**UNIVERSIDADE FEDERAL DE PELOTAS**
**Faculdade de Veterinária**
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Tese

# Suscetibilidade *in vitro* e *in vivo* de *Pythium insidiosum* a quatro óleos essenciais de plantas da família Lamiaceae e proposta de um novo inoculo para testes de suscetibilidade *in vitro*

**Anelise Oliveira da Silva Fonseca**

Pelotas, 2015

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Tese apresentada ao Programa de Pós-Graduação em Veterinária da Faculdade de Veterinária da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área de concentração: Sanidade Animal).

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## Resumo

Fonseca, Anelise Oliveira da Silva, **Suscetibilidade *in vitro* e *in vivo* de *Pythium insidiosum* a quatro óleos essenciais de plantas da família Lamiaceae e proposta de um novo inoculo para testes de suscetibilidade *in vitro***, 2015, 64f. Tese (Doutorado em Ciências) - Programa de Pós Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2015.

*Pythium insidiosum* é um oomiceto aquático e agente etiológico da pitiose, uma doença infecciosa, não contagiosa, de difícil tratamento e prognóstico desfavorável, que acomete mamíferos que habitam áreas pantanosas e alagadas. O presente estudo teve como objetivos: a) avaliar e padronizar um inóculo a partir de cultivo micelial de *P. insidiosum*; b) avaliar a suscetibilidade *in vitro* de *P. insidiosum* aos óleos essenciais de *Origanum vulgare*, *Origanum majorana*, *Mentha piperita* e *Rosmarinus officinalis;* c) investigar a ação antimicrobiana dos óleos essenciais de *O. vulgare* e *M. piperita* sozinhos, associados e em combinação com imunoterapia no tratamento da pitiose experimental. O inóculo foi confeccionado a partir de cultura micelial de *P. insidiosum* e testado frente a antifúngicos azólicos. Os resultados foram comparados aos testes de suscetibilidade empregando o inóculo padrão de zoósporos frente aos mesmos antifúngicos. O inóculo proposto mostrou padrões de suscetibilidade comparáveis ao inoculo padrão, indicando, portanto, que pode ser um método adequado para avaliar-se a suscetibilidade deste oomiceto, particularmente quando não é possível obter-se o inóculo padrão. Os óleos foram analisados por cromatografia gasosa acoplada a espectrometria de massa. Os componentes majoritários dos óleos essenciais foram os seguintes: *O. vulgare*: carvacrol (93,10%) e beta; *O. majorana:* 1,4-terpineol (34,34%); *M. piperita:* mentona (57,53%); R. *officinalis:* 1,8-cineol (64,53%). A atividade antimicrobiana foi determinada pelo método de microdiluição em caldo frente a 22 isolados de *P. insidiosum*. Os óleos foram submetidos a uma série de diluições, obtendo-se concentrações de 14 a 0,025mg/mL. As concentrações inibitórias mínimas para *O. majorana, M. piperita* e *R. officinallis* variaram de 0,11 a 3,5mg/mL e para *O. vulgare* de 0,05 a 1,75mg/mL. Os resultados evidenciaram que os óleos essenciais avaliados apresentaram ação antimicrobiana sobre *P. insidiosum*, ressaltando-se a melhor atividade do óleo essencial de *O. vulgare*. A partir destes dados delineou-se o experimento *in vivo*, no qual foram utilizados 18 coelhos com pitiose experimental, divididos em seis grupos de três animais sendo: grupo 1 controle; grupo 2 tratados com óleo essencial de *M. piperita*; grupo 3 tratados com óleo essencial de *O. vulgare*; grupo 4 tratados com imunoterápico Pitium Vac®; grupo 5 tratados com associação dos óleos de *M. piperita* e *O. vulgare* e grupo 6 tratados com associação dos óleos *plus* imunoterápico. Os óleos foram formulados em creme tópico e as lesões foram tratadas diariamente por 45 dias; os animais dos grupos 4 e 6 receberam uma dose do imunoterápico a cada 14 dias. Os resultados revelaram que a evolução das lesões dos grupos 5 e 6, não diferiram entre si, porém diferiram dos demais grupos. Evidenciou-se que as lesões do grupo 5 aumentaram 3.16 vezes a

cada dia, enquanto àquelas do grupo 6, aumentaram 1.83 vezes, indicando o menor crescimento das lesões quando o tratamento empregou a combinação das terapias. Este estudo é pioneiro no tratamento da pitiose experimental empregando óleos essenciais de plantas e combinação de terapias com óleos em pitiose. Demonstrou que o emprego de óleos essenciais pode se constituir numa alternativa viável de tratamento da pitiose cutânea, particularmente quando utilizados em combinação ou em associação com imunoterapia.

**Palavras-chave:** Oomiceto; pitiose; inóculo; Imunoterapico; *Rosmarinus officinalis; Origanum vulgare; Origanum majorana; Mentha piperita*

## Abstract

Fonseca, Anelise Oliveira da Silva. ***In vitro* and *in vivo* susceptibility of *Pythium insidiosum* to four essential oils from the Lamiaceae plant family and the proposal of a new inoculum for *in vitro* susceptibility testing.** 2015, 64p. Dissertation (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2015.

*Pythium insidiosum* is an aquatic oomycete and etiological agent of Pythiosis, an infectious, non-contagious, difficult-to-treat disease. It affects mammals that inhabit marshy and flooded areas. This study aimed to: a) evaluate and standardize an inoculum from *P. insidiosum* mycelial culture; b) evaluate *P. insidiosum in vitro* susceptibility to *Origanum vulgare, Origanum majorana, Mentha piperita and Rosmarinus officinalis* essential oils; c) investigate the antimicrobial activity of *O. vulgare* and *M. piperita* essential oils separately, associated and in combination with immunotherapy in the treatment of experimental Pythiosis. The inoculum was prepared from *P. insidiosum* mycelial culture and tested against azole antifungals. The results were compared to susceptibility tests by using zoospore standard inoculum against the same antifungals. The proposed inoculum showed susceptibility patterns comparable to standard inoculum, thus indicating that it can be a suitable method to evaluate oomycete susceptibility, particularly when it is not possible to obtain standard inoculum. The oils were analyzed by gas chromatography mass spectrometry. Essential oils main components were as follows: *O. vulgare*: carvacrol (93.10%); *O. majorana*: 4-terpineol (34.34%); *M. piperita*: menthone (57.53%); *A. officinalis*: 1.8-cineole (64.53%). Antimicrobial activity was determined by the broth microdilution method against 22 *P. insidiosum* isolates. The oils were subjected to serial dilutions, and 14-0.025mg/mL concentrations were obtained. *O. marjorana*, *M. piperita* and *R. officinalis* minimum inhibitory concentrations ranged from 0.11 to 3.5 mg/mL, and those for *O. vulgare*, from 0.05 to 1.75 mg/mL. The results showed that the tested essential oils had antimicrobial effect on *P. insidiosum*, with *O. vulgare* essential oil being the most active. The *in vivo* experiment was outlined from these results. Eighteen rabbits with experimental Pythiosis, divided into six groups of three animals, were used: group 1 control; group 2 was treated with *Mentha piperita* essential oil; group 3 was treated with *Origanum vulgare* essential oil; group 4 was treated with commercial immunotherapic; group 5 was treated with a combination of *Mentha piperita* and *Origanum vulgare* oils, and group 6 was treated with a combination of immunotherapy plus oils. The oils were formulated as topical cream and lesions were treated daily for 45 days; animals in groups 4 and 6 received an immunotherapy dose every 14 days. The results revealed that the evolution of lesions in groups 5 and 6 did not differ between each other, but differed from the other groups. Group 5 lesions increased

3.16 fold every day, whereas those in group 6 increased 1.83 fold, thus showing that the smallest lesion growth occurred when an combination of therapies was employed. This is the first study to use essential oils from plants and a combined oil therapy in theexperimental treatment of Pythiosis. It was demonstrated that the use of essential oils can be a viable alternative for skin Pythiosis treatment, particularly when used in combination with or associated to immunotherapy.

**Key-words**: Oomycete; pythiosis; inoculum; immunotherapy; *Rosmarinus officinalis*; *Origanum vulgare*; *Origanum majorana; Mentha piperita*

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**Sumário**

## 1 Introdução

*Pythium insidiosum* é um oomiceto classificado no Reino Stramenopila, Classe Oomycetes, Ordem Pythiales, Família Pythiaceae, Gênero *Pythium* e Espécie *insidiosum* (ALEXOUPOLOS, 1996). Os oomicetos constituem um grupo de micro-organismos que habitam ecossistemas de água doce e salgada, assim como ambientes terrestres. A maioria dos "fungos aquáticos" cresce em água doce, principalmente rios, lagoas e lagos. Dentro destes habitats são mais comumente encontrados em águas rasas, perto das margens. O gênero *Pythium* possui mais de 120 espécies, sendo a maioria habitante do solo. Enquanto algumas espécies causam sérios prejuízos na produção agronômica, outras têm a capacidade de parasitar fungos e larvas de mosquito, e apenas a espécie *P. insidiosum* é patógeno para mamíferos (ALEXOPOULOS et al., 1996).

*P. insidiosum* cresce facilmente em meios de cultura formando colônias transparentes ou esbranquiçadas, submersas com curto micélio aéreo e padrão delicadamente radiado. O diâmetro das hifas cenocíticas varia entre 4 e 10 µm (micrômetros) com ramificações perpendiculares laterais em ângulo reto. Septos são ocasionalmente observados em hifas jovens, porém são abundantes em culturas velhas (DE COCK et al., 1987; MENDOZA et al., 1996). A produção de estruturas assexuadas (zoosporângios e zoósporos) pode ser obtida quando *P. insidiosum* é cultivado em meio líquido entre 28 e 37ºC. Nos estágios iniciais da formação dos zoosporângios não é possível diferenciá-lo da hifa vegetativa, uma vez que apenas observa-se o intumescimento da extremidade da hifa com a formação de uma pequena vesícula. Posteriormente, a movimentação de fluxo citoplasmático dirigindo-se do interior da hifa em direção a vesícula, culmina na formação dos zoosporângios. Estes são hialinos, globosos e medem cerca de 20-60 µm em diâmetro. Por meio de clivagem progressiva, zoósporos biflagelados são formados no interior dos zoosporângios. Os zoósporos mecanicamente rompem a parede do zoosporângio, sendo liberados. Nadam em movimentos espirais ou helicoidais em diferentes direções, por aproximadamente 20 minutos e então se encistam.

