UNIVERSIDADE FEDERAL DE PELOTAS
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Tese

Uso promissor do óleo essencial de *Rosmarinus officinalis* L. e *Origanum majorana* L. na esporotricose cutânea experimental por *Sporothrix brasiliensis* resistente ao itraconazol, e susceptibilidade de isolados aos óleos vegetais

Stefanie Bressan Waller

Pelotas, 2019

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Allan Kardec, *O Evangelho Segundo o Espiritismo,* 1864

Resumo

WALLER, Stefanie Bressan. **Uso promissor do óleo essencial de *Rosmarinus officinalis* L. e *Origanum majorana* L. na esporotricose cutânea experimental por *Sporothrix brasiliensis* resistente ao itraconazol, e susceptibilidade de isolados aos óleos vegetais.** 2019. 140f. Tese (Doutorado/ em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

O itraconazol é o fármaco de eleição no tratamento da esporotricose humana e animal, entretanto, o surgimento de *Sporothrix brasiliensis* com resistência *in vitro* tem incentivado os estudos com plantas da família Lamiaceae como fontes promissoras de moléculas antifúngicas. Dessa família, os óleos essenciais de alecrim (*Rosmarinus officinalis* L.) e manjerona (*Origanum majorana* L.) têm se destacados pela atividade antifúngica e baixa citotoxicidade. Essa tese objetivou avaliar a atividade dos óleos essenciais de plantas da família Lamiaceae em isolados clínicos de *Sporothrix* spp. e na esporotricose cutânea experimental por *S. brasiliensis* resistente ao itraconazol. Quarenta ratos Wistar adultos machos foram inoculados via subcutânea no coxim plantar esquerdo (2×10^5 células/mL) e, dez dias após, receberam tratamento oral (1 mL) por 30 dias nos seguintes grupos experimentais (n=10): controle com solução salina com propilenoglicol 1% (CONT); itraconazol a 10 mg/kg (ITRA); óleos essenciais de alecrim a 90 mg/ml (ALEC) e manjerona a 30 mg/ml (MANJ). Semanalmente, a evolução clínica era acompanhada e a eutanásia realizada para histopatologia e contagem fúngica (retro-isolamento). Ainda, os óleos foram avaliados por cromatografia gasosa com espectrometria de massas, e o mecanismo de ação pelos ensaios de proteção do sorbitol e efeito do ergosterol. Os resultados revelaram que os animais dos grupos ALEC e MANJ apresentaram remissão dos sinais clínicos, com ausência de edema e exsudato no membro inoculado, ao passo que CONT e ITRA mantiveram os sinais da doença. Ainda, a disseminação do *S. brasiliensis* para os órgãos foi menos observada no fígado, baço, linfonodos, rins e testículos dos grupos ALEC e MANJ em comparação ao CONT e ITRA ($P < 0.05$), revelando uma proteção sistêmica a partir da infecção subcutânea. Essa observação foi relacionada com a composição química, cujos compostos prevalentes foram terpineol-4 (34,09%) e γ-terpineno (14,28%) em manjerona, e 1,8-cineol (47,91%) e cânfora (12,47%) em alecrim. Não houve alteração nos valores da concentração inibitória mínima (CIM) quando adicionado o protetor osmótico, demonstrando ausência de atividade à nível de parede celular, ao passo que o uso do ergosterol elevou os valores de CIM, revelando que ambos óleos apresentaram atividade a nível de complexação com o ergosterol. Esse estudo demonstrou a eficácia do uso oral de alecrim e manjerona no tratamento da esporotricose cutânea experimental, sendo promissores como antifúngicos.

Palavras-chave: esporotricose; resistência antifúngica; produtos naturais; alecrim; manjerona

Abstract

WALLER, Stefanie Bressan. **Promising use of the essential oil of *Rosmarinus officinalis* L. and *Origanum majorana* L. in the experimental cutaneous sporotrichosis by an itraconazole-resistant *Sporothrix brasiliensis,* and susceptibility of isolates to plant extracts.** 2019.140f. Thesis (Doctor in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

Itraconazole is the drug of choice for the treatment of sporotrichosis, however, the emergence of *Sporothrix brasiliensis* with *in vitro* resistance has encouraged studies with plants of the family Lamiaceae as promising sources of antifungal molecules. From this family, essential oils of rosemary (*Rosmarinus officinalis* L.) and marjoram (*Origanum majorana* L.) have been highlighted for their antifungal activities and low cytotoxicity. This thesis aimed to evaluate the effectiveness of the essential oils of the plants from Lamiaceae family in clinical isolates of *Sporothrix* spp. and in the experimental cutaneous sporotrichosis by itraconazole-resistant *S. brasiliensis*. Forty male adult Wistar rats were inoculated subcutaneously in the left footpad (2×10^5 cells/mL) and, ten days later, received oral treatment (1 mL) for 30 days in the following experimental groups (n=10): control with solution saline with 1% propylene glycol (CONT); itraconazole at 10 mg/kg (ITRA); essential oils of rosemary at 90 mg/mL (ROSM) and marjoram at 30 mg/mL (MARJ). The clinical follow-up was done weekly, as well as the euthanasia for histopathology and fungal burden (retro-isolation). Furthermore, the oils were evaluated by gas chromatography with mass spectrometry, and the mechanism of action by the assays of sorbitol protection and ergosterol effect. The results showed that the animals from ALEC and MANJ groups presented remission of the clinical signs, with no edema and exudate in the inoculated limb, whereas CONT and ITRA maintained the signs of the disease. Also, the dissemination of *S. brasiliensis* to the organs was less observed in the liver, spleen, lymph nodes, kidneys and testicles of the ALEC and MANJ groups compared to the CONT and ITRA (P <0.05), revealing systemic protection from the subcutaneous infection. This observation was related to the chemical composition, whose prevalent compounds were terpineol-4 (34.09%) and γ-terpinene (14.28%) in marjoram, and 1,8-cineole (47.91%) and camphor (12.47%) in rosemary. There was no change in minimum inhibitory concentration (MIC) values when the osmotic protector was added, showing an absence of activity at the cell wall level, whereas the use of ergosterol increased MIC values, revealing that both oils presented activity at the level of complexion with ergosterol. This study demonstrated the effectiveness of the oral use of rosemary and marjoram in the treatment of experimental cutaneous sporotrichosis, being promising as antifungal.

Keywords: sporotrichosis; antifungal resistance; natural products; rosemary; marjoram

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Sumário

1 Introdução

A esporotricose é uma micose causada por fungos do complexo *Sporothrix schenckii* (MARIMON et al., 2007), a designar as espécies S*. brasiliensis, S. schenckii, S. globosa, S. mexicana, S. pallida, S. luriei* e *S. chilensis*. Dessas espécies, *S. schenckii* e *S. brasiliensis* são consideradas as mais patogênicas e prevalentes nos casos humanos e animais (PEREIRA et al., 2014), sendo que, no Brasil, a espécie *S. brasiliensis* apresenta uma grande distribuição e alta patogenicidade (RODRIGUES et al., 2013).

Para o tratamento em animais, o itraconazol é o fármaco de eleição e atua alterando a permeabilidade da membrana fúngica, impedindo a enzima do citocromo P450 fúngico, *14-α-demetilase,* de converter o lanosterol em ergosterol. Essa conversão é essencial para a célula fúngica, cujo ergosterol tem papel na manutenção da estrutura da membrana celular e, uma vez inibido, acarreta no prejuízo da permeabilidade da mesma, resultando, consequentemente, na morte do agente fúngico (KELLY et al., 1995). Ainda que outros medicamentos possam ser usados em animais, como os iodetos de potássio e os azólicos cetoconazol e fluconazol (GREMIÃO et al., 2017), algumas moléculas de uso humano ainda não são rotineiramente empregadas na rotina veterinária, como terbinafina, anfotericina B, posaconazol e candinas. Esse cenário salienta a carência de opções terapêuticas para o tratamento da esporotricose felina e canina.

As dificuldades na obtenção da cura têm sido relatadas pelos médicos veterinários em felinos (DA ROCHA et al., 2018; GREMIÃO et al., 2009, 2011) e caninos (GUTERRES et al., 2014; VIANA et al., 2017) com a esporotricose, caracterizando casos refratários ao itraconazol. Mesmo nos pacientes em que os tutores cumprem adequadamente com o manejo terapêutico, a falha terapêutica é relacionada à inatividade de determinados produtos à base do princípio ativo itraconazol. De forma agravante, o surgimento de isolados clínicos de *Sporothrix* spp. resistentes a esse fármaco tem ocorrido nos últimos anos em humanos (OLIVEIRA et al., 2015; VETTORATO et al., 2018), inclusive em *Sporothrix brasiliensis* isolados de felinos e caninos no Rio Grande do Sul (WALLER et al., 2017a, 2017b, 2018). O uso

inadequado de medicamentos é um dos fatores contribuintes para o surgimento de resistência pelos patógenos (CHAVES et al., 2013), e essa problemática da resistência antifúngica reforça a necessidade urgente em detectar novas moléculas ativas e seguras para o tratamento e controle da esporotricose.

Sabe-se que o uso de plantas e seus derivativos para fins medicinais é uma prática ancestral usada mundialmente, especialmente nas culturas orientais (TILBURT; KAPTCHUK, 2008). Segundo a Organização Mundial da Saúde, os medicamentos fitoterápicos tradicionais são substâncias naturais, derivadas de plantas, com processamento industrial mínimo ou nenhum, que foram usadas para tratar doenças dentro de práticas de cura locais ou regionais (TILBURT; KAPTCHUK, 2008).

Pesquisas com produtos naturais têm sido realizadas, objetivando a detecção de moléculas promissoras contra patógenos resistentes aos antifúngicos convencionais. Dentre as plantas com potencial investigado, aquelas pertencentes à família Lamiaceae se destacam por sua ampla variedade de espécies botânicas com propriedades antifúngicas ativas (WALLER et al., 2017a), inclusive contra Spo*rothrix brasiliensis* sensíveis e resistentes ao itraconazol, tais como *Rosmarinus officinalis* e *Origanum majorana* (WALLER et al., 2017b), as quais são popularmente conhecidas como alecrim e manjerona, respectivamente. Ainda, é importante verificar a toxicidade e mecanismos ou alvos de atuação desses extratos.

2 Objetivos

2.1 Objetivo Geral

Esse estudo objetivou avaliar a atividade dos óleos essenciais de plantas da família Lamiaceae em isolados clínicos de *Sporothrix brasiliensis*, bem como na esporotricose cutânea experimental por *S. brasiliensis* resistente ao itraconazol.

2.2 Objetivos Específicos

Os objetivos específicos foram:

- Revisar a atividade de plantas da família Lamiaceae em fungos patogênicos de importância médica e veterinária;
- Revisar os fármacos antifúngicos utilizados convencionalmente no tratamento da esporotricose felina e canina;
- Verificar a atividade *in vitro* dos óleos essenciais de alecrim (*Rosmarinus officinalis* Linn.), orégano (*Origanum vulgare* Linn.) e manjerona (*Origanum majorana* Linn.) em isolados clínicos de *S. brasiliensis*;
- Determinar o perfil químico, a citotoxicidade e o mecanismo de ação dos óleos essenciais de alecrim (*Rosmarinus officinalis* Linn.) e manjerona (*Origanum majorana* Linn.);
- Avaliar a eficácia dos óleos essenciais de alecrim (*Rosmarinus officinalis* Linn.) e manjerona (*Origanum majorana* Linn.) na esporotricose cutânea experimental por *Sporothrix brasiliensis* resistente ao itraconazol.

3 Revisão da Literatura

3.1 Esporotricose e o complexo *Sporothrix schenckii*

Esporotricose é uma doença fúngica causada por espécies dimórficas do complexo *Sporothrix schenckii* e que tem distribuição mundial (CHAKRABARTI et al., 2015), afetando tanto humanos (SOTO; MALAGA, 2017; TAKAZAWA et al., 2018), quanto animais (MACÊDO-SALES et al., 2018; POESTER et al., 2018). Por cerca de um século, *S. schenckii* foi conhecido como a única espécie causadora da micose, entretanto, estudo de análise molecular realizado por Marimon et al. (2007) revelou que três novas espécies de *Sporothrix* são clinicamente importantes – *S. brasiliensis, S. globosa* e *S. mexicana* –, além de *S. schenckii sensu stricto*. Essas espécies estão compiladas no complexo *S. schenckii*, sendo *Sporothrix luriei* também conhecido como um patógeno clínico com poucas ocorrências, e de *S. pallida, S. chilensis* e *S. inflata* serem conhecidos como patógenos raros em mamíferos (RODRIGUES et al., 2016).

A esporotricose é transmitida por inoculação traumática na pele de fômites contaminados com os conídios fúngicos, como espinhos de plantas, matéria em decomposição, solo, entre outras fontes ambientais, os quais correspondem à principal via de transmissão da doença na maioria dos países do mundo (GOVENDER et al., 2015; ZHAO et al., 2017). Entretanto, essa via clássica é relatada esporadicamente no Brasil (DE ARAÚJO, et al., 2015), cuja principal via de transmissão é a zoonótica, através de mordidas e arranhaduras de animais doentes, especialmente felinos, constituindo uma enfermidade de grande importância na saúde pública (RODRIGUES et al., 2016).

Estudos sobre a ecologia fúngica demonstraram que essas espécies de *Sporothrix* pertencem a um ramo clínico, os quais *S. schenckii* sensu stricto*, S. globosa* e *S. luriei* são relacionados às sapronoses, ao passo que *S. brasiliensis* é associado às zoonoses (RODRIGUES et al., 2016), os quais apresentam diferentes níveis de impactos clínicos e epidemiológicos. A facilidade de arranhadura e mordedura em outros animais e humanos, e o elevado número de leveduras de

Sporothrix spp. nas lesões (POESTER et al., 2018; YEGNESWARAN et al., 2009) são fatores importantes para o papel dos felinos na esporotricose. Entretanto, um dos fatores epidemiológicos para esse padrão de transmissão no Brasil é o abandono de gatos doentes pelos proprietários, bem como o destino incorreto de felinos que vieram a óbito em decorrência da micose (BARROS et al., 2010). Além disso, o Brasil é o segundo país com o maior número de animais domésticos no mundo, e tem um aumento anual no número de felinos que vivem domiciliados (IBGE, 2014).

Uma vez ocorrida a injúria traumática com conídios de *Sporothrix* spp. dentro da pele, lesões nodulares e ulcerativas podem ocorrer, sendo que as apresentações clínicas podem variar de cutâneas fixas e múltiplas a formas linfo-cutâneas e sistêmicas (LOPES-BEZERRA et al., 2006; OROFINO-COSTA et al., 2017). As apresentações severas estão frequentemente relacionadas às condições imunológicas deficientes desses pacientes (FREITAS et al., 2014; GOVENDER et al., 2015; PAIXÃO et al., 2015). Esses fatores podem afetar a cura, mesmo usando a terapia convencional, o qual tem sido relacionada à resistência de espécies de *Sporothrix* aos principais antifúngicos, como o itraconazol (OLIVEIRA et al., 2015; VETTORATO et al., 2018).

A esporotricose tem sido negligenciada nos últimos anos a ponto de, atualmente, evoluir para a principal zoonose de caráter epidêmico em expansão no País (POESTER et al., 2018). Sua principal ocorrência é no Rio de Janeiro, onde, nas duas últimas décadas, foram registrados pelo menos 4.703 felinos, 4.188 humanos e 244 caninos com a doença pela Fundação Oswaldo Cruz (GREMIÃO et al., 2017). Os demais Estados também vêm registrando casos anuais de esporotricose, como São Paulo (MONTENEGRO et al., 2014), Minas Gerais (STOPIGLIA et al., 2014) e, principalmente, Rio Grande do Sul (POESTER et al., 2018; MADRID et al., 2012; SANCHOTENE et al., 2015), considerado o segundo Estado em alerta de epidemia (BRANDOLT et al., 2018), onde os municípios de Pelotas e Rio Grande são endêmicos com crescentes registros de transmissão zoonótica (BRANDOLT et al., 2018; POESTER et al., 2018;).

Somente na região sul do Rio Grande do Sul, a última década foi marcada por crescentes registros de animais com esporotricose pelo “Centro de Diagnóstico e Pesquisa em Micologia Veterinária”, da Universidade Federal de Pelotas (UFPEL), onde 251 felinos, 70 caninos e 24 humanos foram registrados por transmissão zoonótica entre 2007 e 2017, bem como pela “Secretaria de Saúde de Pelotas”, que

registrou 306 gatos, 58 humanos e 15 cães entre 2013 e 2016, evidenciando a importância em saúde pública de uma doença emergente e endêmica no Rio Grande do Sul e no Brasil.

3.2 Terapias Convencionais em Caninos e Felinos

Considerando as terapias convencionais disponíveis para o tratamento de cães e gatos com esporotricose e a situação atual da suscetibilidade antifúngica *in vitro* dos fungos do complexo *Sporothrix schenckii*, foi elaborado o manuscrito "*Therapeutic approaches for cats and dogs with sporotrichosis and a literature review on perspectives for cases refractory to antifungals*", submetido à revista *Veterinaria Italiana* (submissão nº 1693). O manuscrito está presente na seção "Artigo 1" dessa tese, na página 21.

3.3 Potencial das Plantas da Família Lamiaceae

Diante do surgimento de isolados clínicos do complexo *Sporothrix brasiliensis* com resistência aos antifúngicos convencionais, inclusive ao itraconazol, considerado o fármaco de eleição, foram elaborado dois manuscritos pertinentes ao assunto: "*Plants from Lamiaceae family as source of antifungal molecules in humane and veterinary medicine*", publicado na revista *Microbial Pathogenesis* (vol. 104, p. 232–237, 2017, doi: 10.1016/j.micpath.2017.01.050), bem como o manuscrito "*In Vitro Susceptibility of Sporothrix brasiliensis to Essential Oils of Lamiaceae Family*", publicado na revista *Mycopathologia* (vol. 181, n. 11–12, p. 857–863, 2016, doi: 10.1007/s11046-016-0047-y). Ambos manuscritos estão presentes nas seções "Artigo 2" e "Artigo 3" dessa tese, respectivamente, nas páginas 41 e 48.

Diante dos extratos vegetais oriundos de espécies botânicas da família Lamiaceae, as plantas *Origanum majorana* L. e *Rosmarinnus officinallis* L., popularmente conhecidas por manjerona e alecrim, respectivamente, têm se destacado pela atividade antifúngica contra isolados clínicos de *S. brasiliensis* sensíveis e resistentes ao itraconazol (WALLER et al., 2016b, 2017c, 2018), bem como baixa citoxicidade (WALLER et al., 2016a) em ensaios *in vitro*.

Os resultados obtidos são promissores para o aprofundamento do estudo da eficácia dos mesmos na experimentação animal, sendo elaborado, para este fim, dois manuscritos: “Promising control of the cutaneous sporotrichosis by na itraconazol-resistant *Sporothrix brasiliensis* using marjoram (*Origanum majorana* Linn.) essential oil”, a ser submetido à revista *Journal of Medical Microbiology*, e “Rats experimentally infected by na itraconazol-resistant *Sporothrix brasiliensis* and orally treated with Rosemary (*Rosmarinus officinalis* Linn.) essential oil were protected of the fungal dissemination”, a ser submetido à revista *Medical Mycology*. Ambos manuscritos estão presentes nas seções “Artigo 4” e “Artigo 5” dessa tese, respectivamente, nas páginas 56 e 79.

4 Artigos

4.1 Artigo 1

Therapeutic approaches for cats and dogs with sporotrichosis and a literature review on perspectives for cases refractory to antifungals

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Therapeutic approaches for cats and dogs with sporotrichosis and a literature review on perspectives for cases refractory to antifungals

Running title**: Therapy for animal sporotrichosis and for cases refractory to antifungals**

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Abstract

Sporotrichosis is a mycosis in human and animals caused by *Sporothrix schenckii* species complex. There are few therapies available for treating cats and dogs, complicating the better therapeutic choice by veterinarians. This review addresses the treatments available and the perspectives for cases refractory to antifungals. Azoles, as ketoconazole (KTZ) and ITZ, as well as potassium iodide (KI) are the main antifungals used, being ITZ the drug of choice at a dose of 10 mg/kg orally, once or twice a day, for 3 months minimum with an extension for 30 days after the remission of lesions. The most commonly antifungal used and effective are ITZ and KI associated with ITZ for feline sporotrichosis, whereas in canine sporotrichosis are KTZ and ITZ. The emergence of *Sporothrix* species with resistance to antifungals, as ITZ, is recognized, and animal cases refractory to antifungals has hindered the cure. For cases refractory to antifungals, the therapeutic association can be used, such as ITZ plus terbinafine (TRB) or amphotericin B (AMB), but these options have limited use *in vivo*. Surgical procedures or immunomodulatory substances also can be used in association with antifungals, and thermotherapy may be an option in cases with no possibility for antifungal drugs.

Keywords: antifungal resistance, canine, feline, itraconazole, potassium iodide, *Sporothrix schenckii* complex, sporotrichosis, treatment.

Introduction

Sporotrichosis is a fungal infection caused by the *Sporothrix schenckii* species complex (Marimon *et al*., 2007), being *S. brasiliensis* the most common in human and animal cases in Brazil (Pereira *et al*., 2014; Montenegro *et al*., 2014; Gremião *et al*., 2017; Poester *et al*, 2018). Besides its zoonotic potential (Rodrigues *et al*., 2016; Gremião *et al*., 2017), cats are more affected than dogs (Madrid *et al*., 2012; Pereira *et al*., 2014), and most of the therapeutic information is found for the feline species (Gremião *et al*., 2011; Reis *et al*., 2012; Rossi *et al*., 2013). Both animals can show ulcerated and nodular lesions in inoculated skin and subcutaneous tissue, and respiratory and systemic signs may also occur (Schubach *et al*., 2004; Whittemore and Webb, 2007; Madrid *et al*., 2010), hindering the cure.

