, v. 39, p. 603-613, 2001.

REPETTO, M. **Toxicología Experimental**. Em: Toxicología Fundamental. 3^a ed Editora Diaz de Santos, Madrid, p. 291-300, 1997.

ROCHETTE, F.; ENGELEN, M.; VANDEN BOSSCHE, H. Antifungal agents of use in animal health-practical applications. **Journal of Veterinary Pharmacology and Therapeutics**. v. 26, p. 31-53, 2003.

SAUNTE, D. M.; HASSELBY, J. P.; BRILLOWSKA-DABROWSKA, A.; FRIMODT-MOLLER, N.; SVEJGAARD, E. L.; LINNEMANN, D.; NIELSEN, S. S.; HADRSDAL, M.; ARENDRUP, M. C. Experimental guinea pig model of dermatophytosis: a simple and useful tool for the evaluation of new diagnostics and antifungals. **Medical Mycology**, v. 46, p. 303-313, 2008.

SAYYAH, M.; VALIZADEH, J.; KAMALINEJAD, M. Anticonvulsant activity of the leaf essential oil of *Laurus nobilis* against pentylenetetrazole and maximal electroshock induced seizures. **Phytomedicine**, v. 9, p. 212-216, 2002.

SELL, A. B.; CARLINI, E. A. Anesthetic action of methyleugenol and other eugenol derivatives. **Pharmacology**, v. 14, p. 367-377, 1976.

SHARMA, N. & TRIPATHI, A. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. **Microbiological Research**, v. 163, p. 337-344, 2008.

SIDRIM, J. J. C. & ROCHA, M. F. G. **Micologia médica à luz de autores contemporâneos**. Rio de Janeiro: Guanabara, 2004.

SIMÕES, C. M. O.; SPITZER, V. **Farmacognosia**: da planta ao medicamento. Ed. Universidade/UFRGS. 3^a ed., 1999.

SIMÕES, C. M. O.; SCHENKEL, E. P.; GOSMANN, G.; MELLO, J. C. P.; MENTZ, L. A.; PETROVICK, P. R. Farmacognosia: de planta ao medicamento. 5 ed. Porto Alegre/Florianópolis: Editora da Universidade UFRGS/Editora da UFSC, 2004.

SIQUEIRA, R. J. B.; LEAL-CARDOSO, J. H.; COUTURE, R.; LAHLOU, S. Role of capsaicin-sensitive sensory nerves in mediation of the cardiovascular effects of the essential oil of *Croton zenhtineri* Leaves in anaesthetized rats. **Clinical and Experimental Pharmacology and Physiology**, v. 33, p. 238-247, 2006.

SOARES, L. **Estudo tecnológico, fitoquímico e biológico de *Lippia alba* (miller) n. E. Brown ex britt. & wils. (falsa-melissa) verbenaceae.** Dissertação (Mestrado em Farmácia). Universidade Federal de Santa Catarina. 2001.

SOUZA, M. P. et al. **Constituintes químicos e propriedades biológicas de plantas medicinais brasileiras.** Fortaleza: Editora UFC, 2004.

SOUZA, L. K. H.; OLIVEIRA, C. M. A.; FERRI, P. H.; OLIVEIRA-JÚNIOR, J. G.; SOUZA-JÚNIOR, A. H.; FERNANDES, O. F. L.; SILVA, M. R. Antimicrobial activity of *Hyptis ovalifolia* towards dermatophytes. **Mem. Inst. Oswaldo Cruz**, v. 98, p. 963-965, 2003.

SUAREZ, A. I. Antinociceptive and anti-inflammatory effects of *Croton malambo* bark aqueous extract. **Journal Etnnopharmacol**, v. 88, p. 11-14, 2003.

TORTORA, G. J.; FUNKE, B. R.; CASE, C. L. **Microbiologia.** 6^a Ed, Artes Médicas, 822p, 2003.