São do tipo secundário com forma reniforme apresentando um flagelo anterior (que gera o impulso) e um flagelo posterior (responsável pela direção). Após encistamento, os flagelos são destacados e os zoósporos tornam-se globosos emitindo tubos germinativos (DE COCK et al., 1987; MENDOZA et al., 1993; MENDOZA et al., 1996, AZEVEDO et al., 2012).

Diferente dos fungos verdadeiros, *P. insidiosum* apresenta parede celular constituída por glucanas, celulose e aminoácido hidroxiprolina; talo diplóide; mitocôndria com crista tubular e síntese do aminoácido lisina através de uma rota alternativa (ALEXOPOULOS et al., 1996; MOORE-LANDECKER, 1996). Dentre essas particularidades, a composição da parede celular é a característica mais comumente utilizada na diferenciação desses micro-organismos, uma vez que é composta predominantemente de β (1,3) e β (1,6) glucanas e celulose ao invés de quitina, como é o caso dos fungos. Notáveis diferenças são também observadas quanto à presença do ergosterol na membrana citoplasmática, o qual está ausente nos micro-organismos incluídos no Oomycota (ALEXOPOULOS et al., 1996; MOORE-LANDECKER, 1996).

*P. insidiosum* causa pitiose em mamíferos que habitam regiões de clima tropical, subtropical e temperado, tendo sido relatada em vários países do continente americano, oceania e áfrica (MENDOZA et al., 1996, GAASTRA et al., 2010). Comumente, observa-se que os animais afetados adquirem a enfermidade ao permanecer por longos períodos em contato com águas estagnadas (CHAFFIN et al. 1995). O acúmulo de água em banhados e lagoas, a presença de vegetação aquática e temperaturas entre 30 e 40ºC, são fatores essenciais que influenciam a ocorrência da pitiose (MILLER; CAMPBELL 1982). Nestes ambientes *P. insidiosum* realiza seu ciclo de reprodução formando zoósporos infectantes, que ao serem liberados nas águas são atraídos por quimiotaxia para tecidos danificados, onde se fixam e emitem tubos germinativos dando início a enfermidade (MILLER, 1983, MENDOZA et al. 1996).

A pitiose é uma doença infecciosa, de difícil tratamento, sendo os equinos e caninos, as espécies mais frequentemente afetadas (GAASTRA et al., 2010). No entanto, bovinos (MILLER et al.,1985; SANTURIO et al., 1998; PEREZ et al., 2005), felinos (BISSONNETTE et al., 1991; THOMAS; LEWIS, 1998; RAKICH et al., 2005), ovinos (TABOSA et al., 2004; SANTURIO et al., 2008; PESSOA et al., 2012), caprinos (CARMO et al., 2014), aves (PESAVENTO et al., 2008) e espécies selvagens mantidas em cativeiro, como jaguar (CAMUS et al., 2004), urso (GROOTER, 2003) e

dromedário (WELLEHAN et al., 2004) são também afetadas. Em humanos é uma enfermidade de prognóstico desfavorável, mais comum no Sudeste Asiático, especialmente em trabalhadores de áreas rurais e indivíduos portadores de beta - talassemia (KRAJAEJUN et al., 2006).

No Brasil, a pitiose tem sido bem documentada e ocorre em equinos (SANTOS et al. 1987, MEIRELES et al., 1993; LEAL et al. 2001, SALLIS et al. 2003, FREY et al., 2007; MARCOLONGO-PEREIRA et al., 2012), bovinos (SANTURIO et al., 1998; GRECCO et al. 2009, GABRIEL et al., 2008), ovinos (TABOSA et al. 2004, SANTURIO et al., 2008, PESSOA et al., 2012, CARRERA et al., 2013), caprinos (CARMO et al., 2014), caninos (RODRIGUES et al., 2006; NETTO et al., 2010; FERNANDES et al., 2012; PEREIRA et al. 2010,2013) e humanos (BOSCO et al., 2005; MARQUES et al. 2006), porém a maioria dos casos corresponde a lesões cutâneas em equinos. Nestes animais, a enfermidade caracteriza-se pelo desenvolvimento de lesões ulcerativas granulomatosas, que evoluem rapidamente, formando grandes massas teciduais de bordas irregulares e aparência tumoral. No interior das lesões observa-se abundante tecido conjuntivo fibroso de consistência firme e brancacento, entrecortado por galerias (*sinus*) preenchidas por massas branco-amareladas de 2 a 10 mm de diâmetro, de aspecto arenoso e ramificadas (*kunkers*) que facilmente se desprendem do tecido circunjacente (CHAFFIN et al.,1995; MENDOZA et al., 1996). Os "*kunkers*" além de auxiliarem no diagnóstico da enfermidade, também desempenham papel importante na contaminação de aguadas pelo *P. insidiosum*, uma vez que ao se desprenderem naturalmente das lesões, em ambiente favorável e água estagnada, refazem o ciclo do oomiceto colonizando novas plantas e mantendo a re-contaminação do ambiente (FONSECA et al., 2013).

Nos caninos as infecções manifestam-se como piogranulomas gastrintestinais e cutâneos. As lesões gastrointestinais são as mais comuns e caracterizam-se pela formação de grandes massas nas paredes do estômago e intestino que levam a obstrução da luz do órgão (GROOTER, 2003). Em felinos e ovinos, a doença pode manifestar-se na forma cutânea e gastrointestinal (BISSONNETTE et al., 1991, TABOSA et al., 2004, RAKICH et al., 2005, PESSOA et al., 2012, CARRERA et al., 2013) e em bovinos na forma cutânea (SANTURIO et al., 1998; GRECCO et al., 2009, GABRIEL et al., 2008). Já em humanos, relatam-se três formas clínicas distintas: cutânea, ocular e sistêmica (KRAJEJUN et al., 2006).

Segundo Mendoza & Newton (2005) as várias tentativas de reprodução experimental da enfermidade em espécies naturalmente infectadas resultaram em insucesso. No entanto, a doença pode ser reproduzida em coelhos por meio da inoculação subcutânea de zoósporos de *P. insidiosum*. Os animais evidenciam nódulos que evoluem para fibrogranulomas eosinofílicos após 20-30 dias de inoculação (MILLER; CAMPBELL, 1983). Nos últimos anos, vários estudos têm utilizado coelhos como modelo experimental para avaliar imunoterapias (SANTURIO et al., 2003, PEREIRA et al., 2008) e terapias antifúngicas (PEREIRA et al., 2007; LORETO et al., 2011, ARGENTA et al., 2012; ZANETTE et al., 2013; ZANETTE et al., 2014) como alternativas de tratamento da pitiose.

Dificuldades no tratamento da pitiose são encontradas em todas as espécies afetadas. Todavia, diversos protocolos de tratamento têm sido utilizados, incluindo cirurgia, imunoterapia e terapia com fármacos antifúngicos. Alguns autores sugerem que a melhor opção de tratamento é a cirurgia, entretanto, além das altas taxas de recidiva (45%), esta prática não pode ser realizada em regiões envolvendo áreas anatômicas delicadas (CHAFFIN et al., 1995, GAASTRA et al., 2010; PEREIRA et al., 2013). A partir da década de 1980 denota-se um grande progresso nas pesquisas que visam o desenvolvimento de imunoterápicos para o tratamento da pitiose equina. Mendoza & Newton (2005) afirmam que utilização da imunoterapia é segura, tanto em animais quanto em humanos, curando aproximadamente 60% dos indivíduos tratados e modulando a resposta imune dos mesmos.

A busca por fármacos que possam auxiliar na terapia de pitiose são relatadas desde o ano de 1977 (MCMULLAN et al. 1977). No entanto, a ausência de ergosterol na membrana citoplasmática de *P. insidiosum* explica e justifica os insucessos relatados com as terapias antifúngicas, uma vez que o ergosterol constitui o componente alvo de ação da maioria dos fármacos antifúngicos comumente utilizados na terapêutica clínica (GROOTERS, 2003), assim como os azólicos (itraconazol, cetoconazol, fluconazol, miconazol) e terbinafina que interferem com a biossíntese do ergosterol e anfotericina B que altera a permeabilidade da membrana celular ao ligar-se ao ergosterol (GAASTRA et al., 2010).

Os testes *in vitro* avaliando a suscetibilidade de *P. insidiosum* frente a agentes antimicrobianos teve seu início com SEKHON et al. (1992). No entanto, um dos problemas do desenvovimento de testes *in vitro* com oomicetos era a falta de padronização dos testes pelo CLSI (Clinical Laboratory Standards Institute). Contudo,

Pereira et al. (2007) e Argenta et al. (2008) propuseram a padronização de testes *in vitro* utilizando como inóculo zoósporos de *P. insidiosum*, o que foi adotado nos estudos posteriores que avaliaram a suscetibilidade de *P. insidiosum*. No entanto, como esta técnica apresentava algumas desvantagens como o tempo envolvido na zoosporogênese (aproximadamente 6 dias) e a dificuldade na obtenção do número requerido de zoósporos (ARGENTA et al., 2008), Fonseca et al. ($2014_a$) propuseram a utilização de um novo inoculo utilizando o raspado do micélio do oomiceto cultivado em agar. Como vantagens este método apresentou maior facilidade de obtenção, menor possibilidade de contaminação, bem como melhor mimetizar as características do micro-organismo no tecido infectado.