The therapies available for cats and dogs with sporotrichosis are often based on antifungal medications, mainly by itraconazole (ITZ), which is the drug of choice (Meinerz *et al*., 2007; Whittemore and Webb, 2007; Crothers *et al*., 2009; Madrid *et al*., 2010; Rossi *et al*., 2013). However, therapeutic success is challenging for veterinarians due to the recurrence of lesions after the cessation of therapy (Pereira *et al*., 2010; Chaves *et al*., 2013), as well as to the elevated costs of the antifungals, which many animals are sacrificed due to the lack of treatment or they remain ills until the death due to the disease (Barros *et al*., 2010). Furthermore, the corrected therapeutic management is another point to be considered, since cats often reject oral therapy (Reis *et al*., 2012) and the daily care requires the cooperation of the owner (Gremião *et al*., 2015). In general, the choice of therapy depends on several factors related to the fungus (virulence, pathogenicity, antifungal susceptibility, and resistance) and pharmacological drugs (efficacy and safe use), as well as to the host (clinical signs, immunity, and pregnancy) and the owner (financial condition and ability to administer medications), among others.

These factors should be taken into consideration regarding the choice of therapy, i.e. iodides (Scott *et al*., 1974; Gonzalez *et al*., 1989; Larsson, 2011; Reis *et al*., 2012; Reis *et al*., 2016), terbinafine (TRB, Viana *et al*., 2017), amphotericin B (AMB, Gremião *et al*., 2009; Gremião *et al*., 2011), since other procedures, like surgery (Corgozinho *et al*., 2006; Gremião *et al*., 2006; Souza *et al*., 2015), immunomodulatory therapy (Guterres *et al*., 2014), and thermotherapy (Honse *et al*., 2010) may be appropriate alternative treatments in cats and dogs with sporotrichosis. This review addresses the available treatments for feline and canine sporotrichosis and the therapeutic perspectives for cases refractory to antifungals.

Conventional Therapy with Azoles Drugs

The antifungal activity of azoles occurs via the inhibition of sterol synthesis in fungal cells through binding the cytochrome P450-dependent enzyme *lanosterol 14α-demethylase* (Greene, 2012), conferring good anti-*Sporothrix* spp. activity in most cases. These drugs have good absorption at acidic pH and should be administrated with food. Among the azoles, itraconazole is the drug of choice to treat cats and dogs with sporotrichosis, but ketoconazole (KTZ) and fluconazole (FLZ) can also be used. Since they undergo hepatic metabolism by cytochrome P450 enzymes (Greene, 2012), *transaminase* enzymes should be monitored and, in case of abnormal levels, the therapy should be discontinued until the normalization of signs (Larsson, 2011).

KTZ is recommended for cats at oral doses of 5-10 mg/kg once a day (Schubach *et al*., 2004) and a good drug tolerance with the absence of anorexia have been reported in cats treated with this azole for six months in a Malaysian study (Han *et al*., 2017). In a study with 598 cats treated with KTZ at dosage of 13.5 to 27 mg/kg (once or twice a day), Pereira *et al*. (2010) reported that 28.6% (171) achieved the clinical cure at a median time of four months (28 weeks, ranging of eight to 170 weeks). At a dose of 50 mg/animal, once a day, the skin and ocular lesions were healed in cats treated for 75 days (Silva *et al*., 2008). In dogs, KTZ has been successfully used between 5-10 mg/kg, once (Schubach *et al*., 2006; Schubach *et al*., 2012) or twice a day (Crothers *et al*., 2009), for two to 15 months of treatment (and a median of 3.5 months) in dogs from Brazil (Schubach *et al*., 2006) or for eight to 20 weeks in dogs from United States (Crothers *et al*., 2009).

However, no improvement was reported in Malaysia in feline sporotrichosis after six months of therapy with KTZ at a dosage of 10 mg/kg, per day (Siew, 2017), and cases of disseminated sporotrichosis in humans do not respond well to this azole (Kauffman *et al*, 2007). The therapeutic response in cats treated with KTZ is lower compared to ITZ (Pereira *et al*., 2010) and vomiting, nausea, diarrhea, and inappetence are some of the adverse signs (Crothers *et al*., 2009; Larsson, 2011; Greene, 2012), which are usually observed in this species, which 42% (252/598) showed gastrointestinal signs (Pereira *et al*., 2010). Although no adverse effects were reported in dogs treated with KTZ by Schubach *et al*. (2006), the hepatoxic effects of this antifungal prevent its recommendation for dogs by some authors (Mayer *et al*., 2008; Larsson, 2011), such as vomiting, anorexia, lethargy, diarrhea. Furthermore, variability in antifungal susceptibility among *Sporothrix* species has been noted, since *S. brasiliensis* (Marimon *et al*., 2008; Brilhante *et al*., 2016), *S. schenckii* and *S. globosa* are sensitive, but *S. albicans* and *S. mexicana* are only weakly inhibited (Marimon *et al*., 2008).

Relating to FLZ, there are few reports treating feline sporotrichosis, which was successfully used in a cat with skin lesions and respiratory signs and that was treated at a dose of 10 mg/kg, once a day, for 80 weeks (Crothers *et al*., 2009). In a study with 68 cats that were clinically cured with different therapeutic protocols in Brazil, Schubach *et al*. (2004) reported that 10.3% of these patients received an oral dose of FLZ (50 mg/animal), once a day, associated to ITZ (5-10 mg/kg). In canine cases, only one study was found using FLZ (Crothers *et al*., 2009), with no adverse effects. Despite some cases of clinical cure, the *in vitro* studies showed weak activity of FLZ against *Sporothrix* species (Marimon *et al*., 2008; Rodrigues *et al*., 2014), and, considering the few *in vivo* studies with the *in vitro* tests, FLZ seemed to not be the first recommended therapeutic option for animal sporotrichosis.

Itraconazole: "the antifungal of choice, but…"

In turn, ITZ seems to be less toxic than KTZ because the enzymatic inhibition is more selective for fungal cells (Greene, 2012), besides has better efficacy (Pereira *et al*., 2010). ITZ is largely used to treat cats and dogs with sporotrichosis for a variable period, and the therapy should be continued for more 30 days after the lesion disappears. The average treatment time varies from three to six months in cats treated orally at a dose of 10 mg/kg, once a day, without adverse effects (Meinerz *et al*., 2007; Crothers *et al*., 2009; Madrid *et al*., 2010; Rossi *et al*., 2013). The same dosage in dogs was successfully used for three to

six months (Madrid *et al*., 2007; Whittemore and Webb, 2007), although disseminated presentation requires more time, i.e. an average time of 11.3 months (Rossi *et al*., 2013). A dose of 5 mg/kg, twice a day, also may be successfully used in cats and dogs for a time from three to four months (Crothers *et al*, 2009; Souza *et al*., 2009). Between 5-10 mg/kg, once a day, the daily therapy with ITZ was used in dogs that were cured in a period of time ranged from to two to five months (Schubach *et al*., 2006). However, these authors cited that 16.7% of the dogs treated with ITZ showed anorexia, vomiting, diarrhea and elevated hepatic enzyme levels (Schubach *et al*., 2006).

ITZ has good *in vitro* activity against *S. schenckii* complex species (Brilhante *et al*., 2016; Han *et al*, 2017). However, the susceptibility of *S. brasiliensis* is varied because some strains are less sensitive, showing higher values of the minimal inhibitory concentration (Marimon *et al*., 2008; Rodrigues *et al*., 2014) and the emergence of itraconazole-resistant isolates has been seen in cats and dogs (Waller *et al*., 2017).

Antifungal resistance may make it difficult to obtain a cure, requiring alternative therapies for some cases. The recurrence of lesions after treatment with ITZ has been reported (Gremião *et al*., 2006; Gremião *et al*., 2011; Guterres *et al*., 2014), hindering the cure. Higher doses of oral ITZ (8.3-27.7 mg/kg, once a day) may be an option; it has been successful in 38.3% of cats in Brazil, but gastrointestinal signs were reported (Pereira *et al*., 2010). In Malaysia, three feline cases were reported with clinical cure after higher doses of ITZ, which this regimen were started at an initial dose (10 mg/kg), that was gradually increased (10-20 mg/kg), depending on clinical examination and biochemical tests results in every 1-2 weeks, and, until the higher-dose (up 60 to 80 mg/kg), and no hepatotoxicity was described in the cases (Siew, 2017). However, the mains adverse signs are vomiting, loss of appetite, nausea, anorexia, and hepatotoxicity (Schubach *et al*., 2004; Pereira *et al*., 2010). High dosages can produce teratogenic and embryotoxic effects (Greene, 2012), and its use should be avoided during pregnancy.

Potassium and Sodium Iodides

Potassium and sodium iodides have the advantages of being less costly than others antifungal drugs. Although still unclear, the mechanism of action seems to be related to the immune response of the host and not directly to *Sporothrix* species (Schubach *et al*., 2012). These salts, from the 19th century, were classically used to treat dogs (Scott *et al*., 1974; Larsson, 2011; Schubach *et al*., 2012), but, in the past, they were shown to be less effective in some cats in Japan (Nakamura *et al*., 1996) and Brazil (Larsson, 2011), due to serious toxic effects. However, other studies have reported their successful use in cats in Spain (Gonzalez *et al*., 1989) and Brazil (Schubach *et al*., 2004; Reis *et al*., 2012;).

The classical treatment in dogs is recommended with a supersaturated solution of potassium iodide (SSKI) at a dose of 40 mg/kg (0.4 mL/kg), orally, three times a day, for two months minimum (Schubach *et al*., 2012), or until the remission of lesions, after which it should be continued for 30 days (Larsson, 2011). A similar dosing schedule with a 20% solution of sodium and potassium iodides is recommended in dogs (Larsson, 2011). Although a case reported the successful use of 20% sodium iodide (NaI) solution (1 mL/4.5 kg), three times a day, for three weeks, in three dogs in the United States, which did not show signs

of iodism (Scott *et al*., 1974), this salt seems to be less used, probably due to the easier acquisition of potassium iodide (KI).

In cats, a SSKI is recommended at a dose of 10-20 mg/kg, orally, twice a day, for two months minimum (Schubach *et al*., 2012), as well as 20% KI at a dose of 0.1 mL/kg on the same schedule (Gonzalez *et al*., 1989). A supersaturated solution of sodium iodide also may be used (10 mg/kg, twice a day), for two months minimum (Schubach *et al*., 2012), but in some cats, lesions may worsen after temporary improvement (Nakamura *et al*., 1996; Crothers *et al*., 2009). Felines usually reject oral solutions, so, to facilitate administration, Reis *et al*. (Reis *et al*., 2012) suggested the use of KI in capsules (2.5-20 mg/kg, once a day, for four to five months), with which 47.9% of cats were cured. Furthermore, a combination of KI (2.5-10 mg/kg, once a day) plus ITZ (100 mg/animal, once a day) cured 96.15% of cats in Brazil in a median time of 14 weeks (Reis *et al*., 2016) and the effectiveness of the combined therapy of ITZ and KI capsules may be an option in cases refractory to ITZ (Gremião *et al*., 2015).

In relation to the toxic effects, the signs of iodism can occur, such as loss of weight, anorexia, depression, vomiting, diarrhea, hypothermia, hyperthermia, cardiovascular failure, hyperexcitability, and muscular spasms, among other adverse signs (Crothers *et al*., 2009; Reis *et al*., 2012; Schubach *et al*., 2012). It is recommended that the hepatic enzymes should be monitored due to hepatotoxicity (Reis *et al*., 2012). Dogs can show mucopurulent oculonasal discharges, salivation, and weakness (Larsson, 2011; Schubac *et al*., 2012). In these cases, medication should be discontinued for one week, but recurring or severe disease, another drug should be considered (Larsson, 2011; Schubach *et al*., 2012).

Terbinafine

TRB inhibits ergosterol synthesis via reversibly binding to *squalene epoxidase* (Greene, 2012), conferring good *in vitro* activity against *S. schenckii* complex species (Marimon *et al*., 2008), including in itraconazole-resistant *S. schenckii* and *S. globosa* (Stopiglia *et al*., 2014) However, little is known about its efficacy in cats and dogs with the disease. In feline sporotrichosis in Brazil, oral TRB is given at a dose of 30 mg/animal, once a day, or in combination with oral ITZ, at a dosage of 5-10 mg/kg, once a day (Schubach *et al*., 2004), but there have been few studies in cats.

Two cases of canine sporotrichosis due to *S. brasiliensis* have been treated with TRB, one with a cutaneous presentation, and the other with only respiratory signs. The dogs were orally treated with 25 mg/kg and 30 mg/kg of TRB, respectively, once a day with food, and were cured after 12 and 19 weeks of treatment, respectively (Viana *et al*., 2017). The high *in vitro* activity of terbinafine against the fungal isolate from the canine cases compared to other antifungals (Viana *et al*., 2017) reinforces the importance of antifungal susceptibility testing to make the best choice of antifungal.

Amphotericin B combined with Itraconazole

AMB is a polyenic antibiotic isolated from *Streptomyces nodosus* that binds to membrane steroids in eukaryotic cells, increasing permeability and leading to the leakage of nutrients/electrolytes (Greene, 2012). *In vitro* activity has been reported against the *S. schenckii* species complex (Marimon *et al*., 2008; Rodrigues *et al*., 2014; Stopiglia *et al*., 2014), including in *S. brasiliensis* isolated from cats among the MIC

values of 0.125 to 4 µg/mL (Brilhante *et al*., 2016) and a median MIC of 1 µg/mL (Almeida-Paes *et al*., 2017). Similar MIC values were found against *S. schenckii, S. brasiliensis, S. albicans* and *S. luriei* from animals between 0.03 and 1 µg/mL (Oliveira *et al*., 2011). However, AMB is considered as the second choice for disseminated sporotrichosis due to its toxicity. The lipid formulation is recommended because it is less toxic to the kidneys compared to other formulations (Greene, 2012).

Although few studies have been conducted on cats, adverse effects are common after intravenous and subcutaneous dosing, as the formation of local abscesses has been reported (Greene, 2012), whereas successful intralesional dosing has been reported in feline cases refractory to ITZ (Gremião *et al*., 2009; Gremião *et al*., 2011). Three weekly applications of AMB (1 mg/kg), intralesionally, with oral ITZ (20 mg/kg, once a day), healed a cat with a persistent nodular lesion on the nasal bridge after 9 months of treatment with ITZ (Gremião *et al*., 2009).

In an uncontrolled intervention study with 26 feline cases of sporotrichosis refractory to ITZ, Gremião *et al*. (2011) recommended the dose of 0.5 to 1.5 mL (2.5–7.5 mg) of AMB per application (median 0.7 mL/3.5 mg) per cat and showed that a range of 1 to 5 applications of AMB (median 2 applications) was able to achieve the clinical cure in 84.6% (22/26) of the cats evaluated. All animals received a combination of AMB with oral ITZ (100 mg/animal, once a day) (Gremião *et al*., 2011). However, more studies should be undertaken, and no reports have been published on treating canine sporotrichosis with AMB. The high cost of antifungals and the necessity for anesthesia prior to application should be considered regarding the choice of this therapy.

Although no significant alterations in serum and blood parameters have been in cats (Gremião *et al*., 2009), signs of abscess, edema, lethargy (Gremião *et al*., 2011), and nephrotoxicity (Greene, 2012) may occur. In human pregnancy, AMB is recommended (Briggs *et al*., 2011) and is considered safe even used for 12 months (Kauffman *et al*., 2007). This drug and its lipid formulations are the drugs of choice for systemic treatment in pregnant animals because it is not teratogenic (Greene, 2012); however, there are no studies performed in pregnant animals with sporotrichosis.

Surgical procedure combined with Itraconazole

Surgical excision is an alternative procedure in refractory cases when lesions are at an operable site for resection, such as the scrotal bag (Corgozinho *et al*., 2006; Gremião *et al*., 2006). A cat regularly treated with ITZ for 19 months showed a new lesion on the scrotal bag and the animal was successfully treated again (20 mg/kg, once a day) for five months; however, due to the persistence of the lesion, the animal was submitted to an orchiectomy and two more months of ITZ therapy (Gremião *et al*., 2006). In another case of excision, a cat with an atypical presentation of a severe granulomatous lesion on the scrotal bag and urinary retention was healed after perineal urethrostomy plus oral ITZ (10 mg/kg, once a day) for three months (Corgozinho *et al*., 2006).

Freezing with liquid nitrogen to destroy tissues was successfully used in cases refractory to ITZ (Souza *et al*., 2015). Felines with skin lesions at different body sites, including the nasal region, received oral ITZ (10 mg/kg, once a day) for a variable period, and were submitted to cryosurgery with the maintenance of antifungal therapy for more 30 days after the remission of lesions. Souza *et al*. (2015)

showed a median cure time for ITZ of 32 weeks. The combination with cryosurgery was advantageous because it may be used in the nasal region, which is a body site that complicates surgical interventions.

Immunomodulators combined with Itraconazole

Another alternative is to apply substances containing polysaccharides extracted from fungal cell walls of *Saccharomyces cerevisiae*, called 1,3-β-glucan, which stimulates the phagocytic activity of leukocytes (Freitas *et al*., 2004). The efficacy of this procedure was reported in a dog in southern Brazil with a nasal lesion caused by *S. brasiliensis* that was refractory to conventional doses of ITZ. The treatment was performed using four weekly applications of 1,3-β-glucan (0.5 mg/animal) subcutaneously, combined with oral ITZ at 10 mg/kg, twice a day (Guterres *et al*., 2014). The formation of granulomas in the treated areas was reported as a side effect, but these were reduced within few weeks, and no systemic adverse effects were noted in the hematological and biochemistry assessment, indicating the safe use of this combined treatment. The efficacy of this combined treatment is unknown in feline sporotrichosis, and the cost of the immunomodulator should be considered when making this choice.

Thermotherapy: An Alternative to Pregnancy and Lactation

In pregnancy, the most antifungals are contraindicated due to toxic effects on the fetus. KI and azole drugs are classified in categories D and C by the Food and Drug Administration, respectively, indicating risks of fetal malformation in humans and animals, which may occur when FLZ is used at high levels, and KTZ has teratogenic and embryotoxic effects (Greene, 2012). So, both drugs should be avoided in pregnancy, whereas TRB and AMB belonging to category B, indicating safe use. During lactation, FLZ is considered safe in nursing mothers and infants; however, KTZ, ITZ, AMB, and KI should be evaluated regarding the risk and benefits (Briggs *et al*., 2011). There are no reports on the treatment of lactating females with sporotrichosis, so antifungal therapy should be chosen carefully, preferring low doses with constant monitoring in puppies.

Thermotherapy is an alternative option for these cases and is recommended in human cases through daily applications of heat above 40ºC on lesions (Hiruma *et al*., 1987). Its mechanism of action is based on heat since temperatures higher than 40ºC inhibit the germination of *Sporothrix* spp. conidia (Hiruma and Kagawa, 1983). Temperature control is essential because of the death rate of phagocytized *Sporothrix* spp. is higher at 40ºC compared to 37°C (Hiruma *et al*., 1987). Thermotherapy is recommended in pregnant women, as well as in patients with a single lesion (Kauffman *et al*., 2007), being less costly than antifungals, however, caution should be taken when used in animal sporotrichosis.

Although a cat from Brazil with an ulcerated lesion in the thoracic region was successfully treated with controlled thermotherapy between 40º and 42ºC using a thermal bag, twice a day, for 15 minutes over a period of seven weeks (Honse *et al*., 2010), there are some points to be considered when this procedure is used. Disseminated presentations of sporotrichosis in animals are frequent and the thermotherapy can't be effective in animals with multiple skin and mucosal lesions. Furthermore, thermotherapy requires the

cooperation of the animal, and the lesion must be located at an accessible anatomical site (Honse *et al*., 2010), as well as care to avoid burns.

The antifungal and non-antifungal options available for the treatment of cats and dogs with sporotrichosis are summarized in Table I. The choice of therapy depends on different factors related to the fungal pathogen, the host and the animal owner. In general, conventional therapies such as those with ITZ (Meinerz *et al*., 2007; Whittemore and Webb, 2007; Crothers *et al*., 2009; Madrid *et al*., 2010; Rossi *et al*., 2013) and KI (Scott *et al*., 1974; Gonzalez *et al*., 1989; Schubach *et al*., 2004; Larsson, 2011; Reis *et al*., 2012; Schubach *et al*., 2012; Reis *et al*., 2016) have been successful in most cases, and should be considered carefully regarding dose and time to increase the chances of a cure. Due to the risk of transmission of the disease through scratches or bites from sick cats, these animals should be isolated, and therapeutic management should be carefully performed, due to the zoonotic potential (Madrid *et al*., 2012; Gremião *et al*., 2017). However, the abandonment of therapies without the permission of veterinarians may occur in owners who have difficulties administering drugs, when they observe the remission of lesions (Chaves *et al*., 2013), or due to other reasons. As well as compromising therapeutic success, the inadequate administration of drugs may contribute to antifungal resistance (Rodrigues *et al*., 2014; Stopiglia *et al*., 2014), which also was reported in *S. brasiliensis* isolated from cats and dogs (Waller *et al*., 2017).

In Vitro Antifungal Profile and the Therapeutic Perspectives for Animal Cases

For *in vitro* studies, the antifungal susceptibility tests were developed to guide therapy, as the M38-A2 guideline of Clinical and Laboratory Standards Institute (CLSI, 2008), which a fungal inoculum is directly tested on an antifungal drug in laboratories conditions and the results are expressed as minimum inhibitory concentrations (MIC). This guideline recommends that the *Sporothrix* sp. isolates, which the MIC values of ITZ are higher than 8 µg/ml, are considered as resistant (CLSI, 2008). However, this breakpoint was no defined for the different *Sporothrix schenckii* complex species and, moreover, there are no standardizations of cut-off points for the different antifungals on *Sporothrix* species, hindering the criterion of susceptibility and resistance. In turn, a study with cut-off values in *S. brasiliensis*, proposed by Almeida-Paes *et al*. (2017), suggested that elevated MICs of AMB (≥8 µg/ml), ITZ (≥4 µg/ml), KTZ (≥2 µg/ml), posaconazole (POS) (≥4 µg/ml) and TRB (≥0.5 µg/ml) can classify the fungal strains as antifungal-resistant. Considering this breakpoint, we compiled the *in vitro* studies of antifungal susceptibility testing with *Sporothrix* species isolated only from animal cases (Table II) and the emergence of *Sporothrix* species isolates with antifungal-resistance is notorious.