TUNCA, R.; GUVENÇ, T.; HAZIROGLU, R.; ATASEVEN, L.; OZEN, H.; TOPLU, N. Biological and immunohistochemical investigation of naturally occurring systemic *Candida albicans* infection in dogs. **Turkish Journal of Veterinary Animal Science**, v. 30, p. 545-551, 2006.

VASCONCELOS, A. L. F. C. **Estudo farmacológico e toxicológico do extrato acetato de etila de *Spigelia anthelmia* Linn em animais de laboratório.** Fortaleza. (Mestrado em Ciências Veterinárias). Faculdade de Veterinária, Universidade Estadual do Ceará. 2002.

WU, B. N.; HWANG, T. L.; LIAO, C. F.; CHEN, M I. J. Vaninolol: a new selective beta B-adrenergic antagonist derived from vanillin. **Biochemical Pharmacology**, v. 48, p. 101-109, 1994.

WEBSTER, G. L. Classification of the Euphorbiaceae. **Annals of the Missouri Botanical Garden**, v. 81, p. 3-32, 1994.

WEIG, G. M. & BROWN, A. J. P. Genomics and the development of new diagnostics and anti-*Candida* drugs. **Trends in Microbiology**, v. 15, p. 310-317, 2007.

WRIGHT, D. E.; WHITE, F. A.; GERFEN, R. W.; SILOS-SANTIAGO, I.; SNIDER, W. D. The guidance molecule semaphoring III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. **Journal of Comportamental Neurology**, v. 361, p. 321-333, 1995.

YAYLI, N.; YASAR, A.; GULEC, C.; USTA, A.; KOLAYLI, S.; COSKUNCELEBI, K.; KARAOGLU, S. Composition antimicrobial activity of essential oils from *Centaurea sessilis* and *Centaurea armena*. **Phytochemistry**, v. 66 p. 1741-1745, 2005.

Livros Grátis

(<http://www.livrosgratis.com.br>)

Milhares de Livros para Download:

[Baixar livros de Administração](#)

[Baixar livros de Agronomia](#)

[Baixar livros de Arquitetura](#)

[Baixar livros de Artes](#)

[Baixar livros de Astronomia](#)

[Baixar livros de Biologia Geral](#)

[Baixar livros de Ciência da Computação](#)

[Baixar livros de Ciência da Informação](#)

[Baixar livros de Ciência Política](#)

[Baixar livros de Ciências da Saúde](#)

[Baixar livros de Comunicação](#)

[Baixar livros do Conselho Nacional de Educação - CNE](#)

[Baixar livros de Defesa civil](#)

[Baixar livros de Direito](#)

[Baixar livros de Direitos humanos](#)

[Baixar livros de Economia](#)

[Baixar livros de Economia Doméstica](#)

[Baixar livros de Educação](#)

[Baixar livros de Educação - Trânsito](#)

[Baixar livros de Educação Física](#)

[Baixar livros de Engenharia Aeroespacial](#)

[Baixar livros de Farmácia](#)

[Baixar livros de Filosofia](#)

[Baixar livros de Física](#)

[Baixar livros de Geociências](#)

[Baixar livros de Geografia](#)

[Baixar livros de História](#)

[Baixar livros de Línguas](#)

[Baixar livros de Literatura](#)

[Baixar livros de Literatura de Cordel](#)

[Baixar livros de Literatura Infantil](#)

[Baixar livros de Matemática](#)

[Baixar livros de Medicina](#)

[Baixar livros de Medicina Veterinária](#)

[Baixar livros de Meio Ambiente](#)

[Baixar livros de Meteorologia](#)

[Baixar Monografias e TCC](#)

[Baixar livros Multidisciplinar](#)

[Baixar livros de Música](#)

[Baixar livros de Psicologia](#)

[Baixar livros de Química](#)

[Baixar livros de Saúde Coletiva](#)

[Baixar livros de Serviço Social](#)

[Baixar livros de Sociologia](#)

[Baixar livros de Teologia](#)

[Baixar livros de Trabalho](#)

[Baixar livros de Turismo](#)