A suscetibilidade *in vitro* de *P. insidiosum* tem sido bem estudada e documentada frente aos antifúngicos azólicos, anfotericina B, terbinafina, flucitosina, griseofulvina, caspofungina, anidulafungina e micafungina (SEKHON et al., 1992; SHENEP et al., 1998; PEREIRA et al., 2007; ARGENTA et al., 2008, 2012; CAVALHEIRO et al., 2009; ZANETTE et al., 2014), e a compostos com ação antimicrobiana como rifampicina, tetraciclinas, neomicina, estreptomicina, paromomicina, tigeciclina, macrolídios, metronidazol, ibuprofeno e difenil-disseleneto (SEKHON et al., 1992, CAVALHEIRO et al., 2009, LORETO et al., 2011, 2012, 2014; ARGENTA et al., 2012; MAHL et al.,2012). Além disso, alguns estudos têm avaliado também a associação de fármacos, assim como terbinafina com rifampicina, metronidazol, fluvastatina, ibuprofeno, cetoconazol e caspofungina (CAVALHEIRO et al., 2009); associação de azólicos (ARGENTA et al., 2008); azólicos com caspofungina, ibuprofeno e fluvastatina (ARGENTA et al., 2012); associações de antibacterianos e antifúngicos (LORETO et al., 2014, JESUS et al., 2014) e associação de antifúngicos com quelante de ferro (deferasirox) (ZANETTE et al., 2014). Os resultados desses estudos demonstram que a suscetibilidade *in vitro* do *P. insidiosum* a esses fármacos é variável, assim como os resultados de testes clínicos com alguns desses compostos (PEREIRA et al., 2007; ARGENTA et al., 2012, LORETO et al., 2011; ZANETTE et al., 2014). Corroborando esses dados, relatos de sucesso clínico são descritos, principalmente em caninos e humanos, ao utilizarem terapias com fármacos azólicos (BISSONNETTE et al, 1991; TRISCOTT et al, 1993; SHENEP et al, 1998; GROOTERS, 2003; PEREIRA et al. 2013). Todavia, constata-se que em todos os relatos de cura da pitiose sempre houve associação de terapias (KRAJAEJUN et al., 2006; SANTURIO et al., 2010; HUMMEL et al., 2011;

SUDJARITRUK & SIRISANTHANA, 2011; PEREIRA et al., 2013). A fitoterapia tem sido alvo de diversas investigações científicas relacionando usos, efeitos e propriedades farmacológicas das plantas medicinais. Há um crescente interesse na utilização de óleos essenciais devido as suas propriedades antioxidantes e antimicrobianas. Embora a maior utilização ocorra na área de alimentos (condimentos e aromatizantes de alimentos e bebidas) e cosméticos (perfumes e produtos de higiene), os óleos essenciais também têm sido utilizados na indústria farmacêutica em função de suas propriedades terapêuticas (SIMÕES & SPITZER, 2002). O mecanismo de ação dos óleos essenciais de plantas que incluem alterações da permeabilidade da membrana citoplasmática e modificações no gradiente de íons de hidrogênio, potássio e cálcio, levando a prejuízos dos processos essenciais à sobrevivência das células, tais como transporte de elétrons, transporte de proteínas e interferência na fosforilação (DORMAN & DEANS, 2000; RAO et al., 2010) justifica o interesse por pesquisas que avaliem o uso desses compostos como agentes antimicrobianos tanto na área médica como na área de alimentos.

Estudos têm demonstrado ação antimicrobiana de óleos essenciais de *Rosmarinus officinalis, Mentha piperita, Thymus vulgaris, Origanum vulgare, Origanum majorana, Ocimum basilicum, Glechon spathulata bent*., entre outros sobre bactérias (BOZIN et al., 2007; CELIKTAS et al., 2007; SANTURIO et al., 2007; TYAGI & MALIK 2011; KAČÁNIOVÁA et al., 2014; MONTE et al., 2014); fungos filamentosos (PEREIRA et al., 2006; ARSLAN & DERVIS, 2010; CLEFF et al., 2010; TYAGI & MALIK, 2011; MUGNAINI et al., 2012; MUGNAINI et al., 2013) e leveduriformes (CELIKTAS et al., 2007; COSTA, 2009; CLEFF et al., 2010; TYAGI & MALIK, 2011). Pesquisas avaliando a ação antimicrobiana de vários óleos essenciais, entre eles *Alpinia speciosa* (TAIRA et al. 1994), *Chenopodium ambrosioides* (KUMAR et al. 2007), *Lavandula R. C. hybrid* (ZAMBONELLI et al. 1996), *Mentha piperita* (LEE et al. 2007; ZAMBONELLI et al. 1996), *Ocotea queixos* (BRUNI et al. 2003), *Origanum vulgare* (LEE et al. 2007; WOGIATZI et al. 2009), *Rosmarinus officinalis* (LEE et al. 2007), *Thymus vulgaris* (ZAMBONELLI et al. 1996; LEE et al. 2007) e *Thymus zygius* (PÉREZ-SÁNCHES et al. 2007) sobre espécies fitopatógenas de *Pythium* (*P. irregulare, P. ultimum* e *P. debarynum*) têm evidenciado boa atividade *in vitro* contra esses oomicetos. Adicionalmente, estudos de suscetibilidade de *P. insidiosum*, espécie patógena para mamíferos, frente a óleos essenciais de *Origanum vulgare, Origanum majorana, Mentha piperita* e *Rosmarinus officinalis* demonstraram

resultados promissores (FONSECA, 2011, FONSECA et al., 2014$_b$). SRIPHANA et al., (2013$_a$) SRIPHANA et al., (2013$_b$) também evidenciaram a suscetibilidade de *P. insidiosum* aos compostos extraídos da raiz de *Alyxia schlechteri e Clausena harmandiana.* Similarmente, SUTHIWONG et al., (2014) observaram que compostos cumarinóides extraídos do fruto de *Micromelum falcatum* foram capazes de inibir o crescimento *in vitro* de *P. insidiosum*. Embora esses estudos *in vitro* denotem a promissora eficácia de óleos essenciais de plantas sobre *P. insidiosum,* mais estudos precisam ser realizados com o intuito de avaliar a eficácia *in vivo* desses compostos sobre esta espécie de oomiceto.

## 2 Objetivos

**Objetivo geral**:

Avaliar e padronizar um inoculo preparado a partir de cultivo micelial de *P. insidiosum,* assim como testar a suscetibilidade *in vitro* de *P. insidiosum* a quatro óleos essenciais de plantas da família Lamiaceae, bem como verificar a ação antimicrobiana *in vivo* dos óleos essenciais sozinhos ou em associação.

**Objetivos específicos:**

- Avaliar e padronizar um inoculo de *P. insidiosum* obtido a partir de seu cultivo micelial, comparando-o ao inoculo padrão de zoósporos;

- Verificar a suscetibilidade *in vitro* de isolados de *P. insidiosum* frente a antifúngicos azólicos empregando o inóculo padrão e o inóculo micelial;

- Avaliar a suscetibilidade *in vitro* de isolados de *P. insidiosum* ao óleo essencial de *O. vulgare, O. majorana, M. piperita* e *R. officinalis* assim como avaliar a composição química de cada óleo*;*

- Investigar a ação antimicrobiana *in vivo* dos óleos essenciais de *Origanum vulgare* e *Mentha piperita* sozinhos, associados e em combinação com imunoterapia no tratamento da pitiose experimental.

# 3 Artigos

## 3.1 Artigo 1

**In vitro susceptibility of zoospores and hyphae of *Pythium insidiosum* to antifungal**

**A.O.S. Fonseca, D.I.B. Pereira, F.S. Maia Filho, L.G. Osório, B.P. maronese, J.S.S. Valente, L. Pötter and M.C.A. Meireles**

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# *In vitro* susceptibility of zoospores and hyphae of *Pythium insidiosum* to antifungals

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**Objectives:** The purpose of this study was to compare the *in vitro* susceptibilities of 22 Brazilian isolates of *Pythium insidiosum* to antifungals using a standardized inoculum of zoospores and a proposed novel inoculum prepared from cultured mycelia (hyphae) of *P. insidiosum*.

**Methods:** A zoospore suspension of *P. insidiosum* was obtained by the zoosporogenesis technique. The hyphal inoculum was prepared from a suspension of *P. insidiosum* mycelium. Susceptibility to each drug was evaluated using the CLSI M38-A2 method.

**Results:** Of the 88 MIC comparisons performed, 36 (41%) showed the same MIC value for the two inocula. The agreement (differences not greater than one dilution) between MICs obtained with both types of inocula was 39.8% (35/88). In other MIC comparisons analysed, 17 (19.3%) showed differences of two or three dilutions.

**Conclusions:** We conclude that the use of hyphal inocula of *P. insidiosum* for *in vitro* susceptibility tests could be a suitable method for evaluating antimicrobial susceptibility, particularly when it is not possible to obtain a standardized zoospore inoculum.

**Keywords:** oomycetes, pythiosis, azoles, terbinafine

## Introduction

Members of the genus *Pythium* are fungal-like microorganisms in the kingdom Stramenopila. Although several species of this genus have been described, only *Pythium insidiosum* has the ability to cause disease in animals, including humans.[1]

Infections caused by *P. insidiosum* respond poorly to commonly used therapeutic methods, such as surgery, immunotherapy and antifungal drugs. Although these strategies are occasionally successful, therapeutic failure is more often observed.[1] Thus, the growing number of studies evaluating the susceptibility of *P. insidiosum* to various antimicrobials in recent years is well warranted.[2–7] In light of the fact that the zoospore is the infectious form of *P. insidiosum*, Pereira *et al.*[3] utilized this form to prepare an inoculum for reproducible *in vitro* and *in vivo* studies. However, the preparation of the inoculum from zoospores has several disadvantages, which include the time required for obtaining zoospores (~6 days), a lack of zoosporogenesis in some situations and difficulties in obtaining the number of zoospores needed for experimental studies.[4]

Because *P. insidiosum* infections are characterized by the presence of sparsely septated hyphae within eosinophilic granulomatous lesions in the tissues of infected animals,[8] we believe that using oomycete hyphae could serve as an alternative method of preparing inocula to be used for *in vitro* susceptibility studies.