Few studies have been performed with animal cases, which, among the azoles drugs, ITZ is *in vitro* effectiveness against *Sporothrix* species isolated from feline (Oliveira *et al*., 2011, Brilhante *et al*., 2016; Waller *et al*., 2016a, 2018) and canine (Viana *et al*., 2017; Waller *et al*., 2016a, 2018) sporotrichosis in Brazil and *S. schenckii* from feline sporotrichosis in a Malaysian study (Han *et al*., 2017). Although ITZ is still the antifungal of choice for animal sporotrichosis, the recent cases of sporotrichosis seem to be less sensitive than the ancient cases, when a study with *S. brasiliensis* from Rio de Janeiro (Borba-Santos *et al*. 2015) showed that the human and feline cases between 2011 and 2012 demanded higher MIC values of itraconazole compared to those collected before 2004. This finding highlights for the higher difficulties to

control *Sporothrix* species nowadays, since itraconazole-resistant isolates were recognized in *S. brasiliensis* (Waller *et al*., 2016b, Waller *et al* 2017), *S. schenckii* (Han *et al*., 2017), *S. albicans* and *S. luriei* (Oliveira *et al*., 2011).

Among others azole drugs, KTZ has a good *in vitro* activity against *S. brasiliensis* from cats (Brilhante *et al*., 2016) and dogs (Viana *et al*., 2017), as well as against *S. schenckii* (Oliveira *et al*., 2011; Han *et al*., 2017), *S. albicans* and *S. luriei* (Oliveira *et al*., 2011) from animal cases. Although the MIC values higher or equal to 2 μg/ml suggest emergence of *S. brasiliensis* with antifungal resistance (Almeida-Paes *et al*., 2017), this value was found in ten *S. schenckii* isolates from cats (Han *et al*., 2017) and *S. albicans* and *S. luriei* from a cat and a dog (Oliveira *et al*., 2011). FLZ showed high MIC values (Oliveira *et al*., 2011; Brilhante *et al*., 2016), being the azole with less activity, among the antifungal evaluated (Table II). AMB (Oliveira *et al*., 2011; Brilhante *et al*., 2016; Sanchotene *et al*., 2017; Viana *et al*., 2017) and TRB (Oliveira *et al*., 2011; Sanchotene *et al*., 2017, Viana *et al*., 2017) active were considered active.

Other antifungals are still few studied in *Sporothrix* isolated from animals, as POS (Viana *et al*., 2017), voriconazole (VRZ) and caspofungin (CAS) (Oliveira *et al*., 2011; Brilhante *et al*., 2016). These antifungals were inhibitors for the growth of *Sporothrix* species, but there are few studies in animal cases. POS showed effectiveness in a murine model infected by *S. brasiliensis* and *S. schenckii* (Fernández-Silva *et al*. 2012), including when used in combination with AMB, which the synergistic effect of both antifungals was successfully used in a disseminated experimental sporotrichosis caused by *S. brasiliensis* (Mario *et al*. 2015). However, POS is expensive, and currently has limited availability in some countries with a high prevalence of sporotrichosis, such as Brazil (Borba-Santos *et al*. 2015). Despite *S. brasiliensis* from cats (Brilhante *et al*., 2016) and *S. schenckii*, *S. albicans* and *S. luriei* from cats and dog (Oliveira *et al*., 2011) were inhibited by VRZ in different concentrations. However, VRZ was weakly active in an experimental disseminated sporotrichosis (Fernández-Silva *et al*., 2014), and no synergistic effect was noted when in combination with TRB (Oliveira *et al*., 2015), being few or not recommended this antifungal for treat animal sporotrichosis. Antifungal susceptibility testing with new antifungal should be performed on *Sporothrix* species from animal cases, due to the emergence of antifungal-resistant isolates. Besides that, new active molecules must be discovered as alternative options for the refractory cases, which has been searched on natural sources (Burian *et al*., 2017; Waller *et al*., 2016a, 2016b, 2017, 2018) with potential activity against *Sporothrix* species, as a promising alternative for therapy, but more studies should be performed for this purpose. In this way, veterinarians should advise animal owners regarding correct therapy, aiming for clinical success and to consider the therapies available in cases refractory to antifungals.

Final Considerations

Considering the available therapies for cats and dogs with sporotrichosis, it is important to carry out pharmacological studies to give more therapeutic options in veterinary medicine due to increased cases of the disease in animals, as well as to the reports of antifungal resistance. Azoles and potassium iodide are the main antifungals used, and itraconazole is the drug of choice with dosing over several months with an extension for 30 days after the remission of lesions. For cases refractory to antifungals, drug combinations can be used, as well as surgical procedures and immunomodulatory substances. The choice of the best

therapy depends on the antifungal susceptibility of the Sporothrix sp. isolate, drug efficacy, and safe use, as well as to factors related to the host, such as pregnancy, during which thermotherapy is indicated. Factors related to the animal owner, like cost and therapy management, should also be considered.

Conflict of Interest

The authors declare exist no conflict of interest in this study.

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Table I. Therapeutic information for feline and canine sporotrichosis and their respective dosages and adverse signs, according to the references.

Therapeutic approach	Adverse signs	Clinical cares and observations
Itraconazole ***Cats***: 10 mg/kg, orally, once a day (Meinerz *et al*., 2007; Crothers *et al*., 2009; Madrid *et al*., 2010; Rossi *et al*., 2013); or 5 mg/kg, twice a day (Crothers *et al*., 2009; Souza *et al*., 2009). If hinder the cure, may use 8.3-27.7 mg/kg, once a day (Pereira *et al*., 2010). ***Dogs***: 10 mg/kg, orally, once a day (Crothers *et al*., 2009; Madrid *et al*., 2012; Rossi *et al*., 2013); or 5 mg/kg, twice a day (Crothers *et al*., 2009; Souza *et al*., 2009).	Vomiting, loss of appetite, nausea, anorexia, and hepatotoxicity (Schubach *et al*., 2004; Pereira *et al*., 2010).	Considered the drug of choice (Meinerz *et al*., 2007; Whittemore and Webb, 2007; Crothers *et al*., 2009 Madrid *et al*., 2010; Rossi *et al*., 2013) Should be administered with food (Greene, 2012). Liver enzymes should be monitored (Larsson, 2011; Greene, 2012). Contraindicated in pregnancy (Greene, 2012).
Ketoconazole ***Cats***: 5-10 mg/kg (Schubach *et al*., 2004; Han *et al*., 2017); or 50 mg/animal (Silva *et al*., 2008), orally, once a day. ***Dogs***: 5-10 mg/kg, orally, once (Schubach *et al*., 2012) or twice (Crothers *et al*., 2009) a day.	Vomiting, nausea, diarrhea, inappetence, and hepatoxicity (Silva *et al*., 2008; Crothers *et al*., 2009; Larsson, 2011).	Should be administered with food (Greene, 2012). Liver enzymes should be monitored (Larsson, 2011; Greene, 2012). Contraindicated in pregnancy (Greene, 2012).
Fluconazole ***Cats***: 10 mg/kg, orally, once a day (Crothers *et al*., 2009); or 50 mg/animal plus ITZ (5-10 mg/kg), once a day (Schubach *et al*., 2004).	Nausea, vomiting, diarrhea, and anorexia (Schubach *et al*., 2004; Crothers *et al*., 2009).	Should be administered with food (Greene, 2012). Liver enzymes should be monitored (Larsson, 2011; Greene, 2012). Contraindicated in pregnancy (Greene, 2012).
Potassium iodide alone or combined with itraconazole ***Cats***: 2,5-20 mg/kg, orally, once or twice a day (Reis *et al*., 2012; Schubach *et al*., 2012); or same dosage of KI, once a day, plus ITZ (100 mg/animal), once a day (Reis *et al*., 2016). ***Dogs***: 40 mg/kg, orally, three times a day (Larson, 2011; Schubach *et al*., 2012).	Anorexia, weight loss, lethargy, fever, vomiting, hyperexcitability (Reis *et al*., 2012; Reis *et al*., 2016), and epiphora, nasal discharge, salivation and weakness may occur in dogs (Crothers *et al*., 2009; Schubach *et al*., 2012).	Can be administered with liquids (Sterling and Heymann, 2000). Liver enzymes should be monitored (Reis *et al*., 2012; Reis *et al*., 2016). Contraindicated in pregnancy (Greene, 2012).

Terbinafine alone or combined with itraconazole		
Cats: 30 mg/animal, orally, once a day, or plus ITZ (5-10 mg/kg), once a day (Schubach *et al*., 2004). ***Dogs***: 25-30 mg/kg, orally, once a day (Viana *et al*., 2017).	Vomiting, pruritus, and hepatotoxicity may occur (Greene, 2012).	Should be administered with food (Viana *et al*., 2017). Liver enzymes should be monitored (Greene, 2012).
Amphotericin B combined with itraconazole		
Cats: 1 mg/kg of AMB, via intralesional, plus oral ITZ (20 mg/kg), once a day (Gremião *et al*., 2009) or 0.5 to 1.5 mL (2.5 – 7.5 mg) of AMB/animal, via intralesional, plus oral ITZ (100 mg/animal), once a day (Gremião *et al*., 2011).	Abscess, edema, lethargy (Gremião *et al*., 2011), nephrotoxicity (Greene, 2012).	In humans, it is recommended during pregnancy (Kauffman *et al*., 2007; Briggs *et al*., 2011), although there have been no studies in pregnant females with sporotrichosis.
Immunomodulator combined with itraconazole		
Dogs: 1,3-β-glucan (0.5 mg/animal), via subcutaneous, once every 4 weeks, plus ITZ (10 mg/kg, orally), twice a day (Guterres *et al*., 2014).	Granuloma on application areas may occur, but subsides in a few weeks (Guterres *et al*., 2014).	N.i.
Thermotherapy		
Cats: local heat greater than 40ºC, several times a day, for 15 minutes (Honse *et al*., 2010).	N.i.	Recommended in pregnancy and lactation (Gremião *et al*., 2009). Requires cooperation of the animal (Honse *et al*., 2010). Be careful to avoid burns.

ITZ, itraconazole; KI, potassium iodide; N.i., No informations;

Table II. Minimal inhibitory concentrations (MIC) of the main antifungal drugs against *Sporothrix schenckii* complex species from animal cases.

Sporothrix* species (*n*)	**Animal origin**	**Antifungal agent****	**Range of MIC**	**Country (Reference)**
S. brasiliensis (48)	Feline	AMB	0.125 – 4 µg/ml	Brazil (Brilhante *et al*., 2016)
		ITZ	0.125 – 2 µg/ml	
		VRZ	2 – 64 µg/ml	
		FLZ	62.5 – 500 µg/ml	
		KTZ	0.03 – 2 µg/ml	
		CAS	0.25 – 64 µg/ml	
S. brasiliensis (12)	Feline	ITZ	0.06 – >16 µg/ml	Brazil (Waller *et al*., 2018)
S. brasiliensis (35)	Feline	AMB	0.125 – 8 µg/ml	Brazil (Sanchotene *et al*., 2017)
		ITZ	0.25 - 8 µg/ml	
		TRB	0.0156 - 4 µg/ml	
S. brasiliensis (20)	Feline	ITZ	4 – 32 µg/ml	Brazil (Poester *et al*., 2018)
S. brasiliensis (17)	Canine	ITZ	0.06 – >16 µg/ml	Brazil (Waller *et al*., 2018)
S. brasiliensis (6)	Canine	ITZ	0.5 – 8 µg/ml	Brazil (Waller *et al*., 2016b)
S. brasiliensis (2)	Canine	AMB	4 µg/ml	Brazil (Viana *et al*., 2017)
		TRB	0.06 µg/ml	
		POS	1 µg/ml	
		KTZ	0.25 and 0.5 µg/ml	
		ITZ	0.5 µg/ml	
S. schenckii (44)	Feline	ITZ	0.5 – 4 µg/ml	Malaysia (Han *et al*., 2017)
		KTZ	0.125 – 4 µg/ml	
		TRB	1 – 8 µg/ml	
S. schenckii (6)	Feline	ITZ	00.6 – 0.5 µg/ml	Brazil (Oliveira *et al*., 2011)
		KTZ	0.06 – 2 µg/ml	
		MCZ	0.5 – 1 µg/ml	
		VRZ	1 – 16 µg/ml	
		FLZ	64 – 128 µg/ml	
		TRB	0.06 – 0.25 µg/ml	
		AMB	0.03 – 0.25 µg/ml	
		CAS	8 – 32 µg/ml	
S. albicans (1), *S. luriei* (1)	Feline, Canine	ITZ	32 µg/ml	Brazil (Oliveira *et al*., 2011)
		KTZ	4 µg/ml	
		MCZ	8 µg/ml	

	VRZ	4 and 2 µg/ml, respectively
	FLZ	128 and 64 µg/ml, respectively
	TRB	0.25 µg/ml
	AMB	1 and 0.5 µg/ml, respectively
	CAS	32 µg/ml

*Number of *Sporothrix* species isolates tested through the M38-A2 guidelines (CLSI, 2008); **AMB, amphotericin B; ITZ, itraconazole; VRZ, voriconazole; FLZ, fluconazole; KTZ, ketoconazole; CAS, caspofungin; TRB, terbinafine; POS, posaconazole; MCZ, miconazole.

4.2 Artigo 2

Plants from Lamiaceae family as source of antifungal molecules in humane and veterinary medicine

Stefanie Bressan Waller, Marlete Brum Cleff, Emanoele Figueiredo Serra, Anna Luiza Silva, Angelita dos Reis Gomes, João Roberto Braga de Mello, Renata Osório de Faria, Mário Carlos Araújo Meireles

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Plants from Lamiaceae Family as Source of Antifungal Molecules in Humane and Veterinary Medicine

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Abstract

This work aimed to review the main plants of Lamiaceae family with activity against pathogenic fungi of medical and veterinary interest. Published studies in the main international databases between January 2002 and June 2016 showed that 55 botanical species belonging to 27 genus presented antifungal activity in different forms of extractions, mainly essential oils. Pathogenic fungi of *Aspergillus* spp., *Candida* spp., *Malassezia* spp., *Cryptococcus* spp., *Sporothrix* spp., *Microsporum* spp., *Trichophyton* spp. and *Epidermophyton* spp. genus were *in vitro* sensitive to several plants of Lamiaceae family. Chemical molecules isolated were described as promising use as antifungals in mycoses, highlighting estragole, 1,8-cineole, terpineol-4, γ-terpinene, among others. However, it should be alert to need of universal standardization in the laboratories tests with natural products.

Key words: mycosis; antifungal resistance; natural products; essential oils; extracts.

1. Introduction

In the last years, the increasing impact of mycosis has been related to its therapeutic limitations, such as pharmacological toxicity, side effects, high cost, in addition to the negligence or abandonment of therapy [1–2], which has favored the emergence of antifungal resistance [3–4]. The current scenario has stimulated research to find more efficient antifungal molecules [5] and studies have been conducted with medicinal plants , due to their potential as alternative sources to health care [6]. Among the plants with potential antimicrobial activities, those belonging to Lamiaceae

(Labiateae) family are of great pharmaceutical interest, because they are used in popular medicine against mycosis through macerations, decoctions, infusions and others preparations of its roots, shells, leaves and flowers [7–8]. Composed of aromatic herbs, shrubs and trees with quadrangular branches, around 7500 species are recognized and distributed throughout the world [9–10]. The aromaticpotential of this family must be highlighted, as well asits use in the cosmetic and perfume industries, mainly with essential oils [11].

The antifungal potential of Lamiaceae family has been studied in the last years, including against pathogens with resistance to conventional antifungals [12–15], highlighting different botanical species as potential sources for elaboration of medication. Faced with numerous studies with natural products, there is a need in recognize the plants with potential antifungal application in the pharmacological industry in order to develop in-depth studies. In this sense, this review aimed to compile the information of the past 14 years regarding antifungal activity of different extracts of plants from the Lamiaceae family against pathogenic fungi of importance in humane and veterinary medicine.

2. Research Strategy

Published studies were investigated by three independent examiners in the following databases: Web of Science, SciVerse Scopus, Medline, SciELO, PubMed, LILACS and Science Direct. For investigation, a combination of keywords was used [*antifungal; antimicrobial*] + [*aspergillus; candida; malassezia; cryptococcus; microsporum; trichophyton; dermatophytes; sporothrix*] + [*Lamiaceae*; botanical genus], according to Global Strategy for Plant Conservation [16]. As criteria of inclusion, only papers proving antifungal activity of Lamiaceae plants between January 2002 and June 2016 were including in this review.

3. Universal Standardization with Natural Products

Different methodologies for in vitro tests with natural products were performed, such as disk diffusion assay [5,17-19], agar well difusion [19], agar dilution method [20], agar tube dilution test [7], serial microplate dilution method [21], broth microdilution method [22-26] and broth macrodilution method [27]. The diversity of experimental methodologies make the comparative study of products difficult in relation to antifungal potential and alerts to the urgent need to utilize a universal methodology with natural products. Fennel *et al.* [28] warned about the lack of standardization in antimicrobial tests with herbal extracts, which results in variations in the values of minimal inhibitory concentration. Moreover, it reinforces that the related factors about culture and origin of plant, as well as the extraction methology, may interfere in the majority composition of the active compounds [15], and, subsequently, results in a higher or lower antimicrobial potential.

4. Promising Botanical Species

Botanical species with potential aplicability in pharmaceutical industry against pathogenic fungi are presented in the Table 1. Of the 55 botanical species belonging to 27 genus of Lamiaceae family, the essential oils of *Origanum* spp., such as *O. vulgare* [24,26,47,60-64] and *O. majorana* [14,47,58-59], popularly known as oregano and marjoram, respectively, corresponded to plants with the greatest activity against pathogenic fungi, including those with zoonotical potential, such as dermatophytes of *Microsporum* spp. and *Trichosphorum* spp., and fungi of *Sporothrix schenckii* complex. Different extracts of *Mentha* spp. [6,47,50-54] and *Lavandula* spp. [2,25,44-48] have been promising, mainly as essential oils against dermatophytes and *Candida* spp., as well as oils of *Rosmarinus officinalis*, which were excelled against *Candida* spp. [54], *Aspergillus niger* [65] and *Sporothrix* spp. [64,66].

Candida spp. and *Aspergillus* spp. species constituted the primary target for research [7,13,21-24,32-35,38,40-43,50-54,58,62,68-71], and fungi species that produce toxins, for example, *Aspergillus ochraceus*, showed susceptibility to natural products, such as essential oils of *Hyptis suaveolens* [27]. In *Malassezia* spp., the plants *Ocimum* spp. [53] and *Origanum* spp. [47,61] were promising, as well as *Lavandula* spp. [2,46,48] and *Thymus* spp. [2,70] against *Cryptococcus* spp.

Table 1. Ethnobotanical data of the extracts of medicinal plants from Lamiaceae family with antifungal activity against pathogenic fungi with medical and veterinary interests.

Plant Species	Plant Material (Country Collection)	Extract*	Pathogenic Fungi**	Source
Agastache rugosa	Aerial parts (Korea)	E.O.	*T.er, T.me, T.ru, T.sc, T.so.*	29
	Aerial parts (Korea)	E.O.	*A.fl, A.ni, C.al, C.tr, C.ut, C.ne, T.to.*	17
Ballota nigra	Leaves, stem, root (Pakistan)	BU, CH, E.A., HE	*A.fl, A.fu, A.ni,.*	7
Calamintha nepeta	Aerial parts (Italy, Portugal)	E.O.	*A.ni, A.fl, A.fu, C.al, C.kr, C.gu, C.pa, C.tr, C.ne, E.fl, M.ca, M.gy, T.me, T.ru.*	15
Carum copticum	Commercial formulation (India)	E.O.	*A.fu, A.ni, T.ru.*	30
Clerodendrum glabrum	Leaves (South Africa)	AC	*A.fu, C.al, C.ne.*	21
	Leaves (South Africa)	HE	*C.al, C.ne.*	8
Clerodendrum inerme	Leaves, stems (India)	E.A., HE	*E.fl, T.me, T.ru, T.to.*	31
Clerodendrum phlomidis	Leaves, stems (India)	E.A., HE	*E.fl, T.me, T.ru, T.to.*	31
Colebrookea oppositifolia	Leaves (India)	ME, WA	*C.al.*	32
Coleus aromaticus	Leaves, flowers (India)	E.O.	*A.ni, A.pa, C.al.*	33
Coleus zeylanicus	Leaves, flowers (India)	E.O.	*A.ni, A.pa, C.al.*	33
Coleus barbatus	Leaves (India)	P.E.	*A.fu, A.ni, A.ru, C.al.*	34
Coleus forskohlii	Leaves (India)	P.E.	*A.fu, A.ni, A.ru, C.al.*	34
Cyclotrichium niveum	Aerial parts (Turkey)	ET, WA	*C.al, C.gl, C.pa, C.tr.*	35
Glechon marifolia	Aerial parts (Brazil)	E.O.	*E.fl, T.ru.*	36
Glechon spathulata	Aerial parts (Brazil)	E.O.	*E.fl, T.ru.*	36

Hedeoma drummondii	Aerial parts (Mexico)	HE	*C.al.*	22
Hedeoma multiflora	Leaves, stems (Argentina)	E.O.	*A.f, A.pa.*	9
Hymenocrater longiflorus	Leaves (Iran)	E.O.	*A.fu, C.a.l*	38
Hymenocrater sessilifolius	Whole plant (Pakistan)	ME	*C.al, M.ca, T.ru, T.lo.*	70
Hyptidendron canum	Leaves (Brazil)	ET	*C.al.*	9
Hyptis martiusii	Leaves (Brazil)	ET	*C.al, C.kr, C.tr.*	23
Hyptis suaveolens	Leaves (Brazil) Aerial parts (Thailand)	E.O. E.O.	*A.fl, A.fu, A.ni, A.oc, A.pa.* *C.al, M.gy, T.me, T.ru.*	27 5
Hyssopus officinalis var. *angustifolius*	Aerial parts (Turkey)	E.O.	*A.fl, C.al, T.ru.*	39
*Hyssopus officinalis*var. *pilifer*	Aerial parts (Serbia)	E.O., E.A., ME, WA	*A.fu, A.ni, A.pc, A.ve, C.al.*	40
Hyssopus officinalis	Aerial parts (Bulgaria)	E.O.	*C.al, C.gl, C.kr, C.pa, C.tr.*	41
Lallemantia royleana	Aerial parts (Iran)	E.O.	*A.ni, C.al.*	13
Lamium album	Leaves, flowers (Bulgaria)	WA	*C.al, C.gl.*	42
Lamium tenuiflorum	Leaves, roots (Turkey)	ET	*C.al, C.gu, C.tr, C.la, C.ne.*	43
Lavandula angustifolia	Commercial formulation (N.i.) N.i. (Serbia)	E.O. E.O.	*T.me, T.ru.* *T.me.*	44 45
Lavandula multifida	Aerial parts (Portugal)	E.O.	*A.fl, A.fu, A.ni, C.al, C.gu, C.kr, C.pa, C.tr, C.ne, E.fl, M.ca, M.gy, T.me, T.ru, T.ve.*	46
Lavandula officinalis	Commercial formulation (N.i.)	E.O.	*M.pa.*	47
Lavandula pedunculata	Aerial parts (Portugal)	DI, E.O., E.A., HE, ME, WA	*C.gu, C.ne.*	25
Lavandula stoechas var. *luisieri*	Aerial parts (Portugal)	DI, E.O., E.A., HE, ME, WA	*A.ni, C.al, C.gu, C.ne.*	25

	Aerial parts (Portugal)	E.O.	*A.fl, A.fu, A.ni, C.al, C.gu, C.kr, C.pa, C.ne, E.fl, M.ca, M.gy, T.me, T.ru, T.ve.*	48
Lavandula stoechas	Aerial parts (Italy)	E.O.	*A.fl, A.fu, A.ni, C.al, C.gu, C.kr, C.tr, C.ne, E.fl, M.ca, M.gy, T.me, T.ru, T.ve.*	2
Lepechinia meyenii	Aerial parts (Peru)	ET	*C.al, T.me.*	49
Mentha aquatica	Aerial parts (Yugoslavia)	E.O.	*C.al, E.fl, M.ca, T.me, T.ru, T.to.*	50
Mentha longifolia	Aerial parts (Pakistan)	BU, CH, ET, P.E.	*C.al.*	51
	Aerial parts (Yugoslavia)	E.O.	*C.al, E.fl, M.ca, T.me, T.ru, T.to.*	50
	Aerial parts (Turkey)	ME	*C.al, C.gl, C.tr.*	6
	Leaves (Iraq)	E.O.	*C.al.*	52
Mentha longifologia var. *noeana*	Aerial parts (Turkey)	E.O.	*C.al.*	53
Mentha piperita	Aerial parts (Yugoslavia)	E.O.	*C.al, E.fl, M.ca, T.me, T.ru, T.to.*	50
	Aerial parts (Turkey)	ME	*C.al, C.gl, C.tr.*	6
	Commercial formulation (N.i.)	E.O.	*M.pa.*	47
	Leaves (Brazil)	ME	*C.al, C.du, C.gl, C.gu, C.kr, C.lu, C.pa, C.ru, C.tr, C.ut.*	54
Nepeta curviflora	Leaves (Lebanon)	ME	*C.al.*	18
Ocimum gratissimum	Leaves (Nigeria)	ET	*M.fu.*	55
	Leaves (Brazil)	CH(fr), HE(fr), E.O., ET	*C.ne.*	56
Ocimum sanctum	Leaves (India)	AL, BE, CH, E.A., HE, ME, WA	*E.fl, M.gy, M.na, T.me, T.ru.*	57
Origanum libanoticum	Whole plant (Lebanon)	ME	*C.al.*	18
Origanum majorana	Aerial parts (Tunisia)	E.O.	*C.al, C.gl, C.kr, C.pa, C.tr, M.ca, T.ru, T.me, T.vi.*	58
	Commercial formulation (N.i.)	E.O.	*M.pa.*	47
	Leaves (Mexico)	E.O.	*E.fl, M.ca, M.gy, T.me, T.ru, T.to.*	59
	Aerial parts (Egypt)	E.O.	*S.br, S.sc.*	14
Origanum vulgare	Commercial formulation (N.i.)	E.O.	*C.gl.*	60
	Commercial formulation (N.i.)	E.O.	*M.pa.*	47
	Leaves (Chile and Uruguay)	E.O.	*C.al, C.kr, C.lu, C.pa.*	24

	Leaves (N.i.)	E.O.	*A.fl, A.fu, C.al, M.pa, S.sc.*	61
	Leaves (Chile)	E.O.	*C.al, C.du, C.kr, C.lu, C.pa.*	62
	Aerial parts (Chile)	E.O.	*S.sc.*	63
	N.i. (Brazil)	E.O.	*S.br, S.sc.*	26
	Aerial parts (Chile, Moldavia)	E.O.	*S.br, S.sc.*	64
Rosmarinus officinalis	Aerial parts (Tunisia)	E.O.	*A.ni.*	65
	Leaves (Brazil)	DI, ME	*C.al, C.du, C.gu, C.kr, C.lu, C.pa, C.ru, C.tr.*	54
	Leaves (India)	E.O.	*S.sch*	66
	Aerial parts (Chile, Tunisia)	E.O.	*S.br, S.sch.*	64
Salvia officinalis	Leaves (Thailand)	E.O.	*C.al.*	67
Salvia plebeia	Leaves (India)	ME, WA	*C.al.*	32
Salvia ringens	Aerial parts (Macedonia)	E.O.	*A.fl, A.fu, A.gl, C.al, C.kr, C.pa, T.me.*	68
Stachys officinalis	Aerial parts (Serbia)	E.O.	*A.ni, C.al.*	69
Thymus herba-barona	Aerial parts (Italy)	E.O.	*A.fl, A.fu, A.ni, C.al, C.gu, C.kr, C.tr, C.ne, E.fl, M.ca, M.gy, T.me, T.ru, T.ve.*	2
Thymus schimperi	Aerial parts, leaves (Ethiopia)	E.O.	*A.ni, C.al.*	20
Thymus villosus subsp. *lusitanicus*	Aerial parts (Portugal)	E.O.	*A.fl, A.fu, A.ni, C.al, C.du, C.gl, C.kr, C.pa, C.ne, E.fl, M.ca, M.gy, T.in, T.me, T.ru, T.ve.*	70
Thymus vulgaris	N.i. (India)	E.O.	*A.fu, A.ni, T.ru.*	30
Zataria multiflora	Whole plant (Iran)	ET, ME	*C.al, C.gl, C.pa, C.tr.*	71

N.i. – Not informed in the bibliographic reference consulted; * ***A.fl*** *– Aspergillus flavus,* ***A.fu*** *– A. fumigatus;* ***A.gl*** *– A. glaucus;* ***A.ni*** *– A. niger;* ***A.oc*** *– A. ochraceus;* ***A.pa*** *– A. parasiticus;* ***A.ru*** *– A. ruantii;* ***A.ve*** *– A. versicolor;* ***C.al*** *– Candida albicans;* ***C.du*** *– C. dubliniensis;* ***C.gl*** *– C. glabrata;* ***C.gu*** *– C. guillermondii;* ***C.kr*** *– C. krusei;* ***C.lu*** *– C. lusitaniae;* ***C.pa*** *– C. parapsilosis;* ***C.ru*** *– C. rugosa;* ***C.tr*** *– C. tropicalis;* ***C.ut*** *– C. utilis;* ***C.la*** *– Cryptococcus laurentii;* ***C.ne*** *– C. neoformans;* ***E.fl*** *– Epidermophyton floccosum;* ***M.fu*** *– Malassezia furfur;* ***M.pa*** *– M. pachydermatis;* ***M.ca*** *– Microsporum canis;* ***M.gy*** *– M. gypseum;* ***M.na*** *– M. nanum;* ***S.br*** *– Sporothrix brasiliensis;* ***S.sc*** *– S. schenckii;* ***T.er*** *– Trichophyton erinacei;* ***T.in*** *– T. interdigitale;* ***T.lo*** *– T. longifusus;* ***T.me*** *– T. mentagrophytes;* ***T.ru*** *– T. rubrum;* ***T.sc*** *– T. schoenleinii;* ***T.so*** *– T. soudanense;* ***T.to*** *– T. tonsurans;* ***T.ve*** *– T. verrucosum;* ***T.vi*** *– T. violaceum;* ** **AC** – Acetone, **AL** – Alcoholic; **BE** – Benzene; **BU** – Butanol; **CH** – Chloroform; **CH(fr)** – Chloroform fraction; **DI** – Dichloromethane; **E.O.** – Essential oil; **ET** – Ethanol; **ET(fr)** – Ethanol fraction; **E.A**. – Ethyl acetate; **HE** – Hexane; **HE(fr)** – Hexane fraction; **ME** – Methanol; **P.E.** – Petroleum ether; **WA** – Water.

5. Main Molecules of Pharmaceutical Interest

Some molecules has been identified as major in different extracts and promising for antifungal use (Fig. 1). Estragole is the main chemical compound in essential oil of *Agastache rugosa* and has been active against *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans* [17], and dermatophytes of *Trichophyton* spp. genus [29]. In different species of *Lavandula* spp., the linalool compound was active against *Malassezia pachydermatis* [47] and *T. mentagrophytes* in experimental infections without adverse effects [45], as well as against others dermatophytes, *Candida* spp. and *Aspergillus* spp. [70]. This was even observed in geraniol against yeasts, dermatophytes and *Aspergillus* spp. genus [70] and menthol against dermatophytes, *Candida* spp. and *M. pachydermatis* [47,50,52], being this compound in abundace in essential oils of *Mentha* spp.

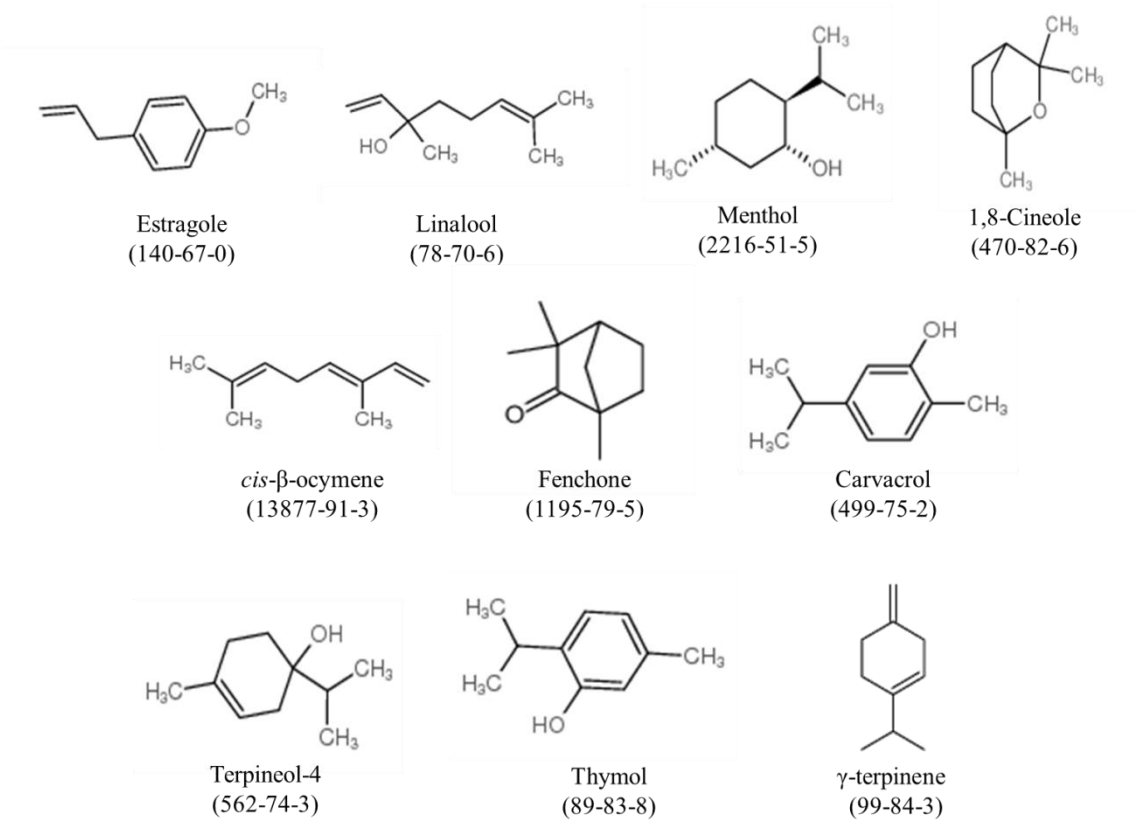

Fig 1. Chemical structure and respective CAS number (unique numerical identifier assigned by Chemical Abstracts Service) of the main compounds found in the extracts of plants from Lamiaceae family with antifungal properties against several pathogenic fungi.

Few studies have been conducted to understand the antifungal mechanism of action of the active molecules derived from natural products [26]. Among them, eucalyptol, which is known as 1,8-cineole, has promoted morphological alterations in *Aspergillus* spp. through decreased formation of conidia and degradation in its plasmatic membrane, resulting in the disruption of cell membrane and, consequently, degeneration of fungal cell wall [27]. This chemical compound has been indentified in essential oils of *Hyptis suaveolens* [27], *Hyssopus officinalis* subsp. *pilifer* [40], *Rosmarinus officinalis* [64,71], *Lavandula luisieri* [48], *Salvia ringens* [68] and *Origanum majorana* [14],

among others. Moreover, the synergic effect between the compounds has been noted due to its better antifungal activity, such as shown in fluconazole-resistant *Candida* ssp., which were more sensitive to essential oil of *Hyssopus officinalis* than to individual major compounds of cis-pinocamphone and β-pinene [41].

The sensibility of *Candida* spp. species, *C. neoformans*, *Aspergillus* spp. and dermatophytes of *Trichophyton* spp., *Microsporum* spp. and *E. floccosum* was tighly excelled by fenchone [2] and cis-β-ocymene, which are major in *Lavandula* spp. oils and has provoked a metabolic prision on fungi, resulting in cell death [46]. Thymol and carvacrol also are promising compounds with activity against *T. mentagrophytes* [72] and *T. rubrum* [30], and others dermatophytes, as well as against *Candida* spp., *C. neoformans*, *Aspergillus* spp. [2] and *M. pachydermatis* [47]. These compounds are major in essential oils of *Hedeoma multiflora* [37], *Lavandula multifida* [46], *O. vulgare* [63-64], *Carum copticum*, *Thymus vulgaris* [30], *Thymus serpillum* [72], *Thymus herba-barona* [2], among others.

Terpineol-4 also has been promising, due its abundance in essential oils of *Origanum majorana* [58-59] and *Origanum vulgare* [61-63] with comproved activity against *Candida* spp., *Cryptococcus neoformans*, dermatophytes and *Aspergillus* spp. [70]. Besides, γ-terpinene also has been highlighted because it was active against *S. schenckii* and *S. brasiliensis* through morphological alterations in hyphae and reduction of conidia, when treated with essential oils of *Origanum vulgare* [26]. This compound also was found as major in others studies [63]. Several isolated molecules of plants from Lamiaceae family are promising for elaboration of antifungals by pharmaceutical industry. Thereby, more studies should be undertaken in order to understand its efficacy and safe use *in vivo* in different mycoses.

5. Conclusions

The botanical species of Lamiaceae family showed antifungal activity against pathogenic fungi of human and veterinary interest, constituting promising candidates as antifungals. Molecules, such as estragole, 1,8-cineole, terpineol-4, γ-terpinene, among others, have been identified as responsible for antifungal activity and their mechanism of action is being studied. New research should be undertaken with the aim of elaborating new antifungals from Lamiaceae plants and utilize them in the treatment of human and animals mycosis.

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4.3 Artigo 3

In Vitro Susceptibility of *Sporothrix brasiliensis* to Essential Oils of Lamiaceae Family

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In vitro susceptibility of *Sporothrix brasiliensis* to essential oils of Lamiaceae family

[Antifungal activity of Lamiaceae oils]

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Abstract

This study evaluated the chemical, cytotoxic and anti-*Sporothrix brasiliensis* properties of commercial essential oils of rosemary (*Rosmarinus officinalis* L.), oregano (*Origanum vulgare* L.) and marjoram (*Origanum majorana* L.). Chemical composition of the oils was identified through gas chromatography with flame ionization detector, and cytotoxicity was performed through MTT assay in Vero cell line. Anti-*S. brasiliensis* activity was performed according to the CLSI M38-A2 guidelines using isolates obtained from cats and dogs. The major compounds found were carvacrol in the oregano oil (73.9%) and 1,8-cineole in rosemary and marjoram oils (49.4% and 20.9%, respectively). All *S. brasiliensis* isolates were susceptible to the plant oils, including itraconazole-resistant ones. Marjoram and rosemary oils showed

MIC_{90} of 0.56 mg ml^{-1} and 1.12 mg ml^{-1}, and MFC_{90} of 4.5 mg ml^{-1} and 9 mg ml^{-1}, respectively. For oregano oil, a strong antifungal activity was observed with MIC_{90} and MFC_{90} values ≤ 0.07 mg ml^{-1}. The weakest cytotoxicity was observed for rosemary oil. Further studies should be undertaken to evaluate the safety and efficacy of these essential oils in sporotrichosis.

Keywords: sporotrichosis; *Origanum vulgare* L.; *Origanum majorana* L.; *Rosmarinus officinalis* L.; cytotoxicity; chemical composition.

Introduction

Sporotrichosis, a worldwide distributed zoonotic mycosis with importance in human and veterinary medicines, is caused by members of the *Sporothrix schenckii* species complex, in which *Sporothrix brasiliensis* is the main etiological agent in feline sporotrichosis outbreaks in Brazil [1 – 3]. The drug of choice for the treatment of sporotrichosis is itraconazole and, despite the use of several therapeutic medications, the emergence of antifungal resistance has been observed [4 – 5].

This alarming situation has stimulated the search for new chemical compounds with antifungal property in medicinal plants. Aromatic plants of Lamiaceae family such as oregano (*Origanum vulgare* L.), marjoram (*Origanum majorana* L.) and rosemary (*Rosmarinus officinalis* L.) are known for their antifungal activity against *Candida* spp., *Cryptococcus* spp., *Microsporum* spp., *Trichophyton* spp. and other pathogenic fungi [6 – 9], as well as against *Pythium insidiosum* [10]. The activity of rosemary [11] and oregano [12 – 13] against *S. schenckii* and *S. brasiliensis* has been previously reported.

In folk medicine, the use of essential oils for therapeutic purposes is assigned to commercial products because of their easy acquisition. Nonetheless, Veiga Jr. *et al*. [14] cautioned for the quality of these products due to the risk of loss of efficacy and appearance of side effects in case of adulteration. The few antimicrobial studies conducted with commercial essential oils of the above-mentioned plants also prove their efficacy against several pathogens [10, 15]. In sporotrichosis, studies are concentrated in oils obtained from plants in natura or that were commercially acquired [6, 11 – 13], and there are no studies with commercial oils of these aromatic plants. Therefore, this study was aimed to investigate the *in vitro* antifungal activity of the commercial oils of oregano, marjoram and rosemary against *S. brasiliensis* and to evaluate their cytotoxic activity and chemical constituents.

Material and Methods

Essential oils used in the experiments

The essential oils of *O. vulgare* L., *O. majorana* L. and *Rosmarinus officinalis* L. were commercially acquired from Ferquima – Indústria e Comércio Ltda (São Paulo, Brazil), with certification of quality.

Chromatographic analysis

The qualitative and quantitative analysis of the essential oils was performed using gas chromatography high-resolution with flame ionization detector (GC-FID). The GC analysis was carried out on a HP 7820A (Agilent®) equipped with HP-5 column (30 m × 0.32 mm × 0.25 mm) at an initial temperature of 70 ºC with addition of 3 ºC min^{-1} up to 240 ºC. The temperature of the injector and of the FID detector was 250 ºC and 260 ºC, respectively. The flow rate of hydrogen, used as carrier gas, was 3 ml min^{-1} and the split ratio 1:30. The oils were diluted in chloroform (1%) and 1 μL was injected into the chromatograph. The identification of oil components war performed by comparing their mass spectra with the Kovats retention index (R.I.). Data were acquired and processed using EZChrom Elite Compact software (Agilent).

Cytotoxic assay

The cytotoxic effects of essential oils were determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [16] in VERO cells grown in RPMI-1640 (Sigma, Steinhiem, Germany) supplemented with L-glutamine at pH 7.2 and free of sodium bicarbonate. The oils were tested at concentrations between 0.078 and 5 mg ml^{-1}. The tests were performed in triplicate and the results were read in a spectophotometer plate reader at 540 nm wavelength. The cell appearance was evaluated by using an inverted light microscope and the results of the oils were expressed as percentage of cytotoxicity relative to the control cells.

Fungal isolates

For *in vitro* tests, 29 *S. brasiliensis* clinical isolates obtained from cats (n=12) and dogs (n=16) with sporotrichosis in southern Brazil were used, as well as a standard strain from a human case (ID codes: Ss177; IPEC 16919 – Instituto de Pesquisa Clínica Evandro Chagas, Fiocruz, Brazil; FMR 8314 – Facultat

de Medicina i Ciencies de la Salut, Reus, Spain). The isolates were identified by PCR-restriction fragment length polymorphism analysis [17].