The purpose of this study was to compare the *in vitro* susceptibilities of 22 Brazilian isolates of *P. insidiosum* to antifungals using standardized inocula of fungal zoospores and our proposed novel inocula prepared from cultured mycelia (hyphae) of *P. insidiosum*.

## Materials and methods

### *Isolates of P. insidiosum*

Twenty-one isolates of Brazilian *P. insidiosum* derived from horses ($n=19$) and domestic dogs ($n=2$) with pythiosis and one standard strain (CBS 101555) were used for these studies. The identities of the isolates were confirmed by PCR and sequencing.[9]

### *Preparation of inocula*

#### *Zoospore inocula*

Each zoospore inoculum suspension contained 20000–30000 zoospores/mL of *P. insidiosum* obtained by the zoosporogenesis method previously described and standardized by Pereira *et al.*[3]

#### *Mycelial inoculum culture (hyphae)*

These inocula were prepared from suspensions of *P. insidiosum* mycelium. These suspensions were obtained by cultivation of the microorganism in yeast agar consisting of agar (20 g/1000 mL; Merck) and yeast extract (1 g/1000 mL; Difco) with incubation for 96 h at 37°C. The cultures were covered with 10 mL of sterile distilled water and the mycelium was scraped with a sterile scalpel blade. Subsequently, this solution was transferred to a test tube, the optical density was determined using a spectrophotometer and the inoculum was adjusted to a transmittance of 80%–85% at 530 nm. To test the viability of this suspension, an aliquot of 100 μL was added to 900 μL of Sabouraud broth and incubated at 37°C for 48 h. The homogeneity of the suspension was evaluated by microscopy. Then, 10 μL of the suspension was placed on a glass microscope slide and subsequently covered with a cover slip and analysed under a microscope (×20 objective).

### *Antifungals*

The drugs tested included miconazole (Pharma Nostra, Rio de Janeiro, Rio de Janeiro, Brazil; 128–0.125 mg/L), ketoconazole (All Chemistry, São Paulo, São Paulo, Brazil; 128–0.125 mg/L), terbinafine (All Chemistry; 128–0.125 mg/L) and itraconazole (EMS Sigma Pharma São Bernardo do Campo, São Paulo, Brazil; 256–0.25 mg/L). The drugs were diluted in DMSO according to protocol M38-A2 of the CLSI.[10]

### *In vitro tests*

The MIC of each antifungal agent was determined by broth microdilution testing according to the CLSI M38-A2 method.[10] After preparation, both types of inoculum were diluted 1:10 in RPMI 1640 glucose and buffered to pH 7.0 with 0.165 M MOPS. The plates were incubated at 37°C for 48 h.

The tests were read by visually observing the growth or lack of hyphae, and the MIC was taken to be the lowest concentration at which no hyphal growth occurred (100% inhibition). All tests were performed in triplicate.

### *Statistical analysis*

MIC values for both inocula were submitted to a normality test regardless of the antifungal drug used. As the response variable did not show normality, data were subjected to the non-parametric $\chi^2$ test and frequency distribution analysis. The analyses were performed using the SAS software package, version 8.2, assuming a 5% probability.

## Results

The method used for preparing the hyphal inoculum in this study yielded highly purified, viable and homogeneous suspensions free of mycelial aggregates.

The *in vitro* susceptibilities of 22 isolates of *P. insidiosum* to individual antimicrobials are listed in Table 1. Of the 88 MIC comparisons performed, 36 (41%) showed the same MIC value for the two inocula. The agreement (differences not greater than one dilution) between MICs obtained with the two types of inocula

**Table 1.** *In vitro* susceptibility of *P. insidiosum* ($n = 22$) to ketoconazole, miconazole, terbinafine and itraconazole determined using hyphae and zoospores as inocula

| | MIC (mg/L), number of isolates (%) | | | | | | | | | | | | | | | | MIC$_{50}$[a] (mg/L) | | MIC$_{90}$[b] (mg/L) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 128 | | 64 | | 32 | | 16 | | 8 | | 4 | | 2 | | | | | | | |
| | H | Z | H | Z | H | Z | H | Z | H | Z | H | Z | H | Z | | | H | Z | H | Z |
| Ketoconazole | 0 | 0 | 0 | 0 | 2 (9.1) | 5 (22.7) | 5 (22.7) | 14 (63.6) | 8 (36.4) | 1 (4.5) | 7 (31.8) | 2 (9.1) | 0 | 0 | | | 8 | 16 | 16 | 32 |
| Miconazole | 0 | 0 | 0 | 0 | 0 | 0 | 6 (27.3) | 4 (18.2) | 2 (9.1) | 4 (18.2) | 6 (27.3) | 8 (36.4) | 8 (36.4) | 6 (27.3) | | | 4 | 4 | 16 | 16 |
| Terbinafine | 0 | 0 | 0 | 0 | 0 | 1 (4.5) | 8 (36.4) | 7 (31.8) | 7 (31.8) | 13 (59.1) | 7 (31.8) | 1 (4.5) | 0 | 0 | | | 8 | 8 | 16 | 16 |
| Itraconazole | 7 (31.8) | 7 (31.8) | 15 (68.2) | 15 (68.2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | 64 | 64 | 128 | 128 |

H, hyphae; Z, zoospores.
[a]Minimal concentration to inhibit the growth of 50% of isolates.
[b]Minimal concentration to inhibit the growth of 90% of isolates.

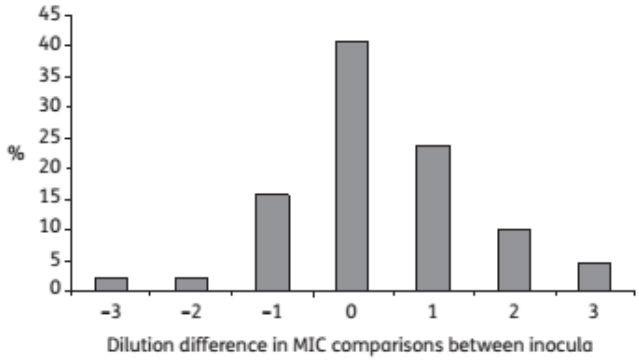


**Figure 1.** Percentage differences in MIC comparisons for two types of inocula of *P. insidiosum* (zoospore inoculum and hyphae inoculum) regardless of antifungal evaluated.

was 39.8% (35/88). In other MIC comparisons analysed, 17 (19.3%) showed differences of two or three dilutions (Figure 1).

The statistical analyses demonstrated that, regardless of the antifungal evaluated, the two inocula had similar MIC values ($P=0.17$). The MIC frequency distribution was also similar for the two inocula ($P=0.45$). Regardless of antifungal and inoculum, more than 60% of isolates of *P. insidiosum* had an MIC between 4 and 16 mg/L.

## Discussion

Because the oomycete *P. insidiosum* is the causative agent of pythiosis, an emerging disease that is very difficult to treat, there has been an increase in the number of studies regarding the *in vitro* susceptibility of *P. insidiosum* in recent years.[2–7,11]

Although the CLSI has not standardized susceptibility testing for this oomycete, Pereira *et al.*[3] and Argenta *et al.*[4] proposed the standardization of *in vitro* testing using *P. insidiosum* zoospores as the inoculum. However, Argenta *et al.*[4] mention several disadvantages of this methodology, including the difficulty of obtaining the required amounts of zoospores and the fact that the zoospore is not a pathological form. Because of the difficulties and the time required to perform the tests using zoospores *in vitro*, the present study proposes the use of a hyphal inoculum of *P. insidiosum*.

The method using hyphal inoculum was less labour intensive and time consuming than the zoosporogenesis method proposed by Pereira *et al.*[3] for testing *in vitro* susceptibility of *P. insidiosum*.

Among previous studies that evaluated the susceptibility of *P. insidiosum*, only Sekhon *et al.*[11] used hyphal inocula to test eight oomycete isolates. Our results are comparable to those of Sekhon *et al.*[11] in some respects because the MICs of itraconazole and ketoconazole were similar in both studies. However, the results for miconazole differed between the two studies. These differences may be due to the Sabouraud broth macrodilution (pH 6.3) method used by Sekhon *et al.*[11] for the development of *in vitro* assays. In addition, they used a hyphal suspension adjusted to a transmittance of 90%, but in our preliminary tests such inoculum densities resulted in non-uniform growth (data not presented). When we performed susceptibility tests using the standard inoculum prepared with zoospores of *P. insidiosum*, the results were similar to those of studies by Cavalheiro *et al.*,[5] which reported MIC ranges of 8–32 mg/L for terbinafine, 16–64 mg/L for ketoconazole and 4–32 mg/L for miconazole, and by Argenta *et al.*,[4,7] which found MICs of 16 to ≥32 mg/L for itraconazole and 0.5–16 mg/L for terbinafine.

Nevertheless, when comparing the MICs obtained with the two inocula tested, it was shown that although 80.7% of MIC comparisons performed had the same MIC value or differed by one dilution, 19.3% of MIC comparisons had a value that differed by two or three dilutions for the two types of inoculum. These findings agree with other studies that reported differences in MIC values when comparing inocula based on hyphae and conidia in antifungal susceptibility testing of *Aspergillus*, *Cladosporium*, *Cladophialophora*, *Paecilomyces*, *Fusarium* and dermatophytes.[12–20] In some of these studies it was shown that hyphae were more susceptible than conidia.[13,14,16,20] However, in other studies the susceptibilities of conidia and hyphae were similar,[15,18] and in yet others conidia were more susceptible.[12,19]

Our results show that it is feasible to test the *in vitro* susceptibility of *P. insidiosum* using as an inoculum a suspension of hyphae prepared from a mycelial culture of *P. insidiosum*. Furthermore, considering that the hyphae of this microorganism colonize cutaneous and subcutaneous tissues, produce intestinal lesions, invade blood vessels and proliferate within bone,[8] the development of susceptibility tests using hyphae could better mimic the characteristics of *P. insidiosum* in infected tissue and could better demonstrate the therapeutic potential of drugs against pythiosis.

We conclude that the use of hyphal inocula of *P. insidiosum* for the development of *in vitro* susceptibility tests could be a suitable method for evaluating antimicrobial susceptibility, particularly when obtaining standardized inocula of zoospores is not possible.

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## Transparency declarations

Conflicts of interest: none to declare.

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## 3.2 Artigo 2

**In vitro suscetibility of Brazilian *Pythium insidiosum* isolates to essential oils of some Lamiaceae Family species**

**A.O.S. Fonseca, D.I.B. Pereira, R.G. Jacob, F.S. Maia Filho, D.H. Oliveira, B.P. Maronese, J.S.S. Valente, S.A. Botton, M.C.A. Meireles**

**Aceito em Mycopathologia**

# *In Vitro Susceptibility of Brazilian* Pythium insidiosum *Isolates to Essential Oils of Some* Lamiaceae *Family Species*

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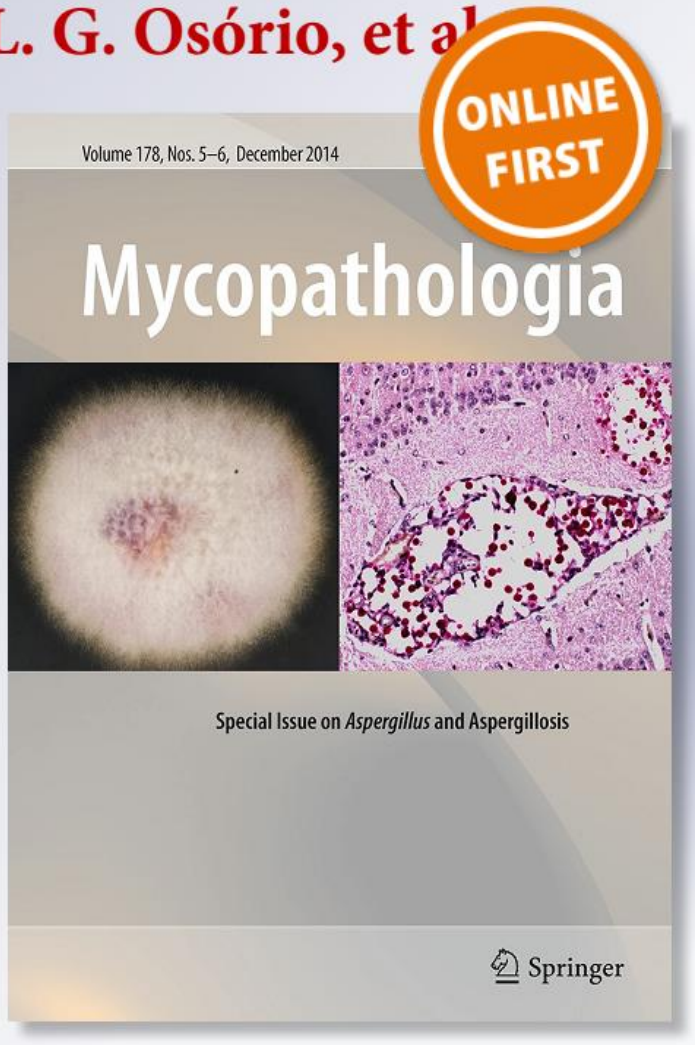


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# In Vitro Susceptibility of Brazilian *Pythium insidiosum* Isolates to Essential Oils of Some *Lamiaceae* Family Species

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**Abstract** The present study aimed to evaluate the in vitro antimicrobial action of *Origanum vulgare*, *Origanum majorana*, *Mentha piperita* and *Rosmarinus officinalis* on *Pythium insidiosum* oomycete zoospores. The antimicrobial activity evaluation was performed by the broth microdilution method according to CSLI M38-A2 documentation adapted to phytopharmaceuticals. Twenty-two *P. insidiosum* isolates were evaluated, and the minimum inhibitory concentration was determined at 100 % growth inhibition. All *P. insidiosum* isolates evaluated showed a minimum inhibitory concentration ranging from 0.05 to 1.75 mg/mL when *O. vulgare* oil was used and from 0.11 to 3.5 mg/mL for *O. majorana*, *M. piperita* and *R. officinalis* oils. The results obtained indicate that the essential oils tested showed antimicrobial activity on *P. insidiosum*, with *O. vulgare* essential oil showing the best performance. These findings emphasize the potential use of plant essential oils as control agents in *P. insidiosum* infections; further research, however, is needed so as the in vivo activity of these oils can also be evaluated.

**Keywords** Oomycete · Rosemary · Peppermint · Oregano · Marjoram

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## Introduction

*Pythium* genus oomycetes belong to the kingdom Stramenopila. Most of these microorganisms inhabit the soil and are plant pathogens, causing serious damage to farming [1]. Among the various species of this genus which have already been identified, *Pythium insidiosum* has the ability to infect mammals that live in tropical and subtropical areas, leading to pythiosis and primarily affecting horses, dogs and humans. This is a severe, difficult-to-treat disease of unfavorable diagnosis which may manifest in its

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cutaneous, gastrointestinal, ophthalmic and systemic forms [2]. Therapeutic treatment includes surgery, immunotherapy and antimicrobial agents, showing varying success rates. Although surgery is still the most employed procedure for pythiosis treatment for animals and humans, high recurrence rates (45 %) have been observed. Immunotherapy has been used as an alternative treatment in equine pythiosis for over 20 years [2]. Though this is thought to be a safe practice with healing rates that range from 60 to 70 % in horses and humans, there have been several non-responsive cases, and the results have been disappointing for some species, such as cats and dogs [3]. Antifungal-based therapies have proved to be difficult due to the absence of ergosterol in the cytoplasmic membrane of *P. insidiosum*, in view of the fact that ergosterol is the action site of the most commonly used antifungals available [2]. The lack of effective therapeutic alternatives for pythiosis has increased the interest in investigating the activity of antifungal [4, 6] and non-antifungal agents [5, 7] and their associations with other agents [8].

Essential oils from aromatic plants have been used as disease control agents in some studies, once these oils tend to show low toxicity levels to mammal cells, and have minor environmental impact and an ever-growing acceptance and use by consumers. Their antimicrobial activity against bacteria and fungi, as well as against insects and nematodes, has been reported as a result of the presence of bioactive chemical substances, which are often used as fragrances and flavorings for food and drinks [9]. In this context, the antioxidant and antimicrobial properties of the plants of the family Lamiaceae—such as *Origanum vulgare* (oregano), *Origanum majorana* (marjoram), *Mentha piperita* (peppermint) and *Rosmarinus officinalis* (rosemary) [10]—are highlighted.

The development of research on phytopathogenic *Pythium* species has shown that these oomycetes are susceptible to different essential oils, among which *O. vulgare* and *R. officinalis* [9]. Susceptibility tests using plant essential oils against *P. insidiosum* have been rarely reported in literature [11].

The present study aimed to determine the chemical composition as well as to evaluate the in vitro antimicrobial action of *O. vulgare*, *O. majorana*, *M. piperita* and *R. officinalis* essential oils against Brazilian *P. insidiosum* isolates.

## Materials and Methods

### Extraction and Chromatographic Analysis of Essential Oils

*Origanum vulgare* (oregano), *O. majorana* (marjoram), *M. piperita* (peppermint) and *R. officinalis* (rosemary) essential oils were purchased from Ferquima Inc. and Com. Ltd. All essential oils were analyzed by gas chromatography–mass spectrometry (GC-MS) at the LASOL-CCQFA (Center of Chemical, Pharmaceutical and Food Sciences of the Federal University of Pelotas) by using a Shimadzu CG/MS QP2010 machine with electron impact ionization (70 eV) with an RTX-5-fused silica capillary column (30 × 0.25 mm × 0.25 µm coated with 5 % phenyl-silicone, 95 % dimethylpolysiloxane and 0.25 µm film thickness). The oven temperature was programmed to increase 60–240 °C at a rate of 4 °C/min. using helium as carrier gas with a flow rate of 3 mL/min. The identification of essential oil components was performed by comparing their mass spectra with those stored in the NIST/EPA/NIH library and also by comparing the Kovats retention indices (KI) recommended in literature. Kovats indices for the oil components were determined in relation to (C8–20 and C10) n-alkanes using a solution of ethylene oxide dissolved in hexane.

### *Pythium insidiosum Isolates*

Twenty-two Brazilian *P. insidiosum* isolates were used for the susceptibility test, of which 21 isolates from clinical cases in horses and dogs in southern Rio Grande do Sul State, Brazil, and a CBS 101555 standard sample kindly provided by the Mycological Research Laboratory at the Federal University of Santa Maria. All samples used were identified by macro- and micromorphology and confirmed by PCR and DNA sequencing [12, 13].

### Inoculum Preparation

The inoculum for the susceptibility test was prepared as described by Pereira et al. [6].

### *In Vitro Susceptibility Test*

The test used the broth microdilution method based on the CLSI M38-A2 document [14] adapted for phyto-

pharmaceutical use [15]. *O vulgare, O. majorana, M. piperita* and *R. officinalis* oil dilution was done by homogenizing 0.45 g essential oil in 8 mL RPMI with the addition of 0.05 mL Tween 80. From this initial solution with a 56 mg/mL concentration, serial dilutions in RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS, ranging between 14 and 0.025 mg/mL, were performed. One hundred milliliter of each essential oil dilution added with an equal volume of inoculum was added to each microdilution plate well. A positive control (100 µL RPMI and 100 µL inoculums) and a negative control (100 µL RPMI and 100 µL essential oil dilution) were determined for all samples. All tests were performed in triplicate. The reading of results was done at 48 h of incubation, and the visual presence or absence of hyphae was taken into consideration. The smallest essential oil concentration that was able to inhibit microorganism growth in the positive control was considered the minimal inhibitory concentration (MIC). The minimum fungicidal concentration (MFC) was determined by transferring 0.1 mL essential oil concentration aliquot equal to or higher than the MIC standard for test tubes containing 0.9 mL Sabouraud broth. After a 48 h incubation period at 37 °C, the smallest essential oil concentration that did not show any growth was considered the MFC.