Anti-*Sporothrix brasiliensis* activity

The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined by following the M38-A2 guidelines of Clinical and Laboratory Standard Institute [18], with adaptations for phytotherapics [6]. Fungal inocula, obtained from colonies grown at 27 ºC for seven days, were resuspended in tubes containing sterile saline and adjusted to 1 McFarland scale and, subsequently, in a ultraviolet-visible spectrophotometer (Spectrum Instruments Co., Shanghai, Chine), with transmittance adjusted to 80-82% at the fixed wavelength of 530 nm. The suspensions were diluted in RPMI-1640 medium buffered with 2% glucose and MOPS [3-(N-morpholino propanesulfonic acid)] (1:50, v/v). To obtain the concentration range of 0.07 - 36 mg ml^{-1}, serial dilutions of the essential oils were prepared using RMPI 1640 medium buffered with MOPS with addition of two drops of Tween 20 to aid solubilization. Aliquots containing 100 µl of the fungal suspension and 100 µl of the diluted oil were added to each well of 96-well plates. All tests included a negative control (only RPMI and essential oil) and a positive control (only fungal inoculum). Itraconazole for veterinary use (Cepav Pharma, São Paulo, Brazil) was diluted using dimethyl sulfoxide, according to the CLSI guidelines, and used as a reference drug. The microplates were incubated in a rotary shaker at 27 ºC for 72 h. For MFC, 10 µl aliquots of wells showing no fungal growth were transferred to Petri dishes containing sabouraud dextrose agar (Acumedia, Michigan, United States) and incubated at 27ºC for 72 h. All experiments were performed in duplicate.

Data analysis

Data were submitted to analysis of variance and geometric means were compared by the Kruskal-Wallis test followed by Dunn`s multiple-comparison test. The statistical software BioEstat®, version 5.3 was used and a p value of 0.05 was considered significant.

Results

Chemical composition

According to the Table 1, 18 compounds were identified in oregano essential oil, with predominance of phenolic compounds (76.9%) and monoterpenes hydrocarbons (9.4%). Among the monoterpenes, carvacrol was the major constituent (73.9%). A prevalence of oxygenated monoterpenes was observed for marjoram (56.9%) and rosemary (69.2%) essential oils, in which 22 and 19 compounds were identified, respectively. In marjoram, 1,8-cineole was the major compound (20.9%), followed by 4-terpineol (20.4%) and γ-terpinene (8.5%). In rosemary, 1,8-cineole (49.4%), camphor (17.8%) and α-pinene (12.2%) were the major compounds detected.

Cytotoxic activity

Cytotoxicity analysis revealed that the three plant essential oils displayed dose-dependent inhibition effects on VERO cell growth. Rosemary oil showed a lower cytotoxicity compared to oregano and marjoram ($p<0.01$). At the concentration of 0.078 mg ml^{-1}, cytotoxicity was lower than 20%, whereas for the other plants was greater than 70%. At concentration of 5 mg ml^{-1}, the three oils presented cytotoxicity greater than 75% (Figure 1).

Anti-*Sporothrix* sp. activity

All *S. brasiliensis* isolates were sensitive to the tested essential oils (Table 2). Concentrations of rosemary between 0.28 mg ml^{-1} and 1.12 mg ml^{-1} inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the overall isolates, and the $MFC_{50,90}$ were eight times the MIC values, between 2.25 mg ml^{-1} and 9 mg ml^{-1}. A similar activity was observed for marjoram oil, with $MIC_{50,90}$ values of 0.14 mg ml^{-1} to 0.56 mg ml^{-1}, and $MFC_{50,90}$ values ranging from 0.28 mg ml^{-1} to 4.5 mg ml^{-1}. The lower active concentrations were observed for oregano oil, with $MIC/MFC_{50,90}$ values ranging from $\leq$0.07 mg ml^{-1} to 0.14 mg ml^{-1}. Rosemary and marjoram oils presented a similar inhibitory and fungicidal activity ($p>0.05$). Notwithstanding, oregano oil showed a better inhibitory and fungicidal activity against *S. brasiliensis* in comparison to others oils ($p<0.05$). The fungal isolates were susceptible to itraconazole, with MIC_{50} values of 1 μg ml^{-1} and 2 μg ml^{-1} for isolates obtained from dogs and cats, respectively. However, theMIC_{90} values was greater than 16 μg ml^{-1}, indicating the presence of itraconazole-resistant isolates. No fungistatic acitivity was observed for this antifungal drug ($MFC_{50,90}$ >16 μg ml^{-1}).

Discussion

In this study, the prevalence of phenolic compounds in oregano oil is in accordance with the study of Maida *et al*. [19], in which carvacrol was also the major compound found. However, these findings differ from those reported by Cleff *et al*. [6, 12], who reported, in a decreasing order, 4-terpineol, thymol and γ-terpinene as the major compounds. The difference observed in phenolic composition can be influenced by the origin of the cultivation area, genetic variability, among others [20]. For Couto *et al*. [13], γ-terpinene was the major compound, followed by carvacrol, constituents also observed in our study, but in inverse order, which demonstrated that they may have a synergistic antifungal activity.

For marjoram and rosemary oils, the antifungal activity may be attributed to the high presence of oxygenated monoterpenes, mainly 1,8-cineole, and to the second major compound found in each oil, camphor and 4-terpineol, respectively. According to Carson *et al*. [21], terpenes insert lipid bilayers between the fatty acyl chains of fungal cells, causing alterations in their permeability and death. In line with the literature, these compounds have been reported to have antimicrobial activity when tested alone [9, 19, 22].

The good antifungal activity of the tested plants was observed mainly in the oregano oil, as observed by the lower MIC and MFC concentrations, and the results are in accordance to the findings of Cleff *et al*. [6, 12] and Couto *et al*. [13] against *S. schenckii* and *S. brasiliensis*, strengthening the promising use of oregano oil in the treatment of sporotrichosis. Couto *et al*. [13] reported that MIC and MFC values of γ-terpinene against *S. brasiliensis* isolates ranged between 125 and 500 μg ml^{-1}. This compound was found to be the second and third most prevalent compound in oregano and marjoram oils, respectively, and its presence may cause a synergistic effect with other major compounds against the studied fungal species. Furthermore, our study also reported the *in vitro* fungicidal activity of oregano oil.

The rosemary oil was also promising as antifungal agent, and is in accordance with the study of Luqman *et al*. [11], in which MIC of 11 mg ml^{-1} was active against *S. schenckii*. However, in our study, the MFC_{90} was observed at concentration of 9 mg ml^{-1} in the overall isolates, whereas Luqman *et al*. [11] showed no fungicidal activity. Moreover, this is the first study on the susceptibility of *S. brasiliensis* to rosemary essential oil. Despite the lack of reports on the anti-*Sporothrix* sp. activity of marjoram oil, our findings are consistent to the reports involving the dermatophytes *Trichophyton* spp. and *M. gypseum* [9]. In another study, 5-fluorocytosine-resistant *C. albicans* isolates and amphotericin B- and fluconazole-resistant *Cryptococcus neoformans* isolates were sensitive to marjoram commercial oil at 160 μl ml^{-1} [7].

This observation reflects in the promising use of marjoram oil in antifungal therapies and the need for more studies with this oil.

In relation to the itraconazole, a fungistatic activity was observed in 50% of the *S. brasiliensis* isolates (Table 2). However, according to the breakpoint suggested by CLSI [18], susceptibility to itraconazole was only observed in 75% of the isolates obtained from cats (09/12) and dogs (12/16), besides the standard strain. The remaining were considered itraconazole-resistant isolates, an emerging problem that has already been reported by Marimon *et al.* [23] and Rodrigues *et al*. [4]. Importantly, all isolates were sensitive to the commercial essential oils of the plants of Lamiaceae family. Studies with these oils were also reported against pathogenic bacteria and fungi with known resistance to antimicrobial drugs [7, 11, 19]. However, despite their promising use in the treatment of diseases, these plant should be evaluated with respect to their possible toxicity in humans and animals.

In this sense, *in vitro* cytotoxicity assays showed a weak toxicity of rosemary oil at lower concentrations, corroborating with the results reported by Vijayan et al. [24], but its *in vitro* antifungal activity was less evident in comparison to marjoram and oregano oils, that presented a better anti-*S. brasiliensis* activity (Table 2) and a higher cytotoxicity (Figure 1). However, it is known that the *in vitro* effects of cytotoxicity are more evident due to the direct exposure of the cells to the products [25]. Indeed, the safe use of the oregano oil was demonstrated in Wistar rats treated orally during 30 days [26]. Hepatoprotective activity of the marjoram oil was also previously reported [27]. These findings indicate the possibility of *in vivo* use of these essential oils in animal models, but more studies should be undertaken to evaluate their safety and efficacy in sporotrichosis.

The results indicate that oregano, marjoram and rosemary oils exhibited anti-*S. brasiliensis* activity, including against itraconazole-resistant isolates. The major compounds were carvacrol for oregano and 1,8-cineole for marjoram and rosemary. The lowest cytotoxicity was observed for rosemary oil and the strongest antifungal activity was observed for oregano oil. The findings support the usefulness of these oils in the treatment of sporotrichosis and make them candidates for application in the pharmaceutical industry.

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Conflict of Interest

The authors declared that there is no conflict of interest in this paper.

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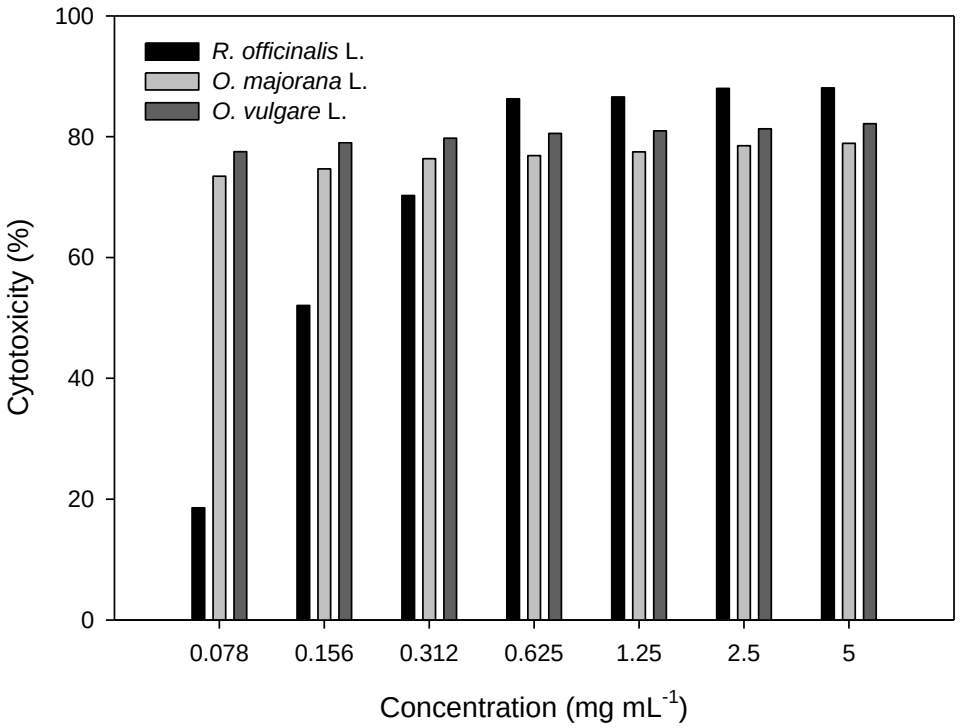

Figure 1 Percentage of cytotoxicity of commercial essential oils of rosemary (*Rosmarinus officinalis* L.), marjoram (*Origanum majorana* L.) and oregano (*Origanum vulgare* L.) after 48 h of incubation by the MTT assay.

Table 1 Chemical composition of the commercial essential oils of *Origanum vulgare* L., *O. majorana* L. and *Rosmarinus officinalis* L.

Constituents	**R.I.***	***Origanum vulgare* L. (%)**	***Origanum majorana* L. (%)**	***Rosmarinus officinalis* L. (%)**
Monoterpenes hydrocarbons				
α-thujene	932		1.1	0.2
α-pinene	941	0.4	2.0	12.2
camphene	946	0.2	0.4	1.1
sabinene	941		6.7	
β-pinene	975	0.9	1.9	9.4
myrcene	984	0.4	1.6	0.5
α-phellandrene	1003	0.1	0.9	0.1
α-terpinene	1015	0.6	4.6	0.1
p-cymene	1019	2.5	7.0	0.4
limonene	1023	0.7	5.3	2.0
β-ocimene	1056		0.1	
γ-terpinene	1089	3.6	8.5	0.1
Oxygenated monoterpenes				
1,8-cineole	1027	1.1	20.9	49.4
trans-sabinene hydrate	1094		2.3	
linalool	1099	2.1	4.4	1.0
camphor	1142	0.8	0.2	17.8
4-terpineol	1175	0.7	20.4	0.2
α-terpineol	1188	0.8	4.7	0.5
linalyl acetate	1261		1.8	
bornyl acetate	1285		2.2	0.3
Phenols				
thymol	1286	3.0		
carvacrol	1293	73.9		
Sesquiterpene hydrocarbons				
α-copaene	1378			0.3
β-caryophyllene	1421	2.8	2.2	3.7
α-humulene	1453	0.9	0.2	0.2
Other		4.5	0.6	0.4

*R.I. – Kovats retention index.

Table 2 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of commercial essential oils of *Rosmarinus officinalis* L., *Origanum vulgare* L. and *O. majorana* L. and itraconazole against *Sporothrix brasiliensis*.

Source (n)*		*R. officinalis* L. (mg ml^{-1})		*O. vulgare* L. (mg ml^{-1})		*O. majorana* L. (mg ml^{-1})		Itraconazole (µg ml^{-1})	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Cats (12)	Range	≤0.07–1.12	≤0.07–18	≤0.07	≤0.07–0.14	≤0.07–0.56	≤0.07–4.5	≤0.12–>16	>16
	50%	0.28	2.25	≤0.07	≤0.07	≤0.07	0.14	2	>16
	90%	1.12	9	≤0.07	0.14	0.56	4.5	> 16	>16
Dogs (16)	Range	≤0.07–4.5	0.28–18	≤0.07	≤0.07	≤0.07–2.25	≤0.07–4.5	≤0.12– >16	>16
	50%	0.28	1.12	≤0.07	≤0.07	0.14	0.28	1	>16
	90%	1.12	9	≤0.07	≤0.07	0.56	2.25	>16	>16
Ss 177 (1)**	Range	0.28	2.25	≤0.07	≤0.07	0.14	0.28	1	>16
Overall (29)	Range	≤0.07–4.5	≤0.07–18	≤0.07	≤0.07–0.14	≤0.07–2.25	≤0.07–4.5	≤0.12– >16	>16
	50%	0.28	2.25	≤0.07	≤0.07	0.14	0.28	1	>16
	90%	1.12	9	≤0.07	≤0.07	0.56	4.5	>16	>16

*50%, MIC/MFC at which 50% of isolates were inhibited/killed; 90%, MIC/MFC at which 90% of isolates were inhibited/killed;
** Others code: IPEC 16919 – Instituto de Pesquisa Clínica Evandro Chagas, Fiocruz, Brazil; FMR 8314 – Facultat de Medicina i Ciencies de la Salut, Reus, Spain.

4.4 Artigo 4

Promising control of the cutaneous sporotrichosis by an itraconazole-resistant *Sporothrix brasiliensis* using marjoram (*Origanum majorana* Linn.) essential oil

Stefanie Bressan Waller, Caroline Bohnen de Mattos, Cristine Cioato da Silva, Cláudia Giordani, Daiane Flores Dalla Lana, Alexandre Meneghello Fuentefria, Rogério Antônio Freitag, Eliza Simone Viegas Sallis, João Roberto Braga de Mello, Renata Osório de Faria, Marlete Brum Cleff, Mário Carlos Araújo Meireles

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Promising control of the cutaneous sporotrichosis by an itraconazole-resistant *Sporothrix brasiliensis* using marjoram (*Origanum majorana* Linn.) essential oil

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Running Head: Marjoram essential oil on experimental sporotrichosis

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Key words: *Sporothrix brasiliensis*, sporotrichosis, itraconazole, antifungal resistance, natural product, *Origanum majorana*

ABSTRACT

Purpose: Due to the emergence of antifungal resistance in *Sporothrix* species and the *in vitro* antifungal activity of *Origanum majorana* L. (marjoram) essential oil, this study evaluated the effectiveness of the plant in the cutaneous sporotrichosis by *Sporothrix brasiliensis*.

Methodology: Marjoram oil was extracted and analyzed by gas chromatographic with mass spectrometry, and the mechanism of antifungal action evaluated by the sorbitol protection and ergosterol effect. An itraconazole-resistant *S. brasiliensis* was used as inoculum (2×10^5 cells ml^{-1}) for the experimental infection of 30 adults male Wistar rats in the subcutaneous of the footpad. Three groups (n=10) received 1 ml of the following daily oral treatments for 30 days: control (saline solution), itraconazole (10 mg kg^{-1}) and marjoram oil (30 mg ml^{-1}). Weekly, a clinical evaluation was performed, as well as the euthanasia for histopathology of the inoculated footpad and the organs (liver, spleen, popliteal lymph node, kidneys, testicles) and for fungal count.

Results: Among the chemical compounds, terpinen-4-ol (34.09%), γ-terpinene (14.28%) and α-terpinene (9.6%) were prevalent. Marjoram oil did not present activity at the cell wall but presented at the level of complexation with the fungal ergosterol. Among the experimental groups, only those treated with marjoram oil showed the alleviation of the clinical signs at the inoculated site with absence of edema, exudate, difficulty in locomotion in the end of the experimental period. All groups showed pyogranulomatous inflammatory infiltrate with polymorphonuclear and mononuclear cells, however, the presence of yeasts cells were lower in marjoram group.

The fungal count in the organs was lower in marjoram group compared to control and itraconazole (P <0.05), showing the lower dissemination of *S. brasiliensis* cells to systemic organs, mainly in livers and spleens.

Conclusion: These findings support the promising use of marjoram oil in the treatment of sporotrichosis by an itraconazole-resistant *S. brasiliensis*.

INTRODUCTION

Sporotrichosis is a fungal disease caused by dimorphic species of the *Sporothrix schenckii* complex [1, 2]. This disease affects humans and animals whose had traumatic contact in the skin with conidia into the soil and plant materials [3] and in scratches and bites from sick animals, especially cats [4–6], characterizing an important zoonosis in public health. The classical clinical signs are cutaneous and subcutaneous lesions in the injured tissue with lymphatic involvement [5–7], and the systemic involvement is related to deeply immunocompromised patients [8]. Among the *Sporothrix* species, the more severe clinical presentations are often related to the infections by *S. brasiliensis* [9, 10], which is recognized as the most virulent agent [11].

Itraconazole is the drug of choice for treatment, according to the guidelines of the *American Society of Infectious Diseases* [12], however, refractories cases in humans [13, 14], cats [15] and dogs [16] has been reported. Moreover, the emergence of *S. brasiliensis* with high minimal inhibitory concentration (MIC) to itraconazole [17–20] has stimulating the searches with promising molecules against sporotrichosis, such as in natural products.

Among the promising natural products as antifungal, the essential oil of *Origanum majorana* Linn. showed *in vitro* anti-*Sporothrix brasiliensis* activity at the MIC values ranged of ≤2.25 mg ml^{-1} to 18 mg ml^{-1}, which 90% of the strains were inhibited at the MIC of 4.5 mg ml^{-1} [21]. This herb is popularly known as marjoram or sweet marjoram, a perennial bushy plant native that is often used as spice in food industry [22]. The *in vitro* efficacy against gram-positive and -negative bacteria, as well as *Candida* spp. and dermatophytes species [23, 24] demonstrate the range of antimicrobial properties of this oil. However, there are no studies of this plant treating sporotrichosis infection.

Considering the promising use of this plant as antifungal, we aimed to evaluate the activity of *O. majorana* L. essential oil in cutaneous sporotrichosis by an itraconazole-resistant *Sporothrix brasiliensis*.

METHODS

Essential oil

The essential oil of *Origanum majorana* L. was obtained by the extraction of the dried aerial parts (*Luar Sul Indústria e Comércio de Produção*, Santa Cruz do Sul/RS, Brazil) via distillation by steam dragging in Clevenger equipment for four hours [25]. The oil was dry over in Na_2SO_4 (anhydrous sodium sulfate, p.a.), concentrated in N_2 (nitrogen ultrapure, 99.99% w/v, White Martins) and stored in amber vial under refrigeration.

Chromatographic analysis

For analysis, it was performed in a high-resolution gas chromatography with mass spectrometry (GC-MS) in a Shimadzu QP® 2010 model equipped with split/splitless injector with a Rtx-5MS RESTEK (30 m × 0.25 mm × 0.25 µm) capillary column. The chromatographic conditions were helium carrier gas, fragments obtained by electron impact at energy of 70 eV, flow rate of 1.2 ml min^{-1}, 1:10 split, injected volume of 1 µl sample. Programmed oven temperature: the initial temperature was 40 °C, with a heating ramp at 5 °C min^{-1} to 280 °C, being stable at that temperature for 10 min, with a total time of 58 min, the injector temperature being 58 °C and interface 200Â °C, the compounds were analyzed based on the NIST08 GC/MS library. The oil was diluted in hexane (analytical grade, ultrapure). This analysis was performed in *Centro de Ciências Químicas, Farmacêuticas e de Alimentos* (Institute of Chemistry and Geosciences, UFPEL, Brazil).

Sorbitol protection and ergosterol effect

The minimal inhibitory concentration (MIC) was evaluated against four *S. brasiliensis* isolates in the absence and presence of sorbitol (0.8 mol l^{-1}) and ergosterol (concentrations of 50 to 200 µg ml^{-1}) [26]. Anidulafungin (Ecalta®, Pfizer, Kalamazoo, Michigan, USA) was used as positive control for sorbitol assay, whereas amphotericin B (Cristália®, São Paulo, Brazil) was for ergosterol assay. For both assays, the tests were performed in triplicate, and the MIC values were measured along three and seven days at 30 ºC of incubation.