## Results

The chromatographic analysis by GC-MS of the essential oils evaluated is described in Table 1. The major component of *O. vulgare* essential oil was found to be carvacrol (93.1 %), whereas the total constituents of *O. majorana* oil that amounted to 63.24 % of its composition were 4-terpineol (34.34 %), *trans*-sabinene hydrate (15.29 %) and γ-terpinen (13.62 %). *R. officinalis* essential oil showed 64.53 % 1.8 cineole, 14.56 % camphor and 12.26 % α-pinene concentrations, corresponding to 95.35 % of total constituents. The chemical compounds found in *M. piperita* which accounted for 81.41 % of total constituents were menthone (57.53 %) and 1.8-cineole (23.88 %).

The MIC of the 22 *P. insidiosum* isolates is described in Table 2. The results obtained revealed that *O. vulgare* essential oil presented the best in vitro antimicrobial activity against oomycete isolates, with MICs ranging from 0.05 to 1.75 mg/mL; furthermore, MIC50 was 0.22 mg/mL, and MIC90 was 0.87 mg/mL for this essential oil. MICs were higher for the other oils tested, with values ranging from 0.05 to 3.5 mg/mL; MIC50 and MIC90, in turn, showed values between 0.44 and 3.5 mg/mL, respectively (Table 2). Minimal fungicidal concentrations were found to be the same as the MIC for all essential oil evaluated in the study (data not shown).

**Table 1** Chromatographic analysis of *Origanum vulgare, Origanum majorana, Rosmarinus officinalis* and *Mentha piperita* essential oils

| Component | *O. vulgare* | *O. majorana* | *R. officinalis* | *M. piperita* |
|---|---|---|---|---|
| *p*-Cymene | 1.78 | | | |
| γ-Terpinene | 2.01 | 13.62 | | |
| Carvacrol | 93.10 | | | |
| β-Caryophyllene | 3.11 | | | 2.65 |
| α-Felantreno | | 0.22 | | |
| α-Pinene | | 0.41 | 12.26 | 0.97 |
| Sabinene | | 6.29 | | |
| Myrcene | | 0.45 | | |
| α-Terpinene | | 7.09 | | |
| *o*-Cymene | | 2.88 | | |
| Limonene | | 3.15 | | |
| α-Terpinolene | | 2.23 | | |
| *Trans*-sabinene hydrate | | 15.29 | | |
| β-Linalool | | 1.65 | | |
| 4-Terpineol | | 34.34 | | |
| Caryophyllene | | 3.21 | | |
| Ethyl linalool | | 4.19 | | |
| Camphene | | | 1.24 | |
| β-Pinene | | | 5.83 | 3.81 |
| 1,8-Cineole | | | 64.53 | 23.88 |
| Camphor | | | 14.56 | |
| Verbenone | | | 1.64 | |
| Menthol | | | | 3.51 |
| Piperitone | | | | 0.95 |
| Menthone | | | | 57.53 |
| Methyl acetate | | | | 6.71 |

The concentration of each compound is expressed as a percentage

## Discussion

The antimicrobial activity of essential oils of plants of the genus *Origanum* is associated with its chemical components, especially monoterpenes such as

**Table 2** In vitro susceptibility of *Pythium insidiosum* ($n = 22$) against *Origanum vulgare, Origanum majorana, Mentha piperita* and *Rosmarinus officinalis* essential oils

| Essential oils | No. of isolates (%) with the indicated MIC (mg/L) | | | | | | | MIC50[a] | MIC90[b] |
|---|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0.11 | 0.22 | 0.44 | 0.87 | 1.75 | 3.5 | | |
| *O. vulgare* | 2 (9 %) | 5 (22.5 %) | 8 (36 %) | 3 (13.5 %) | 1 (4.5 %) | 3 (13.5 %) | 0 | 0.22 | 0.87 |
| *O. majorama* | 1 (4.5 %) | 3 (13.5 %) | 3 (13.5 %) | 6 (27 %) | 2 (9 %) | 3 (13.5 %) | 4 (18 %) | 0.44 | 3.5 |
| *M. Piperita* | 0 | 2 (9 %) | 4 (18 %) | 5 (23 %) | 6 (27 %) | 2 (9 %) | 3 (14 %) | 0.44 | 3.5 |
| *R. officinalis* | 0 | 2 (9 %) | 3 (14 %) | 7 (31 %) | 3 (14 %) | 2 (9 %) | 5 (23 %) | 0.44 | 3.5 |

[a] Minimal concentration to inhibit the growth of 50 % of isolates

[b] Minimal concentration to inhibit the growth of 90 % of isolates

carvacrol, thymol, 4-terpineol and linalool [16, 17]. The *O. vulgare* and *O. majorana* essential oils used in this study included carvacrol and 4-terpineol as their major chemical components, which is in agreement with other studies in literature. The antimicrobial activity of *M. piperita* essential oil is attributed to its monoterpene components, especially menthol and menthone [18, 19]. In this study, the *M. piperita* oil used showed 57.53 % menthone and 23.88 % 1.8-cineole as major components, followed by a lower menthol concentration [3.51 %]. These concentrations partly differ from those found in previous studies, which have demonstrated that some of these components can show either higher or lower concentrations according to the origin and physiological condition of the plant [18, 19].

The antimicrobial activity of *R. officinalis* essential oil results from the presence of 1.8 cineole, camphor, verbenone, α-pinene and borneol; among these, borneol has been reported as showing the greatest activity, followed by camphor and verbenone [20]. In the present study, this oil showed 64.53 % 1.8-cineole as a major component. These values are different from those found by Ganchkar et al. [20], who found piperitone to be the major component of this oil (23.7 %). However, variations between 70 and 98 % in terpene levels can be observed in essential oil total composition [10]. The in vitro susceptibility results revealed that the *O. vulgare*, *O. Majorana*, *M. piperita* and *R. officinalis* essential oils showed antimicrobial activity against the 22 *P. insidiosum* isolates tested. *O. vulgare* presented the best antimicrobial activity against this oomycete. Previous studies have demonstrated the microorganisms of the Oomycota class are susceptible to essential oil action. Fonseca [11], upon testing the antimicrobial activity of *O. vulgare* and *R. officinalis* essential oils against eight *P. insidiosum* isolates, obtained the best antimicrobial activity with the *O. vulgare* oil, with MICs similar to those reported by this study. Similarly, Wogiatzi et al. [21] demonstrated the *Pythium* spp. susceptibility to *O. vulgare* essential oil. Lee et al. [9], on evaluating the activity of 39 essential oils, among which *O. vulgare*, observed that the oregano oil showed the greatest activity against *Pythium ultimum*. The same authors did not detect any *M. piperita* and *R. officinalis* action against the oomycete under evaluation. In this study, *P. insidiosum* was susceptible to these evaluated oils. In agreement to the results of this study, Klimach and Wieczorek [22] stated that *M. piperita* oil had the ability to inhibit the growth of phytopathogenic species of *Pythium* spp. The antimicrobial activity of essential oils is also evidenced on other oomycete genera, as demonstrated by Soylu et al. [16] upon reporting the action of *Origanum syacum bevanii* var. and *R. officinalis* essential oils against *Phytophthora infestans*.

The antimicrobial activity of essential oils on other microorganisms, such as true fungi and bacteria, can also be pointed out [23]. RAO et al. [24] suggested that the activity of essential oils on microorganisms may be due to alterations in the permeability of the cytoplasmic membrane which induce changes in hydrogen, potassium and calcium ion gradient, thus causing the deterioration of essential processes for cell survival, such as electron and protein transport and interference in the phosphorylation process. Studies evaluating the antimicrobial action of *O. vulgare, O. majorana, M. piperita* and *R. officinalis* essential oils against filamentous fungi [11, 19, 25–27] and yeast [15, 18, 28] have proved that these oils have antifungal activity, which can vary according to the genus and species of the evaluated fungus.

## Conclusion

The present study highlights the promising antimicrobial activity of *O. vulgare, O. majorana, M. piperita* and *R. officinalis* essential oils against the *P. insidiosum* oomycete. The potential use of plant essential oils as assisting agents in the control of these important microorganisms is also emphasized. However, in vivo studies are needed so as to evaluate essential oil use in animal pythiosis.

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**Conflict of interest** None of the authors of this manuscript has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## 3.3 Artigo 3

**In vitro suscetibility of Brazilian *Pythium insidiosum* isolates to essential oils of some Lamiaceae Family species**

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**Submetido à revista Veterinary Microbiology**

Treatment of experimental pythiosis with essential oils of *Origanum vulgare* and *Mentha piperita* singly, in association and in combination with immunotherapy (Submetido ao periódico Veterinary Microbiology)

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**Abstract**

This study investigated the *in vivo* antimicrobial activity of the essential oils of *Origanum vulgare* and *Mentha piperita* both singly, associated and in combination with immunotherapy to treat experimental pythiosis. The disease was reproduced in 18 rabbits divided into six groups (n=3): group 1, control; group 2, treated with essential oil of *Mentha piperita*; group 3, treated with essential oil of *Origanum vulgare*; group 4, treated with commercial immunotherapic; group 5, treated with a association of oils of *Mentha piperita* and *Origanum vulgare* and group 6, treated with a combination of both oils plus immunotherapy. Essential oils were added in a topical cream base formula, and lesions were treated daily for 45 days. The animals in groups 4 and 6 received a dose of immunotherapeutic agent every 14 days. The results revealed that the evolution of lesions in groups 5 and 6 did not differ from one another but differed from the other groups. In addition, the results showed that the lesions of group 5 increased 3.16 times every measurement, while those of group 6 increased 1.83 times, indicating that the smallest growth of the lesions occurred when the combination of therapies were used. Additionally, a rabbit from group 5 showed clinical cure at day 20 of treatment. This research is the pioneer in the treatment of experimental pythiosis using essential oils from medicinal plants and a combination of therapies. This study demonstrated that the use of essential oils can be a viable alternative treatment to cutaneous pythiosis, particularly when used in association or in combination with immunotherapy.

**Keywords:** oomycete, *Pythium insidiosum*, oregano, peppermint, immunotherapeutic, rabbits.

## 1. Introduction

*Pythium insidiosum* is an aquatic oomycete and single species of the genus *Pythium* that is pathogenic to mammals (Gaastra et al., 2010). The Stramenopila kingdom comprises a large number of autotrophic and heterotrophic organisms with high ecological importance, such as photosynthetic algae and oomycetes (fungal-like) parasites (Gaulin et al., 2010). As a typical oomycete, *P. insidiosum* inhabits freshwater ecosystems and terrestrial environments and lacks ergosterol in its cytoplasmic membrane (Gaulin et al., 2010). This oomycete causes pythiosis, an infectious disease that is difficult to treat and has poor prognosis in mammals. The affected animals commonly acquire the disease after long periods of contact with stagnant water, where *P. insidiosum* carries out its reproductive cycle (Gaastra et al., 2010). The most frequently affected species are equine, canine and humans, especially those that inhabit regions that are tropical, subtropical or temperate; particularly in marshy and flooded areas. However, other domestic and wild mammals and birds can also be infected by this oomycete. Depending on the species, pythiosis can manifest in cutaneous, gastrointestinal, ophthalmic or systemic form (Gaastra et al., 2010).

Different therapeutic protocols that have been used for the treatment of pythiosis in animals and humans include surgical methods, immunotherapy and antifungal agents, or sometimes a combination of these treatment types (Shenep et al., 1998, Mendoza et al., 2003, Pereira et al., 2013). However, the results may vary considerably (Shenep et al, 1998, Mendoza et al., 2003; Wanachiwanawin et al., 2004., Pereira et al., 2010, Pereira et al., 2013). Due to the need of effective therapeutic options for pythiosis, many investigations have sought alternative therapies with or without antifungal agents. (Pereira et al., 2007, Argenta et al., 2012, Zanette et al., 2014, Jesus et al., 2014a). Among these investigations are *in vitro* studies that evaluate the antimicrobial activity of essential oils (Jesus et al, 2014b, Fonseca et al, 2014), as well as compounds extracted from plants, including root and fruits (Shriphana et al., 2013; Suthiwong et al. 2014). All of these studies show promising results for the treatment of *P. insidiosum in vitro*. Additionally, the use of essential oils in antimicrobial therapies can be an advantageous method as it presents a low toxicity to mammalian cells and a low environmental impact (Fonseca et al, 2014).

The aim of this study was to perform an *in vivo* study to evaluate the therapeutic activity of essential oils from *Origanum vulgare* (oregano) and *Mentha piperita*

(peppermint) both singly, in association, and with combined immunotherapy to treat experimental pythiosis in rabbits.

## 2. Material and methods

### *2.1. Acquisition of essential oils and pharmaceutical formulation of topical product with essential oils*

The essential oils of *O. vulgare* (oregano) and *M. piperita* (peppermint) were obtained commercially from Ferquima Inc. and Com. Ltda, respectively, together with a certificate of purity. Chromatography of the components was previously determined by Fonseca et al. (2014).

To treat the animals, essential oils were formulated in a topical cream with a non-ionic base that was employed using four formulations: cream non-ionic base, cream base with essential oil of *M. piperita* at a concentration of 7 mg/g, cream base with essential oil of *O. vulgare* at a concentration of 7 mg/g, and cream base with an association of both oils at concentrations of 3.5 mg/g per oil.

### *2.2. Animal model*

Initially, to perform all of the *in vivo* tests, pythiosis was experimentally reproduced in 18 female, 90 day-old New Zealand rabbits allocated into six groups (n=3): group 1 (control-cream nonionic base); group 2 (essential oil of *M. piperita*); group 3 (essential oil of *O. vulgare*); group 4 (commercial immunotherapeutic agent); group 5 (association of *M. piperita* and *O. vulgare* essential oils), and group 6 (a combination of both essential oils *M. piperita* and *O. vulgare* plus immunotherapy). All of the animals were inoculated subcutaneously in the right costal region with 1 mL of induction medium containing approximately 20,000 viable zoospores of *P. insidiosum* (CBS 101555) as previously described by Pereira et al. (2007). The animals were kept in individual cages under appropriate conditions of hygiene, light and temperature, receiving water *ad libitum* and food according to body weight. Inspection to assess the growth of lesions was performed daily. After the development of the injuries, the lesions were measured, and the animals were separated into groups. The animals of all groups were treated daily by topical administration of creams on the surface of the lesions. Additionally, the animals in groups 4 and 6 received 2 mL of a commercial immunotherapeutic agent (Pitium-Vac®) in the left costal area by subcutaneous inoculation, every 14 days for a period of 45 days. The evolution of the lesions was measured every 5 days in the horizontal and transverse directions ($cm^2$) using a pachymeter. At the end of the experiment, the animals were submitted to necropsy and representative fragments of subcutaneous lesions were collected, fixed in 10%

formalin, and routinely processed for histopathological analysis. They were then stained with Hematoxylin-Eosin (HE) and Grocott's Methenamine Silver (GMS) stain.

All of the procedures involving animals were previously approved by the Ethics Committee on Animal Experiments of Universidade Federal de Pelotas (Federal University of Pelotas), with protocol number 0475.

## 2.3. Statistical Analysis

The skin lesions of the animals of each group were measured every five days. After each measurement, the averages were calculated for each group, and the areas of injury were converted to percentages using the methodology previously described by Pereira et al. (2007). The data were submitted for normality and homoscedasticity tests. When these assumptions were satisfied, an analysis of variance and F test were performed using a 5% significance level. Responses were also modeled according to the data of the measurements for each treatment using polynomial function up to the third order. In the regression analysis, the choice of the models was based on the significance of the linear and quadratic coefficients using the Student’s "t" test at 5% probability. The linear regression equations obtained for the different treatments were compared using the contrast test among the regression coefficients. The analyses were performed using the SAS statistical software, version 9.4.

## 3. Results

The rabbits developed subcutaneous nodules 25 days after the zoospores inoculation. The areas of the lesions ranged from 0.25 $cm^2$ to 132.2 $cm^2$ at the end of the experiment. An animal from group 5 showed clinical cure 20 days after starting treatment.

Statistical analysis revealed that the rabbits' lesions from the groups treated with the association of *O. vulgare* and *M. piperita* essential oils (group 5), and combination both of the essential oils plus immunotherapy (group 6) did not differ ($P>0.05$); however, these groups differed from the remaining groups ($P<0.05$). It was also observed that the animals from group 1 (control), group 2 (treatment with *M. piperita*) and group 4 (treatment with immunotherapeutic agent) did not differ from one another ($P>0.05$) (Table 1). Regression analysis of the lesions' dimensions (Figure 1) demonstrated that in group 3 (treatment with *O. vulgare*), the treatment was adjusted to a quadratic equation, with the smallest lesion measured 13 days after starting treatment. The treatments of groups 1, 2, 4, 5, and 6 were adjusted for linear regression models (increased manner) (Figure 1, Table 1). It was observed that in group 5 (rabbits treated with both oils in association) the lesions increased 3.16 times every measurement and in group 6 (rabbits treated with both oils combined with immunotherapy) the lesions increased 1.83 times (Table 1). These results indicate the small growth of the lesions following treatment with association and/or combination therapies.

The subcutaneous lesions cut surfaces were multilobulated, hard, white-pinkish and vascularized. The histopathology of the lesions stained with HE showed the presence of multifocal to coalescent necrotic areas delimited by inflammatory infiltrates predominantly constituted by eosinophils. Hyphae-like structures surrounded by irregular, eosinophilic material could be observed in the necrotic areas (Splendore-Hoeppli reactions). Some of these reactive areas were delimited by Langhans' giant cells; some of these cells had hyphae in their inner regions. There was also intense proliferation of fibrous connective tissue cells and the presence of eosinophils, plasma cells, lymphocytes, macrophages, unidentified giant cells and epithelioid cells. Irregularly ramified, scarcely septate hyphae with thick brown walls were visualized with GMS staining, and these structures were preferably located in the periphery of necrotic areas. Although the histological appearance of lesions was similar in all experimental groups as noted by GMS, the distribution of hyphae varied among all of

the groups. It was observed that in the lesions of the rabbits from the control and *M. piperita*-treated groups, hyphae became disseminated by the lesion. However, in the lesions of the rabbits in treatment groups 3, 4, 5, and 6, hyphae of *P. insidiosum* were only observed into the granulomas and giant cells.

## 4. Discussion

The failures of different treatment protocols for pythiosis, as well as the low *in vitro* antifungal susceptibility, present difficult choices for therapeutic treatment of the disease (Gaastra et al., 2010). These challenges motivate the search for new alternative therapies and/or combination of therapeutic protocols. Therefore, both *in vitro* and *in vivo* studies using new antifungal and antimicrobial drugs groups have been evaluated as alternative treatments of pythiosis (Pereira et al., 2007; Argenta et al ., 2012; Zanette et al., 2014; Jesus et al., 2014a). Additionally, *in vitro* research showed that essential oils (Fonseca et al., 2014; Jesus et al., 2014b), compounds extracted from root and fruits (Shriphana et al., 2013; Suthiwong et al. 2014), were capable of inhibiting the growth of *P. insidiosum*, and they are being proposed as promising new therapies.