Sporothrix brasiliensis and inoculum preparation

A *Sporothrix brasiliensis* strain (MV1710/S120) isolated from a cat with sporotrichosis in the city of Pelotas/RS (Southern Brazil) was used for the experimental design due to

the high MIC value to itraconazole (>16μg ml^{-1}) by the broth microdilution assay (CLSI M38-A2) [19]. This feline isolate was previously identified as *S. brasiliensis* based on the by PCR-restriction fragment length polymorphism analysis [27] and stored in potato dextrose agar (PDA) in the mycology collection of the *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* of the Veterinary Preventive Department of the Federal University of Pelotas (UFPEL). For inoculum preparation, *S. brasiliensis* was subculture in PDA and incubated at 27ºC for seven days. Sterile saline solution with a drop of Tween 20 was added to the colonies, which were gently scraped with a scalpel in order to collect the conidia and transferred to a sterile tube. The fungal content was filtered in double-layer sterile gauze, centrifugated (1500 rpm) in 15 min, twice washed in phosphate buffered saline (PBS), homogenized and standardized in 2×10^5 *S. brasiliensis* cells ml^{-1}, using sterile saline solution.

Animals and fungal infection

Seven-weeks-old male Wistar rats (*Rattus norvergicus*) were purchased from the Central Vivarium (UFPEL, Brazil) and housed on controlled conditions of humidity, temperature and 12h/12h light-dark cycle, fed with commercial diet and water *ad libitum* during all the experiment. The procedures used were approved by the Ethics Committee on Animal Experiments (UFPEL, no. 23110.001622/2012-55). The animals were sedated and anesthetized (xylazine at 1–5 mg kg^{-1}; ketamine at 50–100 mg kg^{-1}), intramuscularly, and, subsequently, were infected with the *S. brasiliensis* inoculum in the left hind footpad (0.2 ml, subcutaneous injection).

Experimental design

Ten days after the infection, 30 animals were allocated in three groups and the oral daily treatment (1 ml) was started for 30 days, which received saline solution with 1% of propylene glycol; itraconazole (Sporanox®, Janssen-Cilag Pharmaceutica, Belgium) at 10 mg/kg; and *Origanum majorana* L. (marjoram) essential oil at 30 mg ml^{-1} in saline solution with propylene glycol (1% w/v). For the treatment follow-up, the evolution of the disease was weekly evaluated, and two to three animals were euthanized by intraperitoneal administration of sodium thiopental (150 mg kg^{-1}), after sedation and anesthetization. All procedures followed the good practices for euthanasia in animals, according to the resolution nº 1000 of the Federal Council of Veterinary Medicine [28].

Histopathologic analysis

For the histopathology, the inoculated footpad and the organs liver, spleen, kidney, lymph node and testes were fixed in 10% formaldehyde, included in paraffin. Histological sections were stained with Hematoxylin-Eosin (HE) and Periodic acid-Schiff (PAS). The HE stained sections were used to classify the tissue lesions and cellular infiltrates and the stained with PAS was to enable a better visualization of the fungal structures.

Fungal burden

To estimate the fungal burden, the organs were weighed and submitted to the mechanical homogenization in saline solution, which 100 µl of the homogenate sample was placed onto Mycosel® (Kasvi, Liofilchem®, Italy) and incubated at 25 ºC for 10 days. The colonies were counted to determine the colony-forming units per gram of tissue.

Statistical analysis

For statistical analysis, the analysis of variance of the non-parametric data were performed using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test in the BioEstat® software, version 5.3, which *P* values <0.05 were considered significant.

RESULTS

Chemical composition

The chromatography of the marjoram oil allowed the identification of 23 compounds (Fig. 1), which the main constituents were terpinen-4-ol (**15** – 34.09%), γ-terpinene (**9** – 14.28%) and α-terpinene (**6** – 9.6%). The oxygenated monoterpenes (compounds **10**, **12**, **15**, **16** and **19**) were predominant, representing 48.93% of the total content in the essential oil, followed by monoterpene hydrocarbons (compounds **2–9**, **11**), with 41.41%. The remaining content was represented by sesquiterpene hydrocarbons (**20**, **21**) with 2.26%, and by sesquiterpene alcohol (**22**) with 0.48% of the total content.

Antifungal activity at the level of complexation with ergosterol

We evaluated the sorbitol protection and ergosterol effect in four feline *S. brasiliensis* isolates with low (*n*=2) and high (*n*=2) MIC values to itraconazole. Both isolates were previously tested to marjoram oil by us [18] and were inhibited to growth at the MIC values of ≤0.07 to 2.25 μg ml^{-1}. Considering these values for the mechanism of action assays, no change in the MIC was found in the addition of the sorbitol, indicating no action at the fungal cell wall in the experimental conditions (Table 1). In turn, the addition of exogenous ergosterol to the RPMI-1640 medium showed an increase of 128 times in the MIC values, which was directly proportional to the sterol concentration increasing, with inhibitory concentrations exceeding 8.96 mg ml^{-1} (Table 2). As showed in the selected itraconazole-resistant *S. brasiliensis* used in the animal infection (Fig. 2), these findings supported that marjoram oil presented anti-*Sporothrix brasiliensis* action at the level of complexation with fungal ergosterol.

Marjoram oil alleviated the clinical and histopathological signs of the disease

Since developed the clinical signs of the disease, the animals from control and itraconazole groups showed similar evolution at the inoculated site, featured by a severe edema and ulcerative lesion with suppurative exudation in the days 7 and 14 of treatment, besides the presence of enlarged popliteal lymph node, moderate difficulty in the locomotion, apathy and spiky fur. Although the edema evolved for a moderate to mild intensity in the days 21 and 30 of the treatment with absence of difficulty in locomotion, all animals from both groups kept the clinical signs at the skin lesion in the end of the study. In the animals from marjoram groups, although the exudative lesion and enlarged lymph node were presents in the day 7 of treatment, a quick improvement in the inoculated site was noticed, with a moderate edema and mild difficulty in locomotion. On days 14 and 21, the edema evolved for mild with normal locomotion and absence of exudation. At the 30 days, only the enlarged popliteal lymph node was noticed in the animals, which showed the remission of the cutaneous lesion. An extensive area of pyogranulomatous inflammatory infiltrate by polymorphonuclear and mononuclear cells and necrosis were the histopathological features in the three experimental groups, however, the presence of yeasts cells was severe to moderate in control and itraconazole groups, while it ranged of mild to absent in marjoram group during the study (Fig. 3).

Regarding the systemic involvement, all animals from the three groups showed lymphadenitis, with purulent exudate ranging of whitish and greenish, and none showed gross lesions in kidneys and testes, which showed normal histological features. At the splenic and hepatic levels, nodular and whitish areas were severely found in control and itraconazole groups, besides hepatomegaly and splenomegaly, whereas none animals from marjoram group showed gross alterations in the organs, except only one at the day 14 of treatment, demonstrating the individual response of the host to the treatment. Histopathologically, granuloma and discrete mononuclear inflammatory infiltrate were found in the livers and spleens of the three experimental groups, but the necrotic areas with yeasts cells inside were only found in the control group (Fig. 3).

Marjoram oil decreased the fungal burden in the systemic organs

Animals treated with marjoram oil showed less recovery of the fungal burden in comparison to those from control group at the beginning of the treatment (Fig. 4). At the splenic level, the recovery of the fungal burden was significant lower in comparison to control group in all days evaluated, and to the itraconazole group on the day 30 of treatment ($P < 0.05$). At the hepatic level, a similar finding was observed in comparison to itraconazole group on the day 7, and to control group on the day 30 ($P < 0.05$). In the lymph nodes, no difference was showed among the groups ($P \geq 0.05$), exception on the day 14, which rats from control group showed a significant increase in the fungal count ($P < 0.05$). At the renal and testicular levels, the difference was showed only on the day 7 of treatment comparing to control group ($P < 0.05$). In general, the oral treatment with marjoram oil decreased the fungal burden in the hepatic tissues compared to itraconazole group and to control group in all evaluated tissues.

DISCUSSION

Among the proven pharmacological activities of *Origanum majorana* L. essential oil, the antifungal action was showed against pathogenic fungi in humans and animals, such as *Candida* spp. from urinary [28], oral and dermatological [24] cases, *Malassezia* spp. from dermatitis canine cases [30], and *Microsporum* spp. and *Trichophyton* spp. from human dermatophytosis [23, 24]. Currently, we showed the susceptibility of *S. brasiliensis* and

S. schenckii isolates from human, feline and canine cases by *in vitro* assays [18, 20, 21], which encouraged us to evaluate, for the first time, the *in vivo* efficacy of marjoram oil in experimental infection by an itraconazole-resistant *S. brasiliensis*. The establishment for the concentration of the essential oil was based in the *in vitro* minimal inhibitory and fungicidal concentrations against *S. brasiliensis* [18], in which at least three time the average of these values was chosen for *in vivo* treatment.

We chose the MV1710/S120 clinical isolate due to the high MIC values to itraconazole [19], as well as was derived from a feline case with disseminated presentation and with a refractory historic to itraconazole therapy. Since challenged, all experimental animals developed suppurative subcutaneous nodules with lymphatic involvement, and the histopathology showed pyogranulomatous inflammatory infiltrate, necrosis and severe presence of yeasts at the inoculated site, similarly to the findings of Batista-Duharte *et al*. [10] and Della-Terra *et al.* [11], which also studied murine model infected by *S. schenckii* and *S. brasiliensis*.

As expected, the control and itraconazole treatments showed similar evolution at the end of the treatment period due to the infection by an itraconazole-resistant *S. brasiliensis*, which the animals kept the signs of inflammation in the inoculated site, like edema, whereas those orally treated with marjoram oil not. The alleviation of the signs of the sporotrichosis seemed to be attributed to the chemical compounds, which terpinen-4-ol, γ-terpinene and α-terpinene were the prevalent, as well as in the essential oil of the plant cultivated in Egypt [21], Venezuela [31] and Mexico [23]. This finding highlighted the biological properties, such as the anti-inflammatory effect [32], which was attributed to the presence of γ-terpinene [33], *trans*- and *cis*-sabinene hydrate and terpinen-4-ol [32]. These compounds were presents in our sample and the ability reducing the production of pro-inflammatory cytokines [32, 33] and signs of edema [33] was proven.

Besides the anti-inflammatory activity, the concomitant antifungal activity of marjoram oil improved the control of the disease in the experimental animals, since this plant presented an action at the level of complexation with the ergosterol of an itraconazole-resistant *S. brasiliensis*. The major compounds in our essential oil, terpinen-4-ol and γ-terpinene, seemed to be an important role as antifungal, since studies proven the ability of terpinen-4-ol reducing the ergosterol content of the dimorphic fungi *Coccidioides posadasii* and *Histoplasma capsulatum* [34], as well as *S. brasiliensis*, and γ-terpinene

inhibited the growth of *S. brasiliensis* and *S. schenckii*, changing their morphological structures in hyphae and reducing the conidia numbers [35]. This finding highlighted the effectiveness of the chemical compounds in marjoram oil against *Sporothrix* species and its promising use controlling sporotrichosis.

Furthermore, the systemic dissemination from the subcutaneous infection also was showed in *S. brasiliensis* with different levels of virulence [11], which livers and spleens were highly affected, as well as showed in our study, especially in control group, besides lymph nodes, corroborating to the macroscopic and histological features of the disease in the literature [10, 11, 36, 37]. Kidneys and testicles were the organs little affected in all groups, possibly because of the renal function filtering the blood and contributing to homeostasis of the immune system [38], as well as the immune-privileged testes with the defense mechanism counteracting the invading microbial pathogens [39], representing both organs with lower susceptibility to *Sporothrix* dissemination compared to livers and spleens. However, the dissemination of the *S. brasiliensis* cells to the systemic organs was little observed in animals treated with marjoram oil comparing to the remaining groups, including at the hepatic and splenic levels, highlighting a protective effect of this plant in the organs when orally administered, agreeing with the proven hepatoprotective [40] and nephroprotective [41] activities in marjoram oil, allied to the antifungal activity against *S. brasiliensis*.

Considering the mechanism of action of the amphotericin B [42], it is possible that the action of the marjoram oil is linked to the ergosterol, causing pores in the membrane and, consequently, the essential molecules and ions for the fungal growth are extravasated, resulting in the death of the *S. brasiliensis* cells. Although the mechanisms for antifungal resistance in *Sporothrix* spp. strains are still unclear, which are related to the development of efflux pumps and ATP-binding cassette transporters [43] in the fungal membrane, our study showed the ability of marjoram oil inhibiting the growth of itraconazole-sensitive and itraconazole-resistant *S. brasiliensis* isolates, highlighting it as a promising candidate in light of the emergence of antifungal drug resistance. This study showed the promising use of marjoram oil in the oral treatment of sporotrichosis with the alleviation of the clinical signs of the disease and the decreasing of the *S. brasiliensis* dissemination to the systemic organs. However, more studies should be undertaken in a longer treatment

period, as well as the synergistic effect between marjoram oil and itraconazole for a better understanding of the control of the disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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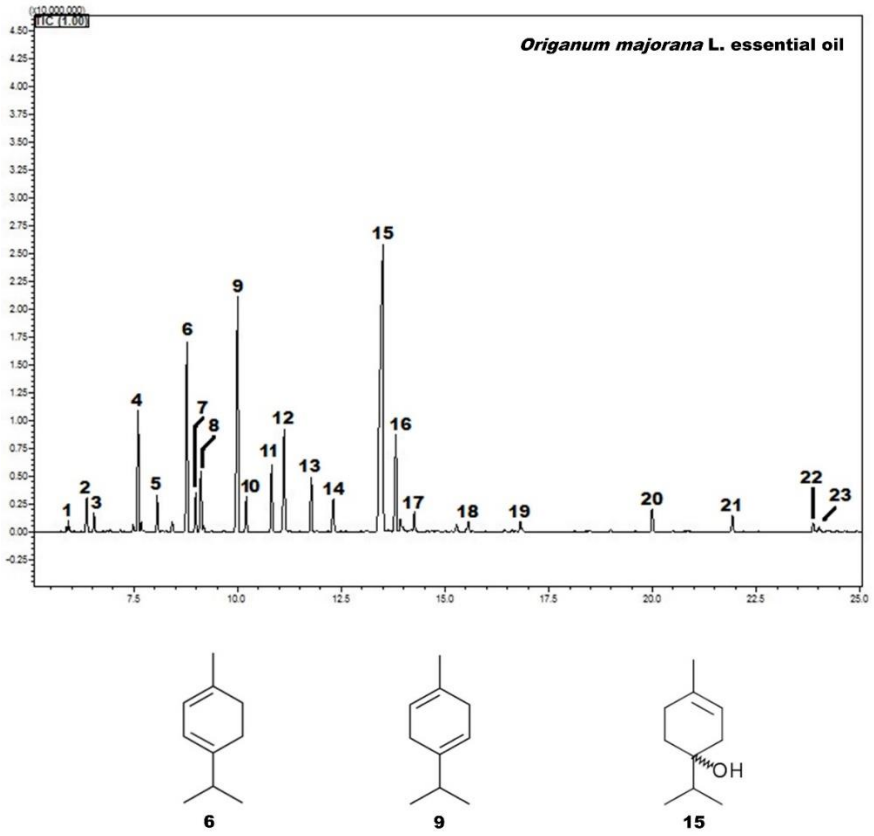

Fig. 1. Chromatogram of the *Origanum majorana* L. (marjoram) essential oil and their respective molecular masses (m/z), retention times (min) and area (%): unknown (**1** – 5.92 min; 0.37%); α-thujeno (**2** – m/z 136.238; 6.36 min; 1.68%); α-pinene (**3** – m/z 136.238; 6.54 min; 0.74%); α-phellandrene (**4** – m/z 136.238; 7.59 min; 5.5%); β-pinene (**5** – m/z 136.238; 8.05 min; 1.57%); α-terpinene (**6** – m/z 136.238; 8.78 min; 9.6%); o-cymene (**7** – m/z 134.222; 8.98 min; 1.74%); β-phellandrene (**8** – m/z 136.238; 9.11 min; 3.03%); γ-terpinene (**9** – m/z 136.238; 9.99 min; 14.28%); trans-sabinene hydrate (**10** – m/z 154.253; 10.10 min; 1.66%); δ-2-carene (**11** – m/z 272.476; 10.82 min; 3.27%); cis-sabinene hydrate (**12** – m/z 138.254; 11.11 min; 6.81%); unknown (**13** – 11.77 min; 2.77%); unknown (**14** – 12.29 min; 1.99%); terpinen-4-ol (**15** – m/z 154.253; 13.49 min; 34.09%); α-terpineol (**16** – m/z 154.253; 13.81 min; 5.86%); unknown (**17** – 14.26 min; 0.95%); unknown (**18** – 15.55 min; 0.57%); 4-terpinenyl acetate (**19** – m/z 196.29; 16.81 min; 0.51%); isocaryophyllene (**20** – m/z 204.357; 19.99 min; 1.38%); elixene (**21** – m/z 204.357; 21.92 min; 0.88%); spathulenol (**22** – m/z 220.356; 23.87 min; 0.48%); unknown (**23** – 24.01 min; 0.27%).

Table 1. Minimal inhibitory concentration (MIC) of the *Origanum majorana* L. (marjoram) essential oil and anidulafungin in the absence and presence of the osmotic protector sorbitol against *S. brasiliensis* from feline cases.

Sporothrix brasiliensis*	**MIC *Origanum majorana* L. oil (mg/mL)**				**MIC Anidulafungin (µg/mL)**			
	3 days		**7 days**		**3 days**		**7 days**	
	S (–)	**S (+)**	**S (–)**	**S (+)**	**S (–)**	**S (+)**	**S (–)**	**S (+)**
Itraconazole-sensitive								
S68	0.28	0.28	0.28	0.28	32	256	32	512
S103	0.07	0.07	0.07	0.07	32	256	32	512
Itraconazole-resistant								
S72	0.07	0.07	0.07	0.07	32	256	32	512
S120**	0.07	0.07	0.07	0.07	32	256	32	512

S (–), absence of sorbitol; S (+), presence of sorbitol;
* All *S. brasiliensis* were came from feline cases in the city of Pelotas/RS (Southern Brazil) and belong to the culture collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária*, Federal University of Pelotas, (UFPEL, Brazil);
**S120 was used for the experimental infection in murine model.

Table 2. Minimal inhibitory concentration (MIC) of the *Origanum majorana* L. (marjoram) essential oil and amphotericin B in the absence (E–) and presence (50–200 µg/mL) of the exogenous ergosterol against *S. brasiliensis* from feline cases.

***Sporothrix brasiliensis* ***	**MIC *Origanum majorana* L. oil (mg/mL)**										**MIC Amphotericin B (µg/mL)**									
	3 days					**7 days**					**3 days**					**7 days**				
	E–	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**
Itraconazole-sensitive																				
S68	0.28	0.28	0.28	0.28	0.28	0.28	0.28	1.12	2.24	>35.84	1	16	>256	>256	>256	1	>256	>256	>256	>256
S103	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.56	0.56	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256
Itraconazole-resistant																				
S72	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.56	0.56	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256
S120**	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.56	0.56	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256

* All *S. brasiliensis* were came from feline cases in the city of Pelotas/RS (Southern Brazil) and belong to the culture collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária*, Federal University of Pelotas, (UFPEL, Brazil);
**S120 was used for the experimental infection in murine model;

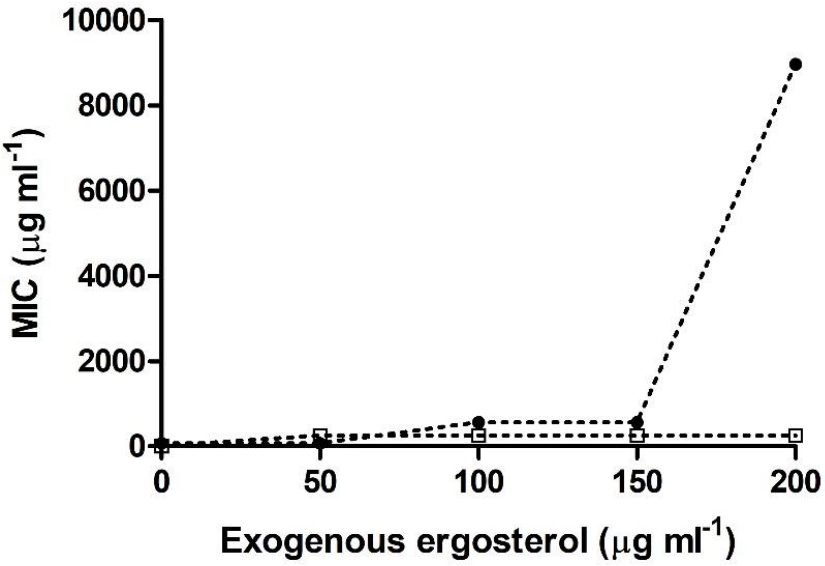

Fig. 2. Effect of different concentrations of exogenous ergosterol (50–200 μg ml^{-1}) on the minimum inhibitory concentration (MIC) of amphotericin B (□) and *Origanum majorana* L. essential oil (●) against an itraconazole-resistant *S. brasiliensis* (S120).