The antimicrobial activity of essential oils against *P. insidiosum* can be attributed to the mechanism of action of these compounds including alteration of the cytoplasmic membrane permeability and changes in the gradient of hydrogen ions, potassium and calcium, which causes damage to essential processes for cell survival such as electron transport, protein transport and interference in phosphorylation (Rao et al., 2010). The antimicrobial action of oils obtained from plants has been known for a long time, and their activities are being reported, in particular, to have an *in vitro* effect against bacteria, true fungi, oomycetes, viruses, and insects (Lee et al., 2007; Koul et al., 2008; Fonseca et al., 2014; Jesus et al., 2014b). However, *in vivo* experiments to test the antimicrobial activity of essential oils of plants are rarely described.

The present study evaluated the action of essential oils from *O. vulgare* and *M. piperita* in an experimental model of pythiosis that was designed from results found in the previous study of *in vitro* susceptibility of Brazilian *P. insidiosum* against the essential oils from the Lamiaceae family plants developed by Fonseca et al. (2014). Those authors showed that the essential oils of *O. vulgare* and *M. piperita* demonstrated a MIC90 of 0.87 mg/ml and 3.5 mg/ml, respectively. Therefore, these results were selected for the treatment of experimental pythiosis in the current research. The effects of this *in vivo* study showed that the use of topical formulation of essential oils as monotherapy had little or no action on the evolution of the disease (Figure 1). These findings show a weak correlation with the *in vitro* results obtained by Fonseca et al. (2014). However, the lack of correlation between the *in vitro* and *in vivo* studies was previously reported by Pereira et al. (2007) and evaluated the

susceptibility of *P. insidiosum* to caspofungin. Afterward, Argenta et al. (2012) employed a different combination of therapies, including terbinafine, itraconazole, caspofungin, ibuprofen, and fluvastatin against *P. insidiosum*.

Conversely, when the therapeutic protocol employed an association of *O. vulgare* and *M. piperita* oils in a topical formulation (group 5) or when used in a combination of immunotherapy plus both oils (group 6), the therapeutic action was higher (Figure 1), including clinical cure of an animal from the group treated with the association of both oils. Recently, Jesus et al. (2014b) suggested that combinations of essential oils of thymol and carvacrol plus antimicrobial agents may be an alternative for treatment of cutaneous pythiosis because it had a synergistic effect *in vitro*. However, these authors did not perform *in vivo* studies with such combinations.

In therapeutic protocols with association of essential oils, similar results were demonstrated by Mugnaini et al. (2012, 2013) to obtain clinical cure of cats and sheep infected with *Trichophyton mentagrophytes* and *Microsporum canis*, respectively, using an essential oil combination of *O. vulgare*, *Rosmarimus officinalis* and *Thymus serpyllum*. Additionally, the use of an association of essential oils from *M. piperita* and *Satureja hortensis* was effective for treating an experimental murine model of cutaneous protothecose (Bouari et al., 2014).

The smallest evolution of lesions in those animals treated with an association and/or combination therapies (rabbits of groups 5 and 6) corroborates the reports of combined therapies that have been proven effective in the treatment of pythiosis in animals and humans (Shenep et al, 1998, Sudjaritruk et al., 2011, Argenta et al., 2012, Pereira et al., 2013). Previously, Gaastra et al. (2010) and Pereira et al. (2013) reported that a combination of different therapies for pythiosis improved the curative effects more effectively than monotherapies.

The present report is a pioneer study involving the treatment of experimental pythiosis in rabbits using essential oils from plants and assessing the association and/or combination of therapies with essential oils against pythiosis. Our results revealed that treatments that associated the essential oils, as well as the protocol combining both oils plus immunotherapy were more effective when compared to the isolated therapies. It is suggested that the use of essential oils can constitute a good alternative to treat cutaneous pythiosis in animals, particularly when used in association of oils and/or in combination with other antimicrobial agents or immunotherapy.

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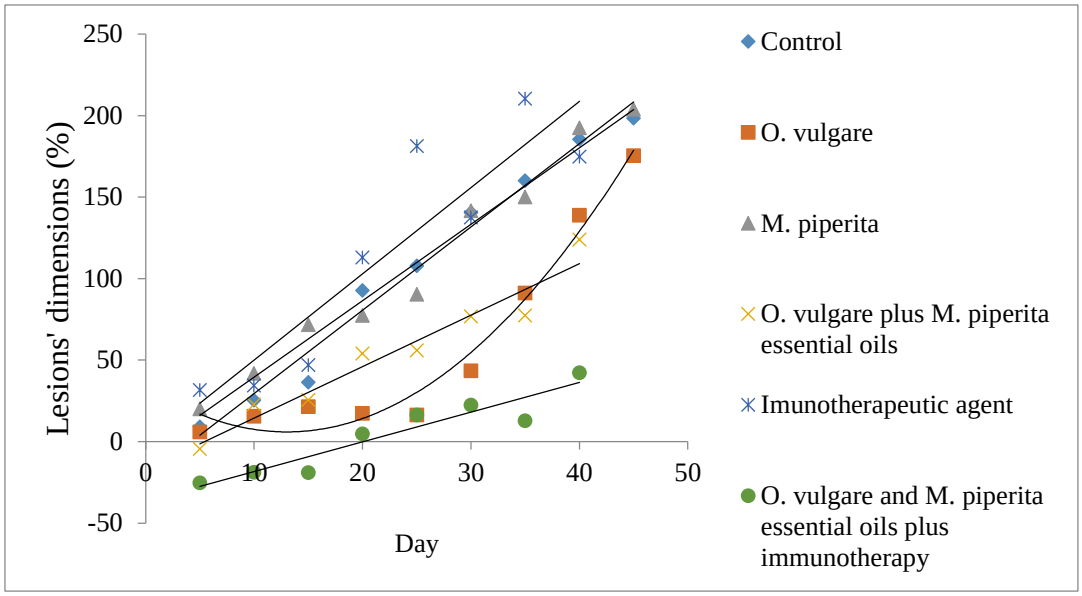


Figure 1: Percent variation of the lesions' dimensions in rabbits experimentally inoculated with zoospores of *Pythium insidiosum* and treated with essential oils from *Origanum vulgare*, *Mentha piperita* singly, both in association and in combination with immunotherapy.

Table 1: Regression equations for the lesions' dimensions in each of the evaluation days for each group of rabbits inoculated experimentally with zoospores of *Pythium insidiosum* and treated with essential oils of *Origanum vulgare*, *Mentha piperita* singly, both in association and in combination with immunotherapy.

| Treatment | Equation | $R^{2*}$ | P** |
|---|---|---|---|
| Group 1 (Control) | $\hat{Y} = -21.543 + 5.110x$ | 98.17% | <0.0001 |
| Group 2 (*M. piperita*) | $\hat{Y} = -7.378 + 4.693x$ | 97.57% | <0.0001 |
| Group 3 (*O. vulgare*) | $\hat{Y} = 34.57 - 4.398x + 0.168x^2$ | 97.24% | <0.0001 |
| Group 4 (Immunotherapeutic agent) | $\hat{Y} = -2.845 + 5.293x$ | 82.46% | 0.0018 |
| Group 5 (Association of *O. vulgare* and *M. piperita* essential oils) | $\hat{Y} = -17.153 + 3.162x$ | 93.95% | <0.0001 |
| Group 6 (Combination of *O. vulgare* and *M. piperita* essential oils plus immunotherapy) | $\hat{Y} = -36.730 + 1.828x$ | 88.90% | 0.0004 |

*Regression coefficient; ** Probability.

## 4 Considerações Finais

Com base nos resultados obtidos neste estudo é possível concluir:

- O inoculo obtido a partir de cultivo micelial de *P. insidiosum* é uma metodologia viável para testes de suscetibilidade *in vitro* deste oomiceto, sendo menos laborioso e consumindo menos tempo na sua preparação, quando comparado ao inóculo padrão. Neste estudo, observou-se que os valores de concentação inibitória mínima obtidos com o inóculo proposto foram comparáveis àqueles obtidos com o inóculo padrão, verificando-se que em 80,8% das comparações de MICs os valores foram iguais ou tiveram apenas uma diluição de diferença em ambos os inóculos.

- A avaliação da composição química dos óleos essenciais empregados no presente estudo revelou que os componentes majoritários de *O. vulgare* foram: carvacrol (71,03%) e gama-terpineno (4,51%); de *O. majorana:* 1,4-terpineol (23,07%) e trans sabineno (16,28%); de *M. piperita:* mentona (46,7%) e 1,8-cineol (13,3%) e de R. *officinalis:* 1,8-cineol (45,8%) e cânfora (12,9%).

- O estudo que avaliou a suscetibilidade *in vitro* de *P. insidiosum* aos óleos essenciais de *O. vulgare*, *O. majorana*, *M. piperita* e *R. officinalis* revelou que os óleos essenciais avaliados apresentam ação antimicrobiana sobre *P. insidiosum* e ressalta a melhor atividade do óleo essencial de *O. vulgare*. Aponta-se este óleo como um potencial agente antimicrobiano para o tratamento da pitiose.

- O experimento *in vivo* revelou que os animais que foram tratados com as terapias associadas (associação de óleos essenciais de *O. vulgare* e *M. piperita* e associação de óleos essenciais *plus* imunoterápico) apresentaram menor crescimento das lesões. Sugere-se o emprego de óleos essenciais como uma alternativa viável de tratamento da pitiose cutânea, particularmente quando utilizados em combinação ou em associação com imunoterapia ou outros fármacos antimicrobianos.

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# Anexos

UFPel

Comissão de Ética em Experimentação Animal

Pelotas, 20 de fevereiro de 2013

**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 projeto intitulado: **"Susceptibilidade *in vitro* e *in vivo* do oomiceto *Pythium insidiosum* a óleos essenciais"**, processo nº23110.000475/2013-87, 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.

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

Salientamos também a necessidade deste projeto ser cadastrado junto ao Departamento de Pesquisa e Iniciação Científica para posterior registro no *COCEPE* (código para cadastro nº **CEEA 0475**).

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

Atenciosamente,

**Prof. Dr. Éverton Fagonde da Silva**

*Presidente da CEEA*

Ciente em: 20/02/2013

Assinatura do Professor Responsável:

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