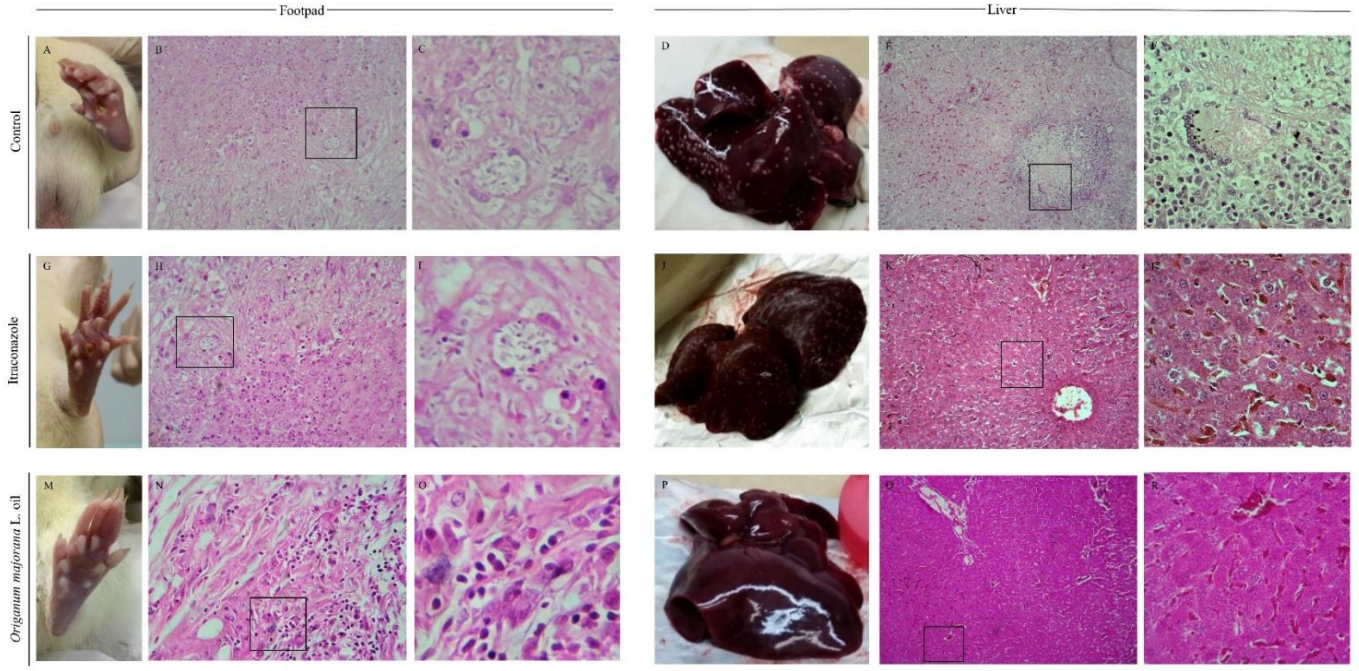

Fig. 3. Footpad and liver of rats experimentally infected by an itraconazole-resistant *Sporothrix brasiliensis* at the end of the oral treatments with control (A–F), itraconazole (G–L) and *Origanum majorana* L. (marjoram) essential oil (M–R). Footpad in control and itraconazole groups showed ulceration with suppurative exudation (A) and edema (G) with a histological feature of pyogranulomatous inflammatory infiltrate, necrosis (B, H) and severe presence of *S. brasiliensis* yeasts cells (C, I), whereas the remission of the lesion (M) with an area of mononuclear inflammatory infiltrate (N, O) was showed in the marjoram group. In the livers of the control and itraconazole groups, whitish and nodular hepatic lesions (D, I) were found with granuloma and Giant multinucleated cells (E, F) and a discrete mononuclear inflammatory infiltrate (K, L), while in marjoram group were found normal macroscopic (P) and histological (Q, R) features (H.E., 40× and 100× magnification).

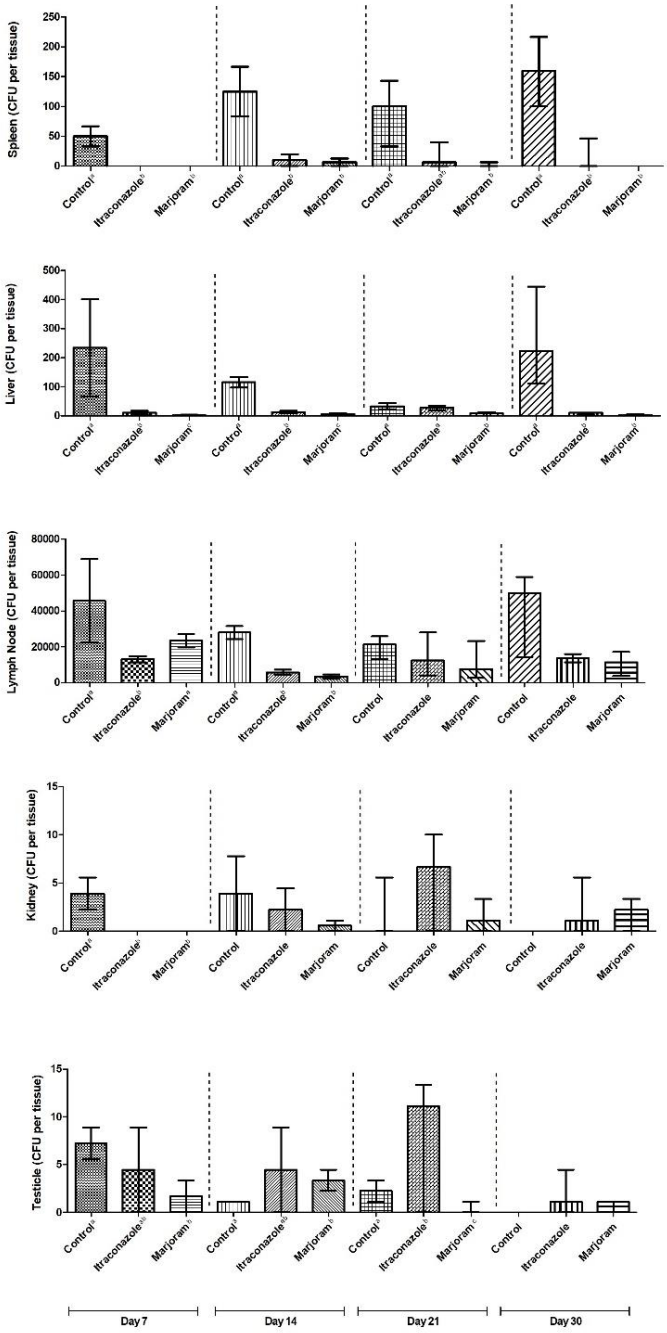

Fig. 4. Comparison of the fungal burden in spleen, liver, lymph node, kidney and testicle (CFU per tissue) of subcutaneously infected rats by an itraconazole-resistant *S. brasiliensis* and receiving the following treatments: saline solution as control, itraconazole and marjoram (*Origanum majorana* Linn.) essential oil. The data were analyzed in the days 7, 14, 21 and 30 of treatment and the median values were compared by Kruskal-Wallis test, followed by Dunn's test, which the different letters $^{(a,b,c)}$ among the treatments are significant ($P < 0.05$) and the absence of letters are non-significant.

4.5 Artigo 5

Rats experimentally infected by an itraconazole-resistant *Sporothrix brasiliensis* and orally treated with rosemary (*Rosmarinus officinalis* Linn.) essential oil were protected of the fungal dissemination

Stefanie Bressan Waller, Caroline Bohnen de Mattos, Daiane Flores Dalla Lana, Karina Guterres, Rogério Antônio Freitag, Eliza Simone Viegas Sallis, Alexandre Meneghello Fuentefria, João Roberto Braga de Mello, Renata Osório de Faria, Marlete Brum Cleff, Mário Carlos Araújo Meireles

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Rats experimentally infected by an itraconazole-resistant *Sporothrix brasiliensis* and orally treated with rosemary (*Rosmarinus officinalis* Linn.) essential oil were protected of the fungal dissemination

"Rosemary oil on the experimental sporotrichosis"

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Key words: sporotrichosis, itraconazole, antifungal resistance, medicinal plant, *Rosmarinus officinalis*.

Abstract

This study aimed to evaluate the effectiveness of rosemary oil on experimental cutaneous sporotrichosis by an itraconazole-resistant *Sporothrix brasiliensis*. Thirty adults male Wistar rats were subcutaneously inoculated in the footpad (2×10^5 cells ml^{-1}) and received daily oral treatment (1 ml) for 30 days in the following groups (n=10): saline solution for control, itraconazole (10 mg kg^{-1}) and rosemary oil (90 mg ml^{-1}). Weekly, the clinical evolution was follow-up and euthanasia were performed for histopathology and fungal burden. Rosemary oil was chemically analyzed by GC-MS, and the sorbitol protection and ergosterol effect were performed for the mechanism of action. 1,8-Cineole, camphor and α-pinene were the compounds prevalent in the rosemary oil, which showed activity at the level of complexion with the fungal ergosterol. Since infected, animals from control and itraconazole groups kept the cutaneous lesions in the footpad at the end of the study, whereas only the rosemary group evolved for absence of edema and exudate in the inoculated point. The footpad histopathology featured of pyogranulomatous inflammatory infiltrate with polymorphonuclear and mononuclear cells and necrosis, however, while the presence of yeasts cells was severe to moderate in control and itraconazole groups, in the rosemary group was mild to absent. Animals treated with rosemary showed the absence of gross lesions in liver and spleen, and low fungal burden in the organs compared to control group ($P < .05$). This study showed the promising use of rosemary oil in the treatment of cutaneous sporotrichosis by *S. brasiliensis*, including protecting the systemic organs from the fungal spread.

Introduction

Sporotrichosis is an infectious disease caused by fungal species of the *Sporothrix schenckii* complex with worldwide distribution.[1–3] In Brazil, it is recognized as epidemic outbreaks in Rio de Janeiro,[4] followed by in Rio Grande do Sul, mainly in southern region.[5] The transmission is by traumatic injury in skin with contaminated plant material or zoonotic contact by scratches and bites of sick animals,[2] whose humans and animals may present fixed and lymphocutaneous manifestations with nodular and ulcerative lesions.[6–9] Among the *Sporothrix* species, *S. brasiliensis* is the most virulent,[10–11] which infections are atypical with severe manifestations.[12–13]

Few antifungal drugs recommended for the therapy, itraconazole still is considered the first option of choice in the human and animal sporotrichosis.[14–15] However, the therapeutic failure has been reported,[16–18] as well as the emergence of *S. brasiliensis* with high MIC values to itraconazole, including from animal cases in Southern Brazil.[19–21] This scenario has harmed the clinical success by the conventional therapy and the search for new active molecules is required against sporotrichosis, being the natural sources by plants from Lamiaceae family considered promising as antifungal.[22]

One of the plants belonging to this botanical family is *Rosmarinus officinalis* Linn., popularly known as rosemary. Essential oil of this plant showed antifungal activities against *Sporothrix schenckii*,[23] including against itraconazole-resistant *Sporothrix brasiliensis* isolates.[22] Little is known about the action of rosemary oil against *Sporothrix* species, encouraging us to investigate it *in vivo* effectiveness in the disease. This study aimed to evaluate the effectiveness of rosemary oil on experimental cutaneous sporotrichosis by an itraconazole-resistant *Sporothrix brasiliensis*.

Material and Methods

Essential oil and chromatographic analysis

Dried aerial parts of *Rosmarinus officinalis* were acquired commercially (*Luar Sul Indústria e Comércio de Produção*, Santa Cruz do Sul/RS, Brazil) and were extracted via distillation by steam dragging in Clevenger equipment for four hours.[24] The oils were dried over in Na_2SO_4 (anhydrous sodium sulfate, p.a.), concentrated in N_2 (nitrogen ultrapure, 99.99%, White Martins), and stored in amber vial under refrigeration. The chemical analysis was performed in a high-resolution gas chromatography with mass spectrometry (GC-MS) in a Shimadzu QP® 2010 model equipped with split/splitless injector with a Rtx-5MS RESTEK (30 m × 0.25 mm × 0.25 µm) capillary column. The chromatographic conditions were the following: helium carrier gas, fragments obtained by electron impact at energy of 70 eV, flow rate of 1.2 ml/min, 1:10 split, injected volume of 1 µl sample. Programmed oven temperature: the initial temperature was 40 °C, with a heating ramp at 5 °C/min to 280 °C, being stable at that temperature for 10 min, with a total time of 58 min, the injector temperature being 58 °C and interface 200Â °C, the compounds were analyzed based on the NIST08 GC/MS library. The oil was diluted in hexane (analytical grade, ultrapure).

Mechanism of action

For understand the mechanism of action, the evaluation of sorbitol protection and ergosterol effect were performed, according to Dalla Lana et al.[25] Itraconazole-sensitive (*n*=2) and itraconazole-resistant (*n*=2) *S. brasiliensis* isolates from feline cases in the city of Pelotas (Southern Brazil) were used. The antifungal susceptibility assay was already performed by Waller et al.[20] and the criteria for the *in vitro* sensitivity and resistance was followed by the M38-A2 guideline,[26] in which the breakpoint is MIC <4 µg/ml and ≥4

μg/ml, respectively. The fungal isolates were stored in the mycology collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (UFPEL, Brazil). For both tests, the MIC was measured in the presence and absence of sorbitol (0.8 mol/l, Sigma-Aldrich, St. Louis, USA) and ergosterol (50–200 μg/ml, Sigma-Aldrich, St. Louis, USA) into the RPMI-1640 culture medium containing L-glutamine, without sodium bicarbonate, buffered to pH 7.0 with MOPS (0.165 mol/l). For the ergosterol effect, this product was previously dissolved in dimethylformamide before dissolution in the culture medium. A solution of rosemary oil was prepared ranged of 256-fold the MIC values to MIC/4 for each fungal isolate. As positive control, anidulafungin (Ecalta®, Pfizer, Kalamazoo, Michigan, USA) and amphotericin B (Cristália®, São Paulo, Brazil) were used, respectively, for the sorbitol and ergosterol assays, and were prepared from a stock solution ranged of 2 to 1024 μg/ml (anidulafungin) and of 2 to 512 μg/ml (amphotericin B). Both tests were performed in triplicate and the MIC was measured along three and seven days at 30ºC of incubation.

Itraconazole-resistant Sporothrix brasiliensis and inoculum

Sporothrix brasiliensis isolated from a cat with sporotrichosis in the city of Pelotas (Rio Grande do Sul state, Southern Brazil) was chosen to reproduce the experimental infection due to the high MIC (>16μg/ml) values to itraconazole[20] in the antifungal susceptibility assay by the M38-A2 guideline of Clinical and Laboratory Standard Institute.[26] Besides that, the isolate showed melanogenic characteristic and was from a disseminated presentation in the host. The isolate was stored in the mycology collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (UFPEL, Brazil) and identified as "MV1710/S120" and was previously identified by PCR-restriction fragment length polymorphism analysis, according to the methodology of Rodrigues et al.[27]

The inoculum was prepared from a young culture of *S. brasiliensis* in Potato Dextrose agar (PDA) at 27ºC for seven days, which was added sterile saline solution with a drop of Tween 20, following by the gently scrap of the colonies with a scalpel in order to collect the conidia. The content was transferred to a sterile tube, filtered in double-layer sterile gauze and centrifugated (1500 rpm) in 15 min. This content was twice washed in phosphate buffered saline (PBS), homogenized and standardized in 2×10^5 *S. brasiliensis* cells/ml per animal, using sterile saline solution.

Animal infection and experimental groups

Immunocompetent seven-weeks-old male Wistar rats (*Rattus norvergicus*) were purchased from the Central Vivarium of Federal University of Pelotas (UFPEL, Brazil) and were housed on controlled conditions of humidity, temperature and 12h/12h light-dark cycle, besides commercial diet and water ad libitum during all the experiment. Before beginning the experimental treatment, all the animals were previously adapted to the handling and orogastric sounding for two weeks. All the procedures involving animals were previously approved by the Ethics Committee on Animal Experiments (UFPEL, no. 23110.001622/2012-55) and followed the recommendations for the care and use of experimental animals.[28]

For the infection, rats were subcutaneously inoculated in the left hind footpad (0.2 ml), after sedation and anesthetization with xylazine (1–5 mg/kg) and ketamine (50–100 mg/kg), both by intramuscular via. Ten days after the animal infection, the rats were randomly divided into groups ($n=10$ each): control (CONT) receiving saline solution with 1% propylene glycol as emulsion vehicle; itraconazole (ITRA) at 10 mg/kg; and *Rosmarinus officinalis* oil (ROSM) at 90 mg/ml in saline solution containing 1% propylene glycol. The treatment was daily administered (1 ml) by the oral route (gavage)

for 30 days, and all products were prepared in a local laboratory of pharmaceutical manipulation.

Treatment follow-up and sample collection

For the treatment follow-up, the evolution of the disease was weekly evaluated throughout the experimental period. Two rats were sacrificed in the first and second weeks of treatment, and three were in the third and fourth weeks. The euthanasia was performed by intraperitoneal administration of sodium thiopental (150 mg/kg), after sedation and anesthetization, and all the procedures of good practices in experimental animals[28–29] were carefully performed for animal welfare.

Histopathology and fungal burden estimation

For the histopathology, the inoculated footpad and the organs liver, spleen, kidney, lymph node and testicles were fixed in 10% formaldehyde, included in paraffin. Histological sections were stained with Hematoxylin-Eosin (HE) and Periodic acid-Schiff (PAS). The HE stained sections were used to classify the tissue lesions and cellular infiltrates and the stained with PAS was to enable a better visualization of the fungal structures. To estimate the fungal burden, the organs were weighed and homogenized in saline solution. From this homogenate, an aliquot of 100 µl was placed onto Mycosel® (Kasvi, Liofilchem®, Italy) and incubated at 25ºC for 10 days. The colonies were counted to determine the colony-forming units per gram of tissue.

Statistical analysis

For statistical analysis, the analysis of variance of the non-parametric data were performed using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test in the BioEstat® software, version 5.3, which P values <0.05 were considered significant.

Results

Rosemary essential oil showed a prevalence of phenolic compounds in the chromatography, and 1,8-cineole/eucalyptol (**7** – 47.91%), camphor (**10** – 17.92%) and α-pinene (**11** – 11.52%) were major into the 16 compounds identified (Fig. 1). When evaluated it mechanism of action through the addiction of sorbitol (Table 1), the *S. brasiliensis* isolates were not inhibited by this osmotic protector because the MIC values were the same for all lectures, showing that rosemary oil seems to do not act on the fungal cell wall. In turn, the ergosterol effect showed a 2-fold increase in MIC on the thirst day, which increased to 128 times on the seventh day of reading (Table 2), supporting an action at the level of complexation with ergosterol.

In the experimental sporotrichosis, all animals showed the characteristics signs of the disease after 10 days of the inoculation, such as edema on the inoculated member, purulent secretion, increased in the popliteal lymph node left, dehydration, piloerection and apathy. Beginning the oral treatment (Table 3), the enlarged lymph node and the presence of exudate was noted in the inoculated point for all animals belonging to control and itraconazole groups during all experimental period, whereas the rats from rosemary group started the regression of the local exudation from the 3rd week of oral treatment. During the firsts three weeks, edema and difficulty in locomotion were also noted in all animals, ranged of moderate to severe for control and itraconazole groups, and of moderate to mild for rosemary group. In the end of the experiment, all animals evolved for the absent of difficulty in locomotion, however, only those from the rosemary group showed absence of edema and exudate in the inoculated point.

At the histopathology, the footpad of rats from control group were featured by a focally extensive area of severe pyogranulomatous infiltrate and necrosis with a severe presence

of yeasts cells during the weeks, besides presence of bacteria structures. Rats from itraconazole group showed a similar feature with moderate pyogranulomatous infiltrate and necrosis and a mild presence of yeasts in the firsts two weeks, which worsened to severe until the end of the experiment. In turn, the footpad of rats treated with rosemary showed severe pyogranulomatous infiltrate with predominance of polymorphonuclear and mononuclear cells with or without necrosis in the first two weeks, but the presence of yeasts cells were little or absent. A similar feature was noted in the 3rd week, which evolved the inflammatory infiltrate to a moderate intensity without necrosis with little or none yeasts cells until the end of the study.

The systemic evaluation showed that lymph node was highly affected in all groups, with lymphadenitis containing purulent exudate. In control and itraconazole groups, the organs spleen and liver were greatly affected in all experimental period, showing hepatomegaly and splenomegaly with gross whitish lesions, which the histopathological features showed granulomas with focal and multifocal areas of pyogranulomatous inflammatory infiltrate with polymorphonuclear cells and presence of necrotic areas, besides a high presence of yeasts cells and bacterial structures. In turn, none rats from rosemary group showed gross or histological alterations in spleen and liver. Lastly, kidneys and testicles were not affected in any experimental groups. The histopathology of the footpad, liver and spleen in the end of the treatment of the experimental groups is showed in Fig. 2.

Regarding to the fungal load (Fig. 3), the lymph node was highly affected in all groups, followed by spleen and liver in control and itraconazole groups. In rosemary group, the fungal load of lymph node and spleen was lower compared to control group ($P < .05$), as well as in the liver, which differentiated to control and itraconazole groups in all weeks ($P < .05$). Although kidneys and testicles had been little affected, the organs from rats

treated with rosemary oil differentiated to those from rats of control group ($P < .05$) in the beginning of the treatment. These findings supported that the oral treatment with rosemary essential oil delayed the spread of an itraconazole-resistant *S. brasiliensis* to systemic organs in the experimental conditions.

Discussion

An itraconazole-resistant *S. brasiliensis* isolate was our research target due to increased reports of antifungal resistance in humane and animal cases in Brazil,[20,30–32] being this species known as the most virulent among the *S. schenckii* complex species.[10,30,33] The inoculum was derived from a feline case in Southern Brazil, a geographical region recognized by the emergence of isolates with high MIC values to this antifungal,[19–21] as previously showed in the selected isolate by us,[20] besides it was a melanin-producing agent, an important virulence factor.

In experimental infection, the decrease of the lesion size by oral itraconazole occur in the fourth week,[34] which it was not showed in our study, due to the virulence of the fungal isolate. Besides that, rats from control and itraconazole groups showed spiky fur and apathy, as described in rats infected by *S. schenckii*.[35] However, these signs were not observed in rosemary group, which the rats showed a better cutaneous recovery of the skin lesion with no adverse effects.

In the necropsy, the systemic involvement was observed from a subcutaneous infection, agreeing with the findings of rats infected with different levels of virulent *S. brasiliensis*,[11] which liver was the organ highly affected. In rats intravenously infected by *S. brasiliensis*[36] and intraperitoneally by *S. schenckii*,[35] gross lesions in liver, spleen and

kidneys were similarly found in the organs in this study, showing the fungal ability to colonize organs tissue, regardless of the route of infection.

Histopathologically, pyogranulomatous inflammation, necrosis, giant cells in granuloma, and yeasts cells found in the tissue injuries were similar to the studies in murine model with sporotrichosis by *S. brasiliensis*[11,36] and *S. schenckii*.[35] The increased influx of inflammatory cells due to the high fungal burden[37] was noted at the site of inoculation, as well as in the systemic organs in the control and itraconazole groups. In rosemary group, few or none yeasts were found in the footpad and, especially, in the organs, showing an organic protection against the dissemination of *S. brasiliensis* from a subcutaneous infection by this treatment.

Rosemary essential oil is a complex mixture of volatile compounds, which the high concentrations of 1,8-cineole, camphor and α-pinene agreed with the composition of this plant cultivated in Serbia,[38] Italy[39] and Tunisia.[40] Considering that itraconazole inhibit the *lanosterol 14-α-demethylase,*[41] an alternative route is used by the chemical compounds in rosemary oil as antifungal against the itraconazole-resistant *S. brasiliensis*. In this study, we showed that the rosemary essential oil seems not to act on the *S. brasiliensis* cell wall, but in the cell membrane through the complexation with the fungal ergosterol.

Considering the similarity with amphotericin B,[42] the compounds in the rosemary link to the fungal ergosterol, creating polar pores in the membrane and causing the output of molecules and ions and, consequently, the fungal death. According to Yu et al.,[43] 1,8-cineole penetrate in the cell membrane, damaging cellular organelles without lesions in this structure, whereas terpinen-4-ol disrupts membranes, and both compounds are synergic between them. Thus, the high concentration of 1,8-cineole and the presence of

terpin-4-ol would be some of the compounds with an important role for the activity of rosemary oil against the itraconazole-resistant *S. brasiliensis*.

Furthermore, the strong DPPH radical scavenging capacity in the rosemary essential oil[38] confer its antioxidant activity,[44] which seems to be indirectly involved in the anti-*Sporothrix brasiliensis* activity. In the pathogenesis of sporotrichosis, a redox imbalance is produced by the intense inflammatory response, causing oxidative stress,[35] especially in *S. brasiliensis*, in which liver and kidneys of infected rats are highly vulnerable.[45] In our study, the histopathological and fungal burden findings of the organs in rosemary group were little or not affected, even if treatment was started 10 days after subcutaneous infection, showing that the components in rosemary oil seems to be important in stabilizing the free radicals liberated during the *S. brasiliensis* infection.

Considering that reactive oxygen species are liberated by defense cells, such as macrophages, as an attempt eliminating the *Sporothrix* spp. infection,[46] the antioxidant activity of 1,8-cineole[47] and camphor[48] seems to be a role in the protection of the systemic organs to membrane injuries, which were found in high concentrations. This activity is due to the ability in donating an electron or a hydrogen atom to the lipid radicals formed in the lipid oxidation, stabilizing the phenoxyl radical that is resulted,[49] decreasing the tissue injury by the excess of free radicals. This ability of the rosemary components in protect tissues also was showed in liver,[38] kidneys[50–51] and testicles[51] experimentally induced to toxicity, being similarly found in the systemic organs of the animals experimentally treated with rosemary essential oil.

The establishment for the concentration of the rosemary oil was based in the *in vitro* minimal inhibitory and fungicidal concentrations against *S. brasiliensis*,[22] in which at least three time the average of these values was chosen for *in vivo* treatment. Furthermore,

the oral treatment was considered safe in the experimental condition, since none rat showed adverse effect by ingestion or died naturally. In experimental studies, different concentrations of rosemary oil reached the highest level of 1,8-cineole in the blood of rats after five minutes of oral ingestion[52] and most of the compounds remained supporting their potential biological activities in the intestine, liver and plasma at substantial concentrations for several hours.[53]

Even if a time is required for metabolization and distribution of the rosemary compounds orally administered for the cutaneous tissues, the antifungal activity by complexation with the ergosterol, as showed in this study, as well as the ability to scavenging radical free,[38] seems to be two important roles in controlling the dissemination of *S. brasiliensis* to the systemic organs from cutaneous infection. Furthermore, it is important to investigate the role of rosemary oil and its components in the oxidative stress by sporotrichosis, and studies of their effects should be undertaken. This study showed that the oral administration of rosemary oil is a promising antifungal in the treatment of sporotrichosis by itraconazole-resistant *S. brasiliensis* and further should be undertaken.

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Conflict of Interest

The authors declare no conflict of interest.

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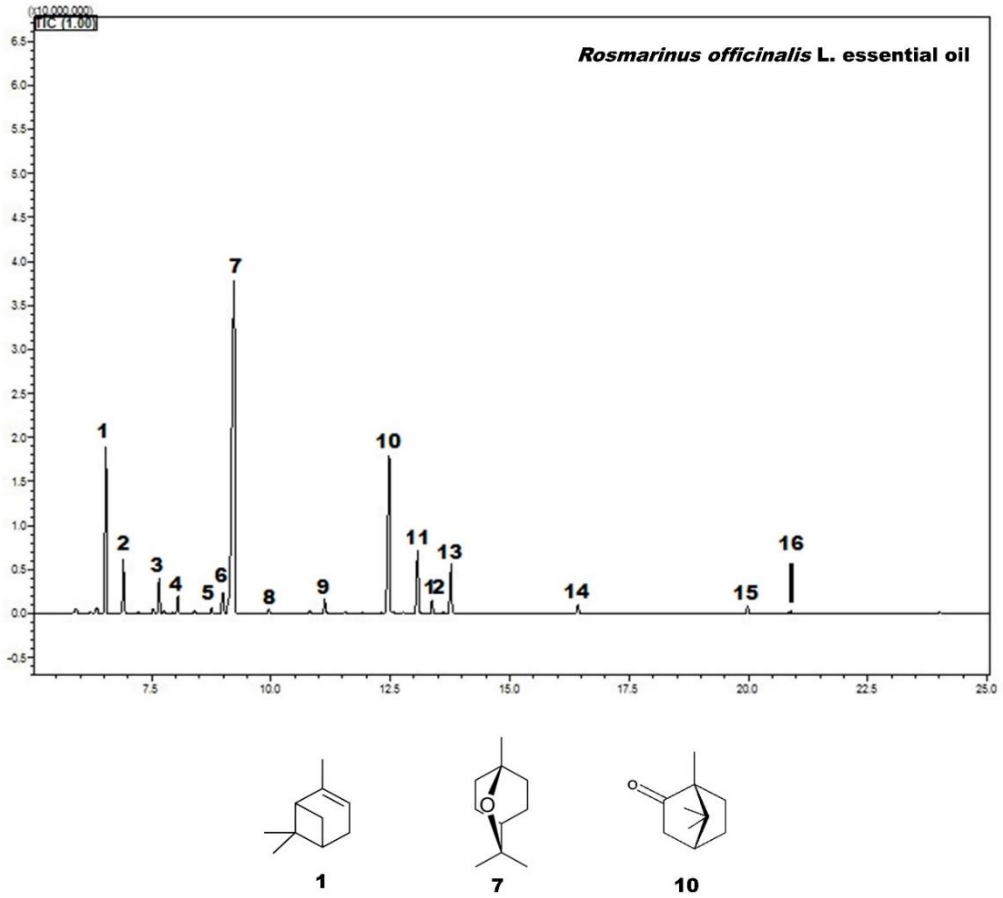

Figure 1. Chromatogram and chemical structures of compounds identified in the essential oil of *Rosmarinus officinalis* and their respective molecular masses (m/z), retention times (min) and area (%): α-pinene (**1** – m/z 136.238; 6.54 min; 11.52%); camphene (**2** – m/z 136.238; 6.91 min; 3.74%); sabinene (**3** – m/z 136.238; 7.65 min; 2.24%); β-pinene (**4** – m/z 136.238; 8.05 min; 1.07%); α-terpinene (**5** – m/z 136.238; 8.75 min; 0.38%); o-cymene (**6** – m/z 134.22; 8.98 min; 2.20%); 1,8-cineole/eucalytpol (**7** – m/z 154.253; 9.23 min; 47.91%); γ-terpinene (**8** – m/z 136.238; 9.95 min; 0.28%); linalool (**9** – m/z 154.253; 11.12 min; 1.09%); camphor (**10** – m/z 152.237; 12.47 min; 17.92%); isoborneol (**11** – m/z 154.253; 13.07 min; 5.43%); terpinen-4-ol (**12** – m/z 154.253; 13.37 min; 0.95%); α-terpineol (**13** – m/z 154.253; 13.77 min; 4.08%); bornyl acetate (**14** – m/z 196.290; 16.42 min; 0.63%); caryophyllene (**15** – m/z 204.357; 19.89 min; 0.51%); α-humulene (**16** – m/z 204.357; 20.84%; 0.05%).

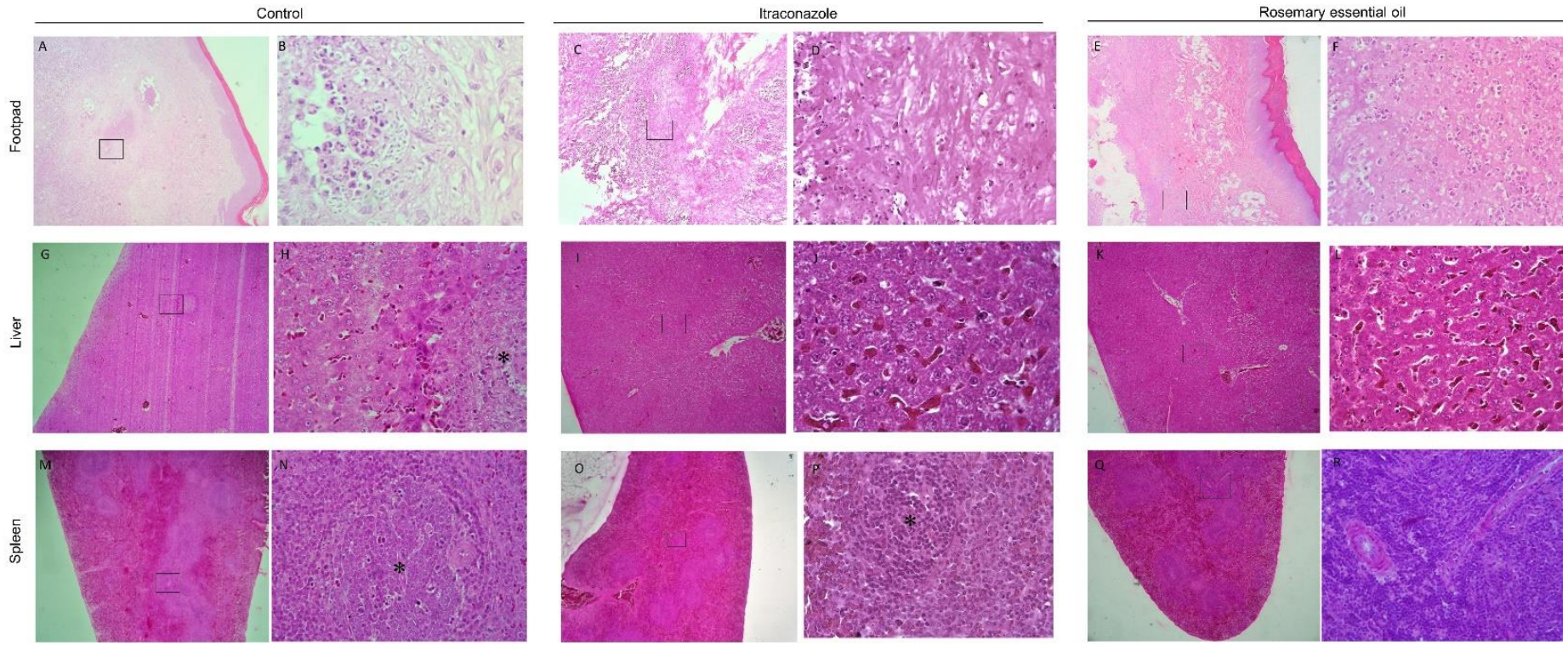

Figure 2. Hematoxylin and eosin-stained from the footpad (A–F), liver (G–L) and spleen (M–R) of rats experimentally infected by an itraconazole-resistant *Sporothrix brasiliensis* in the end of the oral treatment with saline solution as control, itraconazole and rosemary (*Rosmarinus officinalis* L.) essential oil. Footpad of all groups showed pyogranulomatous inflammatory infiltrate with polymorphonuclear leukocyte cells (B, D and F) and necrosis (B and D) but *S. brasiliensis* yeasts cells were severely found only in the footpad of control (B) and itraconazole (D) groups. A same feature was showed in liver and spleen of control and itraconazole groups with formation of granuloma (H, N and P, asterisk), whereas normal histological characteristics were found in these organs in the rosemary group (L and R). Sections at left of each group are in 40× magnification and, at right, 400×.

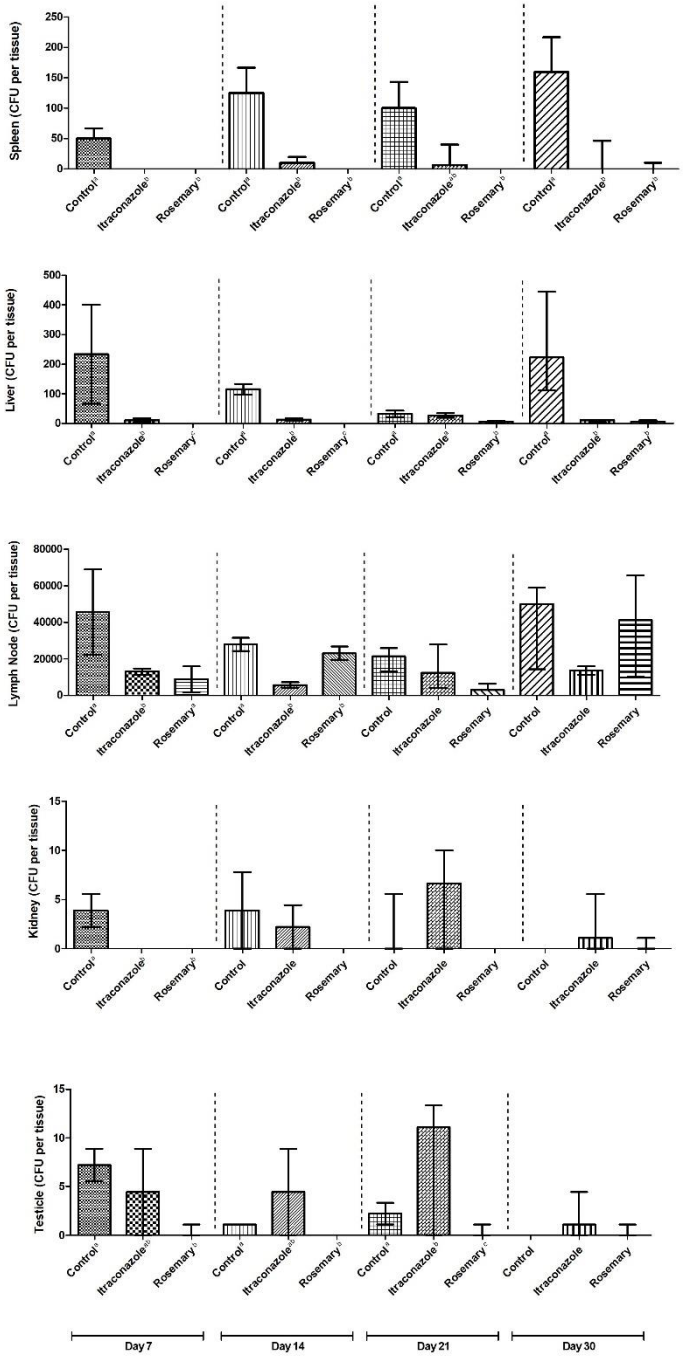

Figure 3. Fungal load in spleen, liver, lymph node, kidney and testicle (CFU per tissue) of rats experimentally infected by an itraconazole-resistant *S. brasiliensis* on the left foot pad and allocated in the following groups: control receiving saline solution, itraconazole and rosemary (*Rosmarinus officinalis* L.) essential oil. Four collects were performed (1st, 2nd, 3rd and 4th weeks of evaluation) and the median values were compared by Kruskal-Wallis test, followed by Dunn's test, which the different letters (^{a,b,c}) among the treatments are significant ($P < .05$), whereas the absence of letters are non-significant ($P \geq .05$).

Table 1. Minimal inhibitory concentration (MIC) of the *Rosmarinus officinalis* essential oil and anidulafungin in the absence and presence of the osmotic protector sorbitol against *S. brasiliensis* from feline cases.

Sporothrix brasiliensis*	**MIC *Rosmarinus officinalis* oil (mg/ml)**				**MIC Anidulafungin (µg/ml)**			
	3 days		**7 days**		**3 days**		**7 days**	
	S (–)	**S (+)**	**S (–)**	**S (+)**	**S (–)**	**S (+)**	**S (–)**	**S (+)**
Itraconazole-sensitive								
S68	0.56	0.56	0.56	0.56	32	256	32	512
S103	0.07	0.07	0.07	0.07	32	256	32	512
Itraconazole-resistant								
S72	0.28	0.28	0.28	0.28	32	256	32	512
S120**	0.07	0.07	0.07	0.07	32	256	32	512

S (–), absence of sorbitol; S (+), presence of sorbitol; * All *S. brasiliensis* were came from feline cases in the city of Pelotas/RS (Southern Brazil) and belong to the culture collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária*, Federal University of Pelotas, (UFPEL, Brazil); **S120 was also used for the experimental infection in murine model and is also named as “MV 1710”.

Table 2. Minimal inhibitory concentration (MIC) of the *Rosmarinus officinalis* essential oil and amphotericin B in the absence (E–) and presence (50–200 µg/mL) of the exogenous ergosterol against *S. brasiliensis* from feline cases.

***Sporothrix brasiliensis* ***	**MIC *Rosmarinus officinalis* oil (mg/ml)**										**MIC Amphotericin B (µg/ml)**									
	3 days					**7 days**					**3 days**					**7 days**				
	E–	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**
Itraconazole-sensitive																				
S68	0.56	0.56	0.56	0.56	1.12	0.56	0.56	0.56	0.56	>71.68	1	16	>256	>256	>256	1	>256	>256	>256	>256
S103	0.07	0.07	0.07	0.07	0.14	0.07	0.07	0.07	0.07	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256
Itraconazole-resistant																				
S72	0.28	0.28	0.28	0.28	0.56	0.28	0.28	0.28	0.28	>35.84	1	16	>256	>256	>256	1	>256	>256	>256	>256
S120**	0.07	0.07	0.07	0.07	0.14	0.07	0.07	0.07	0.07	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256

* All *S. brasiliensis* were came from feline cases in the city of Pelotas/RS (Southern Brazil) and belong to the culture collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária*, Federal University of Pelotas, (UFPEL, Brazil);
**S120 was also used for the experimental infection in murine model and is also named as “MV 1710”;

Table 3. Effect of control, itraconazole and rosemary (*Rosmarinus officinalis* L). essential oil in the clinical signs of infected rats by an itraconazole-resistant *S. brasiliensis* under different time of the treatment.

Clinical signs*	**Control**	**Itraconazole**	**Rosemary**
1st week			
Edema	+++	+++	++
Enlarged lymph node	Yes	Yes	Yes
Presence of exudate	Yes	Yes	Yes
Difficulty in locomotion	++	++	+
2nd week			
Edema	+++	+++	+
Enlarged lymph node	Yes	Yes	Yes
Presence of exudate	Yes	Yes	Yes
Difficulty in locomotion	++	++	–
Other alterations	Spiky fur	Spiky fur	–
3rd week			
Edema	++	++	+
Enlarged lymph node	Yes	Yes	Yes
Presence of exudate	Yes	Yes	No
Difficulty in locomotion	+	+	–
4th week			
Edema	+	+	-
Enlarged lymph node	Yes	Yes	Yes
Presence of exudate	Yes	Yes	No
Difficulty in locomotion	–	–	–
Other alterations	–	–	–

*Information based on the mean of the animals in each group; edema and difficulty in locomotion expression according to the following clinical lesion score: absent (–), mild (+), moderate (++) and severe (+++).

5 Considerações Finais

Diante dos resultados observados, o surgimento de *Sporothrix brasiliensis* com resistência *in vitro* ao itraconazol é notório em casos clínicos de humanos, cães e gatos com esporotricose. As plantas da família Lamiaceae são fontes promissoras de moléculas antifúngicas, a destacar os óleos essenciais de alecrim (*Rosmarinus officinalis* L.) e manjerona (*Origanum majorana* L.). Na esporotricose cutânea experimental por *Sporothrix brasiliensis* resistente ao itraconazol, os sinais clínicos da doença foram amenizados nos animais tratados oralmente com os óleos essenciais de alecrim e manjerona, comparado aos grupos controle e itraconazol, além de apresentarem uma baixa ou nenhuma disseminação fúngica para os órgãos sistêmicos. A cromatografia gasosa permitiu detectar a presença de 23 e 16 compostos químicos, respectivamente, nos óleos essenciais, os quais os compostos terpineol-4 e γ-terpineno (manjerona) e 1,8-cineol e cânfora (alecrim) foram prevalentes. A atividade anti-*Sporothrix brasiliensis* de ambos óleos essenciais foi associada à atividade dos compostos químicos a nível de complexação com o ergosterol. Dessa forma, esse estudo demonstrou o efeito do uso oral de alecrim e manjerona no tratamento da esporotricose cutânea experimental por *Sporothrix brasiiensis* resistente ao itraconazol, sendo promissores como antifúngicos.

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Anexos

Anexo I - Documento da Comissão de Ética e Experimentação Animal

UFPel

Pelotas, 03 de setembro de 2014

De: Prof. Dr. Éverton Fagonde da Silva

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Professor Mário Carlos Araújo Meireles

Faculdade de Veterinária

Senhor Professor:

A *CEEA* analisou o adendo ao projeto intitulado: **"Prospecção de plantas da família Lamiaceae para o tratamento e controle da esporotricose"**, processo nº23110.001622/2012-55, sendo de parecer FAVORÁVEL a sua execução, considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos, com prazo de execução até 31/12/2014.

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Éverton Fagonde da Silva

Presidente da CEEA

Ciente em 03/09/2014

Assinatura do Professor Responsável: