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Carlos Garrido Pinheiro

Hesperozygis ringens (Benth.) Epling: CARACTERIZAÇÃO DOS ÓRGÃOS VEGETATIVOS, ANÁLISE HISTOQUÍMICA E ATIVIDADE ANTIFÚNGICA DO ÓLEO ESSENCIAL

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Engenharia Florestal, Área de Concentração em Silvicultura, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Engenharia Florestal.**

Orientadora: Prof[a]. Dr[a]. Berta Maria Heinzmann

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Não se deve ir atrás de objetivos fáceis. É preciso buscar o que só pode ser alcançado por meio dos maiores esforços.

(Albert Einstein)

RESUMO

Hesperozygis ringens (Benth.) Epling: CARACTERIZAÇÃO DOS ÓRGÃOS VEGETATIVOS, ANÁLISE HISTOQUÍMICA E ATIVIDADE ANTIFÚNGICA DO ÓLEO ESSENCIAL

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ORIENTADORA: Berta Maria Heinzmann

Lamiaceae se destaca por apresentar espécies com potencial para produção de óleos essenciais (OEs) e, entre elas, encontra-se *Hesperozygis ringens* (Benth.) Epling. Além de estudos envolvendo a produção de um óleo essencial (OE) e suas possíveis bioatividades, é fundamental o conhecimento sobre a caracterização estrutural da espécie, bem como as classes de constituintes produzidas pela mesma. Portanto, esse estudo teve como objetivos: caracterizar estruturalmente os órgãos vegetativos de *H. ringens*; detectar as classes de constituintes produzidas; otimizar um método para extração de ergosterol de fungos apodrecedores da madeira; avaliar o potencial antifúngico do OE de folhas e seu constituinte majoritário frente aos mesmos fungos. Foram coletadas folhas, inflorescências, caules e raízes. A caracterização estrutural ocorreu por análise microscópica de materiais incluído e fresco. Folhas e pétalas foram utilizadas para testes histoquímicos visando detectar as classes de constituintes disponíveis. Os OEs de folhas e inflorescências foram extraídos e descritos quimicamente. Para os bioensaios, foram selecionados os fungos de podridão-branca *Ganoderma applanatum* e *Trametes versicolor*, e os de podridão-parda *Gloeophyllum trabeum* e *Lentinus lepideus*. Os fungos foram cultivados em placas de Petri contendo meio de cultura coberto por membrana de celofane. O micélio de cada fungo foi coletado para verificação da massa úmida, sendo na sequência transferido para tubo de ensaio, onde foram adicionados hidróxido de potássio alcoólico a 25%, água destilada e *n*-heptano. As camadas de *n*-heptano foram coletadas e analisadas por espectrofotometria. Após extração e análise química do OE de folhas, a pulegona foi isolada através de coluna cromatográfica. OE e pulegona foram diluídos em etanol e testados nas concentrações de 0,5; 0,75; 1,00; 1,25 e 1,50 µL mL^{-1} (OE) e 0,35; 0,71 e 1,05 µL mL^{-1} (pulegona). Também foram considerados controles negativo e positivo, avaliando-se: inibição de crescimento micelial e índice de velocidade de crescimento micelial (OE) e inibição de crescimento micelial (pulegona). A influência do OE e pulegona sobre a concentração do ergosterol nos fungos foi analisada. A caracterização de órgãos vegetativos evidenciou a presença de tricomas tectores e glandulares do tipo peltado em folha e caule. Na raiz foram encontrados idioblastos fenólicos. Dois tipos de cristais foram detectados: inulina em folha e caule; hesperidina em raiz. Através dos testes histoquímicos foram detectadas as seguintes classes: lipídios totais, ácidos e neutros; OEs; oleorresinas; proteínas; compostos fenólicos; flavonoides; alcaloides. Os OEs extraídos de folhas e inflorescências apresentaram a pulegona como constituinte majoritário. As quantidades de ergosterol calculadas demonstraram que o método otimizado para extração de ergosterol foi bem sucedido. Os resultados de atividade antifúngica para o OE e a pulegona evidenciaram o efeito fungistático do extrativo sobre *T. versicolor*. A pulegona apresentou efeito fungistático sobre *G. applanatum* e fungicida sobre *T. versicolor*. O OE e a pulegona interferiram nas concentrações de ergosterol dos fungos de podridão-branca. Os resultados encontrados evidenciaram o potencial do OE de *H. ringens* e da pulegona para desenvolvimento de pesticidas botânicos. Os resultados descritos podem servir de estímulo para pesquisas objetivando verificar outras bioatividades das classes de constituintes produzidas pela planta.

Palavras-chave: Anatomia vegetal. Testes histoquímicos. Pulegona. Monoterpenoide. Fungos apodrecedores da madeira. Ergosterol.

ABSTRACT

Hesperozygis ringens (Benth.) Epling: CHARACTERIZATION OF VEGETATIVE ORGANS, HISTOCHEMICAL ANALYSIS AND ANTIFUNGAL ACTIVITY OF THE ESSENTIAL OIL

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Lamiaceae stands out for presenting species with potential to produce essential oils (EOs), and among them, *Hesperozygis ringens* (Benth.) Epling is classified. In addition to studies about the production of an essential oil (EO) and its possible bioactivities, the knowledge about the structural characterization of the species is fundamental, as well as the classes of constituents produced by the same. Therefore, this study had the following objectives: to characterize structurally the vegetative organs of *H. ringens*; to detect the classes of constituents produced; to optimize a method for extracting ergosterol from wood rot fungi; to evaluate the antifungal potential of the leaves EO and its major constituent against the same fungi. Leaves, inflorescences, stems and roots were collected. The structural characterization occurred by microscopic analysis of included and fresh material. Leaves and petals were used for histochemical testes aiming to detect the available classes of constituents. Leaves and inflorescences EOs were extracted and chemically described. For the bioassays, the white-rot fungi *Ganoderma applanatum* and *Trametes versicolor*, and the brown-rot ones *Gloeophyllum trabeum* and *Lentinus lepideus* were selected. The fungi were cultivated in Petri plates containing culture medium covered by cellophane membrane. Mycelia of each fungus was collected to verify the wet weight and they were transferred to test tubes where 25% potassium hydroxide, distilled water and *n*-heptane were added. The *n*-heptane layers were collected and analyzed through spectrophotometry. After the EO extraction and chemical analysis, pulegone was isolated through column chromatography. EO and pulegone were diluted in ethanol and tested at concentrations of: 0.5, 0.75, 1.00, 1.25 and 1.50 µL mL^{-1} (EO) and 0.35, 0.71 and 1.05 µL mL^{-1} (pulegone). Negative and positive controls were also considered, evaluating: mycelial growth inhibition and mycelial growth rate (EO) and mycelial growth inhibition (pulegone). The influence of EO and pulegone on fungi ergosterol contents was analyzed. The characterization of vegetative organs evidenced the presence of non glandular and glandular trichomes of peltate type on leaf and stem. In root, phenolic idioblasts were found. Two types of crystals were detected in the plant: inulin in leaf and stem; hesperidin in root. By histochemical tests, the following classes were detected: total, acid and neutral lipids; EOs; oil-resins; proteins; phenolic compounds; flavonoids; and alkaloids. The EOs extracted from leaves and inflorescences presented pulegone as major constituent. The calculated ergosterol contents showed that the optimized method for ergosterol extraction was successful. The results of antifungal activity for EO and pulegone evidenced the fungistatic effect of the extractive against *T. versicolor*. Pulegone presented fungistatic effect against *G. applanatum* and fungicide effect against *T. versicolor*. The EO and pulegone interfered in ergosterol contents of the white-rot fungi. The results obtained evidenced the potential of *H. ringens* EO and pulegone for the development of botanical pesticides. The described results can serve as stimulus for research aiming to verify other bioacitivities of the constituent classes produced by the plant.

Keywords: Plant anatomy. Histochemical tests. Pulegone. Monoterpenoid. Wood-decay fungi. Ergosterol.

.

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1.1 INTRODUÇÃO

O Brasil possui uma vasta diversidade de espécies vegetais nativas com valor econômico (MÜGGE et al., 2016), e a utilização de seus recursos de maneira inadequada está ocasionando sua degradação (CANSIAN et al., 2010), prejudicando a sobrevivência desses organismos. Dentre os produtos que podem ser extraídos de diferentes órgãos das espécies vegetais, estão os óleos essenciais (OEs) (BIZZO; HOVELL; REZENDE, 2009), os quais ocorrem em um grande número de espécies nativas, sendo que o Brasil apresenta uma vasta disponibilidade de biomassa capaz de proporcionar sua produção (JACOB et al., 2017).

Considerando as espécies nativas com potencial para produção de óleo essencial (OE), *Hesperozygis ringens* (Benth.) Epling é uma excelente produtora deste extrativo. Segundo Von Poser et al. (1996), essa espécie é uma erva lenhosa, de nome popular ‘‘espanta-pulga’’ que ocorre em campos rochosos no sudeste do Rio Grande do Sul. Uma das características marcantes da espécie em seus locais de ocorrência é o odor mentolado liberado por suas partes aéreas. O óleo essencial (OE) da espécie apresenta como constituinte majoritário o monoterpenoide pulegona, encontrado em altas concentrações (PINHEIRO et al., 2016; PINHEIRO et al., 2017), substância caracterizada por apresentar diversas bioatividades descritas na literatura (BOŽOVIĆ; RAGNO, 2017; GONZÁLES-CHAVEZ et al., 2011; MUCCIARELLI et al., 2001; RIBEIRO et al., 2010; ROSSI; CANAVOSO; PALACIOS, 2012).

Além da descoberta de novas utilizações para o OE de *H. ringens*, é fundamental o conhecimento das características estruturais da espécie. A descrição das características dos órgãos vegetativos da planta fornece informações sobre folha, caule e raiz de uma representante de *Hesperozygis* Epling. Uma vez que pouco conhecimento foi encontrado acerca do gênero, os relatos sobre caracterização estrutural da espécie em estudo possivelmente auxiliarão na identificação da planta e na compreensão de sua sobrevivência nos locais de ocorrência. Apesar do OE de *H. ringens* ter sua composição química e variabilidade sazonal estudada (PINHEIRO et al., 2016), a literatura ainda carece de informações sobre as estruturas produtoras e secretoras de OE nos órgãos vegetais da espécie e do gênero ao qual ela pertence. O estudo sobre a anatomia das estruturas secretoras e de armazenagem do OE em *H. ringens* poderá proporcionar importantes informações, possibilitando a identificação e classificação dessas estruturas, as quais podem influenciar na

volatilização do extrativo. Essas informações, por sua vez, poderão auxiliar a escolha do processamento pós-colheita do material vegetal, com o objetivo de otimizar o rendimento desse extrativo. *Hesperozygis ringens* é reconhecida pela produção de OE em suas folhas (PINHEIRO et al., 2016; PINHEIRO et al., 2017; SILVA et al., 2014; TONI et al., 2014). A extração e análise do extrativo produzido pelas inflorescências da planta possibilitará a confirmação de que a espécie produz OE em outros órgãos vegetais. Adicionalmente, a realização de testes histoquímicos propiciará a detecção de outras substâncias derivadas do metabolismo secundário, biossintetizadas pela folha e pétalas da espécie.

As características e bioatividades relatadas para o OE de *H. ringens* (PINHEIRO et al., 2017; RIBEIRO et al., 2010; SILVA et al., 2014) sugerem a possibilidade desse extrativo ser utilizado para controle de pragas, com o intuito de substituir produtos sintéticos. Durante o controle de pragas com esses produtos, dois grandes problemas são considerados: a forma com que esses produtos são manipulados; e os possíveis resíduos prejudiciais liberados por eles (PINTO JÚNIOR et al., 2010). Por esses motivos, o desenvolvimento de novos agentes biológicos para o controle de pragas, de menor impacto ambiental e que atuem sem afetar significativamente seres humanos, animais, ecossistema, além de gerar produtos inovadores, de interesse para o mercado, pode levar à substituição dos praguicidas sintéticos. Como possibilidades aparecem os pesticidas botânicos, produtos biodegradáveis, que muitas vezes são desenvolvidos a partir de OEs (EL-WAKEIL, 2013). Segundo o autor, esses extrativos são compostos por diversos constituintes químicos e apresentam menor impacto ambiental quando comparados aos pesticidas convencionais.

Uma vez que os OEs apresentam um futuro promissor na área de biopesticidas (REGNAULT-ROGER; VINCENT; ARNASON, 2012), a avaliação de atividades biológicas como a antifúngica, de um extrativo e seu constituinte majoritário, possibilita a descoberta do real potencial do produto. A exploração de potenciais utilizações para o OE de *H. ringens* e seu componente majoritário, adicionada à propagação dessas informações, poderá incentivar pesquisas e programas de preservação e reprodução da espécie, uma vez que, devido à perda do habitat, *H. ringens* se encontra incluída na lista oficial das espécies ameaçadas de extinção (GOVERNO DO ESTADO DO RIO GRANDE DO SUL, 2014). A partir da necessidade de maiores informações a respeito das substâncias químicas produzidas pela espécie e a caracterização dos órgãos vegetativos da planta, bem como as possíveis aplicações para o seu OE, este estudo foi realizado.

A presente tese está estruturada na forma de quatro artigos científicos. O Artigo 1 caracteriza estruturalmente folha, caule e raiz de *H. ringens* a partir de amostras obtidas de

uma população em Santa Maria, Rio Grande do Sul, Brasil. As amostras foram desidratadas em série etílica, pré-infiltradas em metacrilato e etanol absoluto e infiltradas em metacrilato. A caracterização dos cristais encontrados na planta também é relatada nesse artigo. Secções dos órgãos vegetativos foram obtidas com auxílio de micrótomo e foram submetidas à ação de Azul de Toluidina. Os materiais foram analisados através de microscopia.

O Artigo 2 caracteriza quimicamente folha e pétala frescas de *H. ringens*, obtidas de uma população em Santa Maria, Rio Grande do Sul, Brasil. Testes histoquímicos foram realizados, visando detectar lipídios, OEs, resinas, oleorresinas, proteínas, compostos fenólicos, polissacarídeos, flavonoides, taninos e alcaloides. Além disso, foram analisados rendimentos e composições químicas dos OEs extraídos de folhas e inflorescências da espécie. O referido artigo foi publicado no periódico *Flora*. Na presente tese, o artigo é apresentado na sua formatação original, constante no periódico no qual foi publicado.

O Artigo 3 descreve a otimização de um método para a extração e quantificação do ergosterol presente na membrana plasmática dos fungos apodrecedores da madeira. Foram selecionadas as espécies de podridão-branca *Ganoderma applanatum* (Pers.) Pat. e *Trametes versicolor* (L.) Lloyd, e aos representantes de podridão-parda *Gloeophyllum trabeum* (Pers.) Murrill e *Lentinus lepideus* (Fr.) Fr.

O Artigo 4 analisa o potencial antifúngico do OE de folhas de *H. ringens*, coletadas de uma população encontrada em São Francisco de Assis, Rio Grande do Sul, Brasil, frente a fungos apodrecedores da madeira. As espécies analisadas no referido estudo são as mesmas utilizadas no Artigo 3. Adicionalmente, a composição química do extrativo utilizado é descrita, o constituinte majoritário foi isolado e testado contra as mesmas espécies fúngicas. Também foram avaliados os possíveis efeitos do OE e da substância sobre a concentração de ergosterol na membrana plasmática dos fungos apodrecedores da madeira.

Todos os trabalhos estão redigidos em inglês. A tese ainda apresenta o tópico Discussão, onde os resultados encontrados nos quatro artigos são interpretados, interligados e fundamentados a partir de argumentos obtidos na literatura. A tese é finalizada com os tópicos Conclusão e Referências.

1.2 REFERENCIAL TEÓRICO

1.2.1 Família Lamiaceae Martinov

A família Lamiaceae abrange 236 gêneros e, dentre os mais representativos, destacam-se *Salvia* L. e *Clerodendrum* L., com 900 e 500 espécies, respectivamente (HARLEY et al., 2004). Segundo Hedge (1992 apud HARLEY et al., 2004), a América do Sul é uma das regiões onde existe uma vasta diversidade de espécies dessa família. No Brasil, são encontrados representantes de gêneros como *Hesperozygis* Epling, *Scutellaria* L., *Ocimum* L., *Cunila* D. Royen, *Glechon* Spreng. e *Peltodon* Pohl (PEREIRA; PEREIRA, 1973).

Lamiaceae é conhecida pela importância econômica de suas espécies. *Tectona grandis* L. f, além de representantes de *Vitex* L. e *Gmelina* L., são conhecidas pelo valor comercial de suas madeiras (HARLEY et al., 2004). Segundo os mesmos autores, a família ainda apresenta muitos gêneros constituídos de espécies aromáticas utilizadas na culinária (*Salvia* L., *Mentha* L., *Thymus* L., *Origanum* L.), na produção de perfumes e produtos de higiene pessoal (*Lavandula* L.) e na elaboração de bebidas e chás (*Salvia*, *Mentha*, *Sideritis* L.). Lamiaceae ainda apresenta espécies utilizadas para fins medicinais como *Mentha pulegium* L. (poejo), *Ocimum americanum* L. (manjericão), *Ocimum gratissimum* L. (alfavaca) e *Rosmarinus officinalis* L. (alecrim) (AZEVEDO; SILVA, 2006).

Uma das características marcantes presente em espécies dessa família é o indumento composto por tricomas glandulares e tectores (HARLEY et al., 2004). Diversos gêneros são conhecidos pela presença de tricomas glandulares em suas espécies, podendo ser citados *Salvia*, *Mentha*, *Teucrium* L., *Origanum*, dentre outros (DINÇ et al., 2008; ERBANO et al., 2012; WERKER, 1993). Essas estruturas podem ser encontradas em diferentes órgãos vegetais como folha, caule e flor (HARATYM; WERYSZKO-CHMIELEWSKA, 2017; KREMER et al., 2014). Apesar da capacidade de biossintetizar uma vasta quantidade de constituintes, as representantes de Lamiaceae são conhecidas principalmente pela produção de OEs nessas estruturas glandulares (HARLEY et al., 2004).

Estudos realizados com a família demonstram o potencial de suas representantes para produção de OEs, como já relatado para *H. ringens*, *Lavandula dentata* L., *Origanum elongatum* E. & M., *Plectranthus grandidentatus* Gürke, e *Thymus satureioides* C. & B. (DRIS; TINE-DJEBBAR; SOLTANI, 2017; MOTA et al., 2013; PINHEIRO et al., 2016; RAMZI et al., 2017). Caracterizações químicas dos extrativos produzidos por espécies de Lamiaceae demonstram a presença de uma série de classes como hidrocarbonetos

monoterpênicos, monoterpenoides oxigenados, hidrocarbonetos sesquiterpênicos, entre outras (CUNHA et al., 2017; PINHEIRO et al., 2016; RICCI et al., 2005). A literatura ainda apresenta relatos de bioatividades já detectadas para os OEs da família, como acaricida (RAMZI et al., 2017), alelopática (PINHEIRO et al., 2017), anestésica (CUNHA et al., 2017), larvicida (DRIS; TINE-DJEBBAR; SOLTANI, 2017), inseticida (ÇALMAŞUR; ASLAN; ŞAHIN, 2006) e fungicida (BOULOGNE et al., 2012).

1.2.2 Gênero *Hesperozygis* Epling

Um dos gêneros pertencentes à família Lamiaceae, que apresenta espécies nativas com elevado potencial para produção de OE, é *Hesperozygis* Epling. Nesse gênero está classificada *Hesperozygis marifolia* Epling., descrita no México (CANTINO; SANDERS, 1986) e em cujo OE foram identificados como constituintes majoritários a (R)-pulegona, isomentona e mentona (GONZÁLES-CHAVEZ, 2011). No mesmo estudo, foi relatado que o extrativo apresenta atividade fungicida contra *Aspergilus flavus* Link.

O gênero ainda apresenta sete espécies nativas do sul e sudeste do Brasil (BRÄUCHLER; MEIMBERG; HEUBL, 2010; PEREIRA; PEREIRA, 1973). *Hesperozygis rhododon* Epling pode ser localizada no topo de montanhas, em São Paulo e Paraná, na formação da Serra do Mar (VON POSER et al., 1996). A composição química do OE da espécie, descrita pelos autores, apresenta como constituintes majoritários mentona e pulegona. No mesmo estudo ainda foi possível constatar que o extrato alcoólico da planta possui potencial alelopático frente a sementes de alface.

Outra espécie do gênero presente no Brasil, que teve seu OE estudado, é *Hesperozygis myrtoides* (St. Hill ex Benth.) Epling, encontrada em Minas Gerais (CASTILHO et al., 2016; MARTINI et al., 2011). Em estudo realizado por Martini et al. (2011), a composição química do extrativo apresentou como constituintes majoritários a pulegona, seguida de isomentona. Já em estudo realizado por Castilho et al. (2016), o OE da espécie demonstrou a pulegona como componente majoritário, com teores oscilando entre 19,8 e 57,3%. O gênero ainda apresenta *Hesperozygis spathulata* Epling, com distribuição geográfica em Santa Catarina, e *Hesperozygis nitida* (Benth.) Epling, com distribuição de Minas Gerais ao Rio Grande do Sul (PEREIRA; PEREIRA, 1973).

1.2.3 Espécie *Hesperozygis ringens* (Benth.) Epling

Hesperozygis ringens é uma espécie destacada por apresentar um alto potencial para produção de OE, em comparação com outras espécies vegetais, mesmo no inverno, estação que proporcionou rendimentos abaixo de outono, primavera e verão (PINHEIRO et al., 2016). A composição química do extrativo é caracterizada pela presença de pulegona como constituinte majoritário. Estudo envolvendo o efeito sazonal sobre a composição química do OE detectou esse monoterpenoide oxigenado como o mais representativo em todas as estações (PINHEIRO et al., 2016). Essa substância demonstrou as bioatividades fungicida contra *Aspergillus flavus* Link (GONZÁLES-CHAVEZ et al., 2011), acaricida (RIBEIRO et al., 2010), inseticida (ROSSI; CANAVOSO; PALACIOS, 2012) e alelopática sobre *Cucumis sativus* L. (MUCCIARELLI et al., 2001), já relatadas.

Pinheiro et al. (2016) ainda detectaram variações individuais na composição química do OE produzido por *H. ringens* na população analisada. Em estudo sobre a variabilidade genética de quatro populações da planta, através do uso de marcadores moleculares, Fracaro e Echeverrigaray (2006) observaram variabilidade tanto interpopulacional quanto intrapopulacional da espécie.

Embora apresente um pequeno número de indivíduos na região onde ocorre, *H. ringens* é a espécie dominante nos locais onde existe, indicando atividade alelopática (VON POSER et., 1996). No mesmo estudo, os autores relataram o efeito fitotóxico do OE da espécie sobre sementes de *Lactuca sativa* L. Recentemente, Pinheiro et al. (2017) constataram essa atividade, em estudo com o OE da espécie aplicado sobre sementes de espécies bioindicadoras *Avena strigosa* Schreb. e *Lactuca sativa* L., plantas daninhas *Lolium multiflorum* Lam. e *Bidens pilosa* L. bem como de culturas agrícolas *Oryza sativa* L. e *Glycine Max* (L.) Merr. Foi observado pelos autores que as sementes de plantas daninhas apresentaram maior susceptibilidade ao efeito do extrativo, em comparação a sementes de culturas agrícolas. Além do potencial alelopático, o OE de *H. ringens* apresenta outras atividades biológicas descritas, como a acaricida, anestésica e larvicida (RIBEIRO et al., 2010; SILVA et al., 2013; SILVA et al., 2014; TONI et al., 2014).

1.2.4 Histoquímica

Segundo Ventrella et al. (2013), a histoquímica é composta por técnicas histológicas unidas a métodos físico-químicos que proporcionam a identificação, localização e

quantificação de compostos ou grupos de compostos químicos presentes em células e tecidos. Este conjunto de técnicas tem se tornado uma estratégia para pesquisas envolvendo detecção de componentes celulares e extracelulares (ZUBER; TAATJES; ROTH, 2007). Durante o procedimento da reação histoquímica, quando necessário, pode-se optar por um pré-tratamento do material vegetal, consistindo de fixação da amostra, visando preservar a estrutura celular (FIGUEIREDO et al., 2007). Os testes histoquímicos podem ser realizados em material fresco, fixado e incluído em parafina ou metacrilato (resina), mesmo alguns casos apresentando limitações em relação ao processamento (VENTRELLA et al., 2013).

Existe uma grande quantidade de metabólitos vegetais que podem ser caracterizados pela presença de diferentes grupos químicos, identificáveis através de testes histoquímicos. Lipídios totais podem ser detectados a partir de utilização de reagentes como Sudan Black B (PEARSE, 1972), Sudan III (PEARSE, 1972), Vermelho Neutro (KIRK, 1970) e Sudan Red 7B (BRUNDRETT; KENDRICK; PETERSON, 1991), enquanto que lipídios ácidos e neutros podem ser detectados por Azul do Nilo A (CAIN, 1947). Dentro do grupo dos lipídios são encontrados os OEs, resinas e oleorresinas, possíveis de serem visualizados a partir da aplicação do reagente de NADI (DAVID; CARDE, 1964).

As proteínas podem ser detectadas pela utilização de Azul de Comassie (O'BRIEN; MCCULLY, 1981), enquanto que o amido pode ser visualizado a partir do uso de Lugol (GABE, 1968). Compostos fenólicos podem ser detectados através de reagentes como dicromato de potássio (GABE, 1968) e cloreto férrico (JOHANSEN, 1940). Para a detecção de taninos, pode ser utilizada a vanilina hidroclorídrica (MACE; HOWELL, 1974), enquanto que para flavonoides a literatura apresenta como opção o cloreto de alumínio (CHARRIÈRE-LADREIX, 1976). A presença de alcaloides pode ser observada a partir da aplicação do reagente de Wagner no material vegetal (FURR; MAHLBERG, 1981).

Quando testes histoquímicos são realizados, Ventrella et al. (2013) ressaltam a importância de avaliações em material fresco para servirem como controle, pois esse material não está sujeito a alterações proporcionadas pelo meio, nem possíveis interações com o produto utilizado para a inclusão. Também é interessante a comparação dos resultados obtidos com cortes histológicos sem aplicação de reagentes, para evitar enganos em relação à coloração natural das estruturas e a coloração proporcionada pelo produto aplicado (VENTRELLA et al., 2013). A literatura também indica a realização de controles positivo e/ou negativo para a detecção de algumas classes de constituintes, como lipídios, proteínas, alcaloides, entre outras (FIGUEIREDO et al., 2007).

1.2.5 Metabolismo secundário e óleos essenciais

Uma espécie vegetal é capaz de produzir metabólitos não obrigatoriamente relacionados à manutenção de sua vida, contudo, essas substâncias proporcionam vantagens para sua sobrevivência e perpetuação (SANTOS, 2003). Essas substâncias podem ser produzidas e secretadas por diferentes estruturas, dentre elas, os tricomas glandulares (TIAN et al., 2017). Alguns desses metabólitos secundários, como terpenoides e flavonoides, são capazes de proporcionar atividades biológicas, indicando funções de proteção contra ataque de herbívoros e patógenos (FUMAGALI et al., 2008; STRATMANN; BEQUETTE, 2016).

Além de potenciais biológicos contra pragas, os metabólitos secundários também atuam na atração de agentes polinizadores (BRAZ FILHO, 2010; DUDAREVA et al., 2012). Os teores desses metabólitos nas plantas são variáveis, podendo oscilar dependendo de fatores externos como sazonalidade, disponibilidade hídrica, temperatura, radiação UV, método de moagem do material vegetal, estágio de desenvolvimento e métodos de secagem (GOBBO-NETO; LOPES, 2007; PIMENTEL et al., 2012; TISCHER et al., 2017). Além desses, a variação de metabólitos secundários também pode ocorrer dependendo da espécie, quimiotipo ou local de crescimento (FERREIRA; AQUILA, 2000). Metabólitos primários e secundários apresentam distribuições diferenciadas no reino vegetal. Diferente dos metabólitos primários, os secundários são restritos e não estão presentes em todo reino vegetal (TAIZ; ZEIGER, 2009).

Os OEs são obtidos a partir de fontes botânicas (SCHWAB; DAVIDOVICH-RIKANATI; LEWINSOHN, 2008) e são consideradas misturas complexas, podendo conter diversos componentes (PAVELA, 2015). Esses extrativos são constituídos majoritariamente por fenilpropanoides, monoterpenoides e/ou sesquiterpenoides (BIZZO; HOVELL; REZENDE, 2009). As características específicas dos componentes do extrativo são capazes de proporcionar sabores e aromas distintos às espécies produtoras (PAVELA, 2015). Essas misturas complexas podem ser biossintetizadas em diferentes órgãos vegetais, como frutos, flores, folhas, caules e raízes (BAKKALI et al., 2008). Segundo os mesmos autores, o extrativo pode ser secretado por diferentes estruturas como dutos e tricomas glandulares.

O OE está relacionado às vantagens que proporciona à espécie produtora, visando sua sobrevivência no ambiente (SHARIFI-RAD et al., 2017; SIANI et al., 2000). As características aromáticas desses extrativos possibilitam funções como atração de agentes polinizadores, proteção contra frio e calor, e defesa contra pragas e micro-organismos prejudiciais (PAVELA, 2015). Na indústria, os OEs podem ser utilizados em perfumaria,

cosméticos, produtos de higiene, nas indústrias farmacêutica e sanitária, alimentação, química fina (BAKKALI et al., 2008; REGNAULT-ROGER; VINCENT; ARNASON, 2012). Em relação aos métodos de obtenção desses extrativos, pode ser citada a hidrodestilação, a destilação por arraste de vapor, destilação seca, ou obtenção por prensagem mecânica (extração a frio) (PAVELA, 2015), entre outros. Já para a sua detecção em cortes histológicos, podem ser utilizados os reagentes para lipídios totais, como Vermelho Neutro (KIRK, 1970), Sudan Red 7B (BRUNDRETT; KENDRICK; PETERSON, 1991), Sudan III e Sudan Black B (PEARSE, 1972), e mais especificamente, o reagente de NADI (DAVID; CARDE, 1964).

1.2.6 Fungos apodrecedores da madeira

A madeira, por ser um material orgânico, fica sujeita à deterioração e decomposição, processos esses acelerados por agentes físicos, químicos e biológicos (OLIVEIRA; TOMASELLO FILHO; SILVA, 2005). Dentre esses agentes, está o grupo de fungos apodrecedores no qual se inclui a classe de basidiomicetos, responsáveis pelas podridões branca e parda (OLIVEIRA et al., 2005). Esses organismos são considerados os xilófagos mais importantes no mundo (OLIVEIRA; TOMASELLO FILHO; SILVA, 2005), desempenhando um importante papel no ecossistema, uma vez que uma diversidade de espécies animais e vegetais que ocorrem nas florestas depende da energia emanada do apodrecimento da madeira (SCHWARZE, 2007).

Ainda que apresentem benefícios ao ecossistema, os fungos apodrecedores da madeira são capazes de proporcionar uma série de prejuízos quando o componente arbóreo está em utilização. Fungos que ocorrem em árvores ainda em pé podem enfraquecer galhos, ramos ou raízes, possibilitando problemas que envolvem falhas mecânicas, colocando em risco a integridade física de pessoas próximas a esses locais (SCHWARZE, 2007).

As espécies que compõe o grupo de fungos apodrecedores da madeira apresentam distinções importantes em alguns aspectos como morfologia dos organismos, diferenças nos sistemas bioquímicos utilizados para degradação, e respostas diferentes a fatores ambientais extremos (SCHWARZE, 2007). Condições ambientais como umidade, temperatura, teor de nitrogênio presente no substrato e microclima podem influenciar a seleção de ligninas a serem degradadas por fungos de podridão-branca (TUOR; WINTERHALTER; FIECHTER, 1995). Fungos de podridão-branca e parda também apresentam diferenças quanto às árvores hospedeiras. Enquanto os fungos de podridão-branca degradam preferencialmente a madeira

de espécies folhosas (angiospermas), os de podridão-parda atacam principalmente a madeira de coníferas (gimnospermas) (TUOR; WINTERHALTER; FIECHTER, 1995).

Um dos critérios fundamentais para que ocorra o crescimento dos fungos, é a necessidade da presença de oxigênio e pH baixo (DE BELIE et al., 2000). Fungos de podridão-branca apresentam como característica a degradação de celulose e lignina, removendo a coloração marrom da última, deixando a primeira fibrosa (MCCARTHY; COOKSON; SCOWN, 2009). A madeira atacada por esses fungos apresenta coloração de aspecto claro (OLIVEIRA et al., 2005). Fungos de podridão-branca são capazes de produzir bainhas de hifas, que apresentam polissacarídeos na composição química (GUTIÉRREZ et al., 1995). Segundo os mesmos autores, os polissacarídeos extracelulares podem apresentar funções na biodegradação da lignina. Mais de 600 fungos do grupo de podridão-branca são reconhecidos como lignolíticos (KUMAR; GUPKA, 2006).

Fungos de podridão-parda apresentam como característica principal a degradação da celulose, deixando a lignina quebradiça quando a madeira é seca (MCCARTHY; COOKSON; SCOWN, 2009). Fungos de podridão-parda aparecem com frequência em construções expostas à umidade (DE BELIE et al., 2000). A madeira atacada por fungos de podridão-parda terá os polissacarídeos da parede celular decompostos, apresentando coloração pardacenta (OLIVEIRA et al., 2005). Segundo Schwarze (2007), devido à ação de decomposição dos carboidratos, a madeira prejudicada adquire consistência frágil, apresenta rachaduras em forma de cubos, se desintegrando e tornando-se pó.

Constituintes de baixo peso molecular e radicais livres são indicados como fatores responsáveis por permear a madeira e iniciar os processos de apodrecimento (LEONOWICZ et al., 1999). Dentre esses constituintes, podem ser encontrados os oxalatos. Compostos metálicos de oxalato podem ser formados com cálcio, cádmio, cobre e manganês, comuns de serem encontrados em fungos de podridão-parda e branca (FERNANDES et al., 2005).

1.3 PROPOSIÇÃO

O presente estudo tem como proposta contribuir para a caracterização estrutural e histoquímica de *H. ringens*, bem como avaliar o potencial antifúngico do OE e seu componente majoritário. Para isso, os objetivos específicos do trabalho foram:

- caracterizar estruturalmente folha, caule e raiz de *H. ringens*;

- realizar testes histoquímicos em folha e pétala de *H. ringens*, visando detectar lipídios, OEs, resinas, oleorresinas, proteínas, amido, compostos fenólicos, flavonoides, taninos e alcaloides;

- analisar as composições químicas dos OEs extraídos de folhas e inflorescências de *H. ringens*;

- padronizar um método para extração e quantificação do ergosterol presente nas membranas plasmáticas dos fungos apodrecedores da madeira;

- testar o potencial antifúngico do OE extraído de folhas de *H. ringens* frente a fungos de podridão-branca *Ganoderma applanatum* e *Trametes versicolor*, e podridão-parda *Gloephyllum trabeum* e *Lentinus lepideus*, bem como analisar a composição química do extrativo utilizado;

- testar o potencial antifúngico da pulegona frente a fungos de podridão-branca *G. applanatum* e *T. versicolor*, e podridão-parda *G. trabeum* e *L. lepideus*;

- testar os efeitos do OE de *H. ringens* e da pulegona sobre o ergosterol dos fungos de podridão-branca *G. applanatum* e *T. versicolor*, e podridão-parda *G. trabeum* e *L. lepideus*.

1.4 MATERIAIS E MÉTODOS

Uma vez que *H. ringens* está presente na lista de espécies ameaçadas de extinção (GOVERNO DO ESTADO DO RIO GRANDE DO SUL, 2014), para a elaboração da presente tese, nosso grupo de pesquisa obteve uma autorização para coletar amostras da espécie, com o objetivo de realizar atividades científicas, pelo ‘‘Sistema de Autorização e Informação em Biodiversidade’’ (SISBIO, número 44197-2). Também foi possível obter autorização de acesso ao patrimônio genético pelo Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq, número 010191/2014-3).

1.4.1 Caracterização estrutural de órgãos vegetativos

As análises foram realizadas no Laboratório de Botânica Estrutural, do Departamento de Biologia da Universidade Federal de Santa Maria, em colaboração com o Prof. Dr. João Marcelo Santos de Oliveira. Amostras de lâmina foliar, pecíolo, caule e raiz de *H. ringens* foram coletadas aleatoriamente de uma população localizada em Santo Antão (S 29° 37’; O 53° 52’), Santa Maria, Rio Grande do Sul, Brasil, em abril de 2016. Amostras de lâminas foliares, pecíolos e de caules foram coletadas de 10 indivíduos. Considerando os riscos de

extinção da espécie, amostras de raízes foram coletadas de apenas 3 indivíduos. Uma exsicata foi depositada no Herbário do Departamento de Ciências Florestais (HDCF 6720), da UFSM.

As amostras foram analisadas e dissecadas com auxílio de navalhas, pinças e um estereomicroscópio. Logo após, os materiais foram fixados em glutaraldeído 1% e formaldeído 4% em tampão de fosfato de sódio 0,1M, com pH 7,2 (GABRIEL, 1982; MCDOWELL; TRUMP, 1976). Na sequência, as amostras foram submetidas a vácuo, seguido de um procedimento de lavagem no mesmo tampão fixador por 15 min, de acordo com método adaptado de Gabriel (1982). Após, os materiais vegetais foram lavados em água destilada por 15 min., imersos em 2 mL/L de Tween 20, seguindo método adaptado (FREUDENSTEIN; HARRIS; RASMUSSEN, 2002), e submetidos à rotação pelo período de 15 dias, até que ocorresse a remoção de ceras epicuticulares. Na sequência, a desidratação das amostras foi realizada em série etílica (10, 30, 50, 70, 90, 100%) por 15 min. em cada concentração, e posteriormente, pré-infiltração em (2-hidroxietil) metacrilato (HEMA) e etanol absoluto, seguido de infiltração em HEMA. No procedimento seguinte, as amostras foram colocadas em moldes incluídos contendo HEMA, até que ocorresse a polimerização (GERRITS; SMID, 1983). Os processos de histologia ocorreram ao menos 3 vezes para cada material vegetal.

A caracterização dos cristais encontrados em *H. ringens* foi realizada em materiais frescos e incluídos. As amostras dos órgãos vegetativos foram submetidas à floroglucina alcoólica por 2 min., seguindo montagem em HCl concentrado, para que ocorresse a identificação de inulina, baseada na formação de precipitado em forma de mancha (GAHAN, 1984). Inulina também foi identificada através de desidratação de material fresco em etanol e reação de cristais de fenol e H_2SO_4 (ZARLAVSKY, 2014). O HCl nas concentrações de 7 e 25% foi utilizado para solubilização de cristais e eventual liberação gasosa, permitindo assim, a identificação de oxalato de cálcio ou carbonato de cálcio (ZARLAVSKY, 2014).

A partir dos materiais incluídos, secções de 4 μm foram obtidas através da utilização de micrótomo rotatório Leica RM2245. Essas foram coradas com Azul de Toluidina O (FEDER; O'BRIEN, 1968) em tampão de benzoato de sódio a 0,05%, pH 4,4 (SIDMAN; MOTTLA; FEDER, 1961). Secções da lâmina foliar contendo tricomas glandulares foram coradas com Sudan Red 7B (BRUNDRETT; KENDRICK; PETERSON, 1991). Sudan Black B também foi aplicado em secções de caule, visando à detecção de substâncias lipofílicas na endoderme (PEARSE, 1972). Iodo dissolvido em iodeto de potássio (reagente de lugol) foi aplicado para detecção de amido (JOHANSEN, 1940). A análise das lâminas ocorreu com auxílio de microscópio de luz Leica DM2000 em campo claro e luz polarizada para a detecção

de cristais. A dissecação do material vegetal e os registros fotográficos foram realizados através da utilização de estereomicroscópio Leica DM80. Os registros fotográficos foram obtidos pelo uso do sistema de captura digital Leica DFC 295 e o software LAS (Leica™), assim como pelo microscópio Zeiss Axio Imager A2, o sistema de captura digital Zeiss MCr e o software ZEN (Zeiss™).

1.4.2 Caracterização histológica e histoquímica de folhas e pétalas de *H. ringens*

1.4.2.1 Análises histológicas e histoquímicas

As análises histoquímicas foram realizadas no Laboratório de Botânica Estrutural, do Departamento de Biologia da UFSM, em colaboração com o Prof. Dr. João Marcelo Santos de Oliveira. Folhas e flores de *H. ringens* durante a antese foram coletadas aleatoriamente de uma população em Santa Maria (Santo Antão), Rio Grande do Sul, Brasil, entre os meses de agosto e dezembro de 2014. Uma exsicata foi depositada no Herbário do Departamento de Ciências Florestais (HDCF 6720), UFSM, Brasil.

Cortes transversais à mão livre nas porções medianas de folhas e pétalas foram realizadas visando investigar as principais classes de constituintes e suas respectivas localizações no material vegetal. As classes observadas foram detectadas seguindo os testes histoquímicos descritos de acordo com as respectivas referências, considerando controles negativos e positivos: Sudan Black B, Sudan III, Neutral Red e Sudan Red 7B para lipídios totais (BRUNDRETT; KENDRICK; PETERSON, 1991; KIRK, 1970; PEARSE, 1972), Azul do Nilo A para lipídios ácidos e neutros (CAIN, 1947), reagente de NADI para OEs e oleorresinas (DAVID; CARDE, 1964), Azul de Comassie para proteínas (O'BRIEN; MCCULLY, 1981), lugol para amido (GABE, 1968), dicromato de potássio e cloreto férrico para compostos fenólicos (GABE, 1968; JOHANSEN, 1940), vanilina hidroclorídrica para taninos (MACE; HOWELL, 1974), cloreto de alumínio para flavonoides (CHARRIÈRE-LADREIX, 1976) e reagente de Wagner para alcaloides (FURR; MAHLBERG, 1981). Materiais testemunhas dos cortes histológicos de folhas e pétalas também foram analisados.

As análises dos testes histoquímicos e as observações de fotomicrografia das lâminas foram realizadas em campo claro e luz polarizada utilizando um microscópio óptico Leica DM 2000. Análises, dissecação e registros fotográficos também foram obtidos com uso de estereomicroscópio Leica M80. Para ambos os microscópios mencionados, os registros fotográficos foram realizados através do sistema digital de captura Leica DFC 295 e do

software LAS 4.0 (Leica^{TM}). Adicionalmente, as lâminas foram observadas através da utilização do microscópio Zeiss AxioImager A2, e os registros fotográficos foram obtidos com o sistema de captura digital Zeiss MCr e do software ZEN (Zeiss^{TM}).

1.4.2.2 Extração e caracterização química dos óleos essenciais de folhas e inflorescências

Os OEs de folhas e inflorescências frescas foram extraídos em triplicata por hidrodestilação utilizando Clevenger por 2 h, e seus rendimentos foram calculados por % m/m em base fresca (PINHEIRO et al., 2016; 2017). Também foram calculadas as densidades dos extrativos (g mL^{-1}). A composição química de cada extrativo foi analisada utilizando um cromatógrafo gasoso Agilent 7890 acoplado a um detector seletivo de massas Agilent 5975C. Os OEs foram caracterizados quantitativamente por cromatografia com detector de ionização de chamas (CG-DIC), e qualitativamente por cromatografia gasosa acoplada à espectrometria de massas (GC-MS), utilizando as mesmas condições de equipamentos relatadas por Pinheiro et al. (2016). Os constituintes químicos detectados nos OEs foram identificados por comparação dos índices de retenção e os padrões de fragmentação dos espectros de massas obtidos com os dados da literatura (ADAMS, 2009; NIST, 2010).

1.4.3 Padronização de método para análise da concentração de ergosterol no micélio das espécies fúngicas em estudo

1.4.3.1 Crescimento fúngico em BDA com e sem membrana de celofane

Os testes foram realizados no Laboratório de Fitopatologia do Departamento de Defesa Fitossanitária, CCR/UFSM, em colaboração com a Profa. Dra. Marlove Fátima Brião Muniz, e no Laboratório de Extrativos Vegetais. Os dois fungos de podridão-branca *Ganoderma applanatum* e *Trametes versicolor*, e os representantes de podridão-parda *Gloeophyllum trabeum* e *Lentinus lepideus* foram obtidos com a colaboração do Dr. Fernando Nunes Gouveia pelo Laboratório de Produtos Florestais/ IBAMA, Brasília, DF.

As espécies foram cultivadas em placas de Petri (9 cm de diâmetro) contendo meio BDA (batata- dextrose- ágar) coberto por membrana de celofane (BENTO et al., 2014) e sem membrana, com o objetivo de verificar se haveria influência no crescimento micelial. Um disco micelial (11 mm de diâmetro) de cada espécie fúngica foi transferido para placa. Após, as placas foram seladas com parafilme e permaneceram em câmara BOD (Biochemical

Oxygen Demand) a 25°C (±1), com fotoperíodo ajustado em luz/ escuro (12h/12h). Os testes foram realizados em três repetições. A primeira avaliação ocorreu no sétimo dia após o início do experimento, através de duas medidas diametralmente opostas das colônias fúngicas. Os testes foram finalizados quando o crescimento fúngico cobriu completamente o meio de cultura de cada placa (BADAWY; ABDELGALEIL, 2014). A normalidade dos dados e a homogeneidade de variâncias foram verificadas e foi realizada ANOVA de uma via, com comparação de médias pelo teste de Tukey através do software SigmaPlot 11.0.

1.4.3.2 Análise da quantidade de ergosterol na membrana plasmática dos fungos apodrecedores da madeira

As quatro espécies de fungos apodrecedores foram inicialmente cultivadas por 15 dias em placas de Petri contendo BDA em BOD, nas mesmas condições de temperatura e fotoperíodo descritas anteriormente. Discos (11 mm de diâmetro) contendo o micélio de cada espécie fúngica foram transferidos para as placas contendo BDA coberto por membrana de celofane (BENTO et al., 2014). Após, as placas foram seladas e transferidas para câmara BOD, onde permaneceram por 5 dias. Todos os testes foram realizados em três repetições.

Para a extração do ergosterol do micélio dos fungos, foi desenvolvida uma adaptação dos métodos de Arthington-Skaggs et al. (1999) e Tian et al. (2012). Após o período de incubação, o micélio de cada espécie fúngica foi coletado e sua massa úmida foi verificada. 5,0 mL de hidróxido de potássio alcoólico a 25% foram transferidos para tubos de ensaio contendo os fungos. O conteúdo dos tubos foi agitado em vórtex (Velp Scietifica, Vortex Mixer, Wizard X) por 5 min e então, os tubos forma transferidos para ultrassom (Ultra Cleaner 1450A com aquecimento. Unique Ultrasonic Clean), onde permaneceram por 5 min. Após, os tubos foram incubados a 85ºC for 4 h. 2 mL de água destilada estéril e 5 mL de *n*-heptano foram adicionados a cada tubo de ensaio e, então, esses permaneceram em ultrassom por 2 min. As camadas orgânica (de *n*-heptano) e aquosa foram separadas em funis de separação por 1 h à temperatura ambiente. A camada de *n*-heptano de cada tubo foi coletada e analisada por espectrofotometria (Biospetro sp-220) entre 230 e 300 nm. A absorbância em 282 nm caracteriza a presença de ergosterol, enquanto que as leituras em 230 e 282 nm sugerem a presença do esterol intermediário 24 (28) dehidroergosterol (TIAN et al., 2012). Também foi analisado um controle negativo (branco) contendo apenas *n*-heptano.

O cálculo da quantidade de ergosterol foi realizada baseada na massa úmida do micélio (g) de cada fungo e os valores de absorbância obtidos, considerando as fórmulas

adaptadas descritas por Tian et al. (2012): *% 24(28) dehidroergosterol = (A230/518)/massa úmida micelial*; *% ergosterol + %24 (28) dehidroergosterol = (A282/290/massa úmida micelial*. Para as fórmulas, 290 e 518 são considerados valores de E (%/cm) para o ergosterol cristalino e 24 (28) dehidroergosterol. Os gráficos e análises foram realizadas através do software SigmaPlot 11.0.

1.4.4 Bioensaios antifúngicos do óleo essencial e pulegona

1.4.4.1 Extração e caracterização química do óleo essencial de folhas de H. ringens

Folhas de *H. ringens* foram coletadas de quatro indivíduos em uma população em São Francisco de Assis (S 29° 35' 43, 1''; W 055° 07' 33, 4''), Rio Grande do Sul, Brasil na primavera de 2012. Uma exsicata foi depositada no Herbário do Departamento de Biologia (SMDB 13.427), UFSM, Brasil. O OE de cada indivíduo foi extraído de folhas frescas, em triplicata por hidrodestilação utilizando um aparelho Clevenger, pelo período de 2 horas (PINHEIRO et al., 2016). Após o procedimento de extração, as quatro amostras de OE foram reunidas e armazenadas a -4°C até a realização dos testes antifúngicos. A análise qualitativa do OE ocorreu por CG-MS, enquanto que a análise quantitativa foi realizada por CG-FID, utilizando-se o mesmo equipamento e parâmetros descritos anteriormente.

1.4.4.2 Isolamento da pulegona

O isolamento do constituinte majoritário do OE ocorreu através de uma coluna cromatográfica (CC) em sílica gel 60 0.063- 0.2 mm/ 70- 230 MESH ASTM (Macherey-Nagel). Inicialmente, 10 g do OE foram aplicados sobre 700 g de adsorvente (58 x 6,3 cm) e eluídos com hexano:acetona (95:5), em fluxo *flash*. Foram coletadas frações de 50 mL. Foram agrupadas as frações 42-52, totalizando 2,605 g de uma fração enriquecida contendo pulegona. Após, uma fração contendo 0,9 g dessa fração enriquecida foi aplicada sobre 90 g de CC (60 cm x 2,5 cm) e eluída com hexano:acetato de etila em gradiente (99:1; 98:2; 97:3; 96:4) com fluxo de 1 mL. min^{-1}. Foram coletadas frações de 5 mL, e o intervalo de 160-202 foi agrupado, totalizando 0,707 g da substância isolada. Visando obter maior quantidade de pulegona, um terceiro procedimento foi adotado. Uma fração enriquecida com pulegona contendo 0,9 g foi aplicada sobre 90 g de CC (60 cm x 2,5 cm), eluída com hexano:acetato de etila em gradiente (96:4), com fluxo de 1 $mL.min^{-1}$. Foram coletadas frações de 5 mL, e o

intervalo entre as frações 79-95 foi agrupado, totalizando 0,721 g da substância isolada. Em ambos os casos, a pulegona isolada foi analisada por CG-MS / CG-DIC, e sua estrutura foi confirmada por espectros de ressonância magnética nuclear de prótons e carbono 13.

1.4.4.3 Avaliação das atividades antifúngicas do óleo essencial e da pulegona

Os testes para a avaliação da atividade antifúngica foram realizados no Laboratório de Fitopatologia do Departamento de Defesa Fitossanitária, CCR/UFSM, em colaboração com a Profa. Dra. Marlove Fátima Brião Muniz. As atividades antifúngicas do OE e da pulegona foram testadas contra os fungos de podridão-branca *G. applanatum* e *T. versicolor*, e as espécies de podridão-parda *G. trabeum* e *L. lepideus*. As quatro espécies fúngicas foram cultivadas inicialmente em placas de Petri (9 cm de diâmetro) contendo meio BDA, e seladas com parafilme por um período de quinze dias, em incubadora BOD a 25°C (±1), com fotoperíodo ajustado em luz/ escuro (12h/12h).

Para os testes antifúngicos, um controle absoluto (T0) foi considerado, assim como um controle negativo, contendo apenas a concentração de etanol utilizada como diluente (1 µL mL^{-1}) (T1). O OE foi testado nas seguintes concentrações: 0,5; 0,75; 1,00; 1,25 e 1,50 µL mL^{-1} (T2, T3, T4, T5 e T6, respectivamente). Também foi avaliado um controle positivo, contendo o antifúngico comercial Propiconazole, na concentração de 1,50 µL mL^{-1} (T7). As concentrações escolhidas para o constituinte majoritário do OE foram proporcionais as suas concentrações no extrativo. Logo, a pulegona foi avaliada nas concentrações de 0,35; 0,71 e 1,05 µL mL^{-1}. Tanto o OE quanto a pulegona foram previamente diluídos em etanol e então adicionados ao meio, enquanto este ainda estava em estado líquido. Posteriormente, as soluções foram transferidas para placas de Petri. Para cada espécie fúngica, discos do micélio medindo 11 mm de diâmetro foram removidos das culturas de 15 dias de idade, e então, transferidos para as placas de Petri contendo BDA com os tratamentos mencionados. Na sequência, as placas foram seladas e transferidas para a incubadora BOD, onde foram armazenadas nas mesmas condições de temperatura e fotoperíodo citados anteriormente.

Para os tratamentos caracterizados pela presença de OE e os grupos controle, a avaliação do crescimento dos fungos começou 24 horas após o início dos experimentos, através de medições das colônias fúngicas. Duas medidas diametralmente opostas foram realizadas a cada dois dias em cada placa. Para os tratamentos caracterizados pela presença de pulegona, as medições foram realizadas ao fim dos experimentos. Os experimentos foram finalizados quando os fungos nos controles cobriram completamente os meios contidos nas

placas (BADAWY; ABDELGALEIL, 2014). Quando algum tratamento caracterizado pela presença de OE ou pulegona inibiu completamente o crescimento do fungo, o disco do micélio foi re- inoculado em meio BDA fresco, e seu eventual crescimento foi observado por sete dias (KEDIA et al., 2014). Esse método foi utilizado para diferenciar atividades fungistáticas de fungicidas.

O cálculo para inibição de crescimento micelial (ICM) foi realizado a partir da fórmula usada por Badawy e Abdelgaleil (2014): $ICM(\%) = [(DC - DT)*100] / DC$. Onde DC e DT são considerados as médias dos diâmetros do controle e dos tratamentos, respectivamente. A partir das medições de crescimento foi realizado o cálculo do índice de velocidade de crescimento micelial (IVCM), utilizando a fórmula proposta por Oliveira (BARBOSA; VIEIRA; TEIXEIRA, 2015): $IVCM = \Sigma(D-Da)/N$. Onde D e Da são as médias dos diâmetros das colônias fúngicas atuais, e as médias das colônias do dia anterior, respectivamente. N é considerado o número de dias após o início dos experimentos.

Foi considerado o delineamento inteiramente casualizado, onde todos os tratamentos foram realizados em três repetições (BADAWY; ABDELGALEIL, 2014) com cada repetição constituída por duas unidades experimentais. ANOVA de uma via foi realizada e as médias foram comparadas pelo teste Tukey ($P<0,01$) ou pelo teste não paramétrico de Kruskall Wallis ($P<0,01$), quando necessário. Para cada espécie fúngica, a regressão linear foi analisada considerando os resultados obtidos para as curvas de concentração do OE (0,5; 0,75; 1,00; 1,25 e 1,50 µL mL^{-1}) e pulegona (0,35; 0,71 e 1,05 µL mL^{-1}). As concentrações inibitórias médias (IC50) de OE e pulegona foram calculadas por análise de probito e após, o teste t ($P<0,05$) foi efetuado. Todas as análises estatísticas foram realizadas utilizando Microsoft Excel e SigmaPlot 11.0.

1.4.4.4 Efeito do óleo essencial e da pulegona sobre a quantidade de ergosterol na membrana plasmática dos fungos apodrecedores da madeira

As espécies *G. applanatum*, *T. versicolor*, *G. trabeum* e *L. lepideus* foram cultivadas nas mesmas condições descritas anteriormente. Visando verificar os efeitos de ambas as amostras sobre a quantidade de ergosterol na membrana plasmática, o OE foi testado a 0,5 µL mL^{-1}, enquanto a pulegona foi avaliada a 0,35 µL mL^{-1}. Ambos foram diluídos previamente em etanol (1 µL mL^{-1}). Um controle negativo também foi considerado. Todos os tratamentos foram realizados em três repetições. As soluções foram adicionadas ao meio como descrito anteriormente, e o inóculo dos discos contendo o micélio dos fungos ocorreu de acordo com

método adaptado de Bento et al. (2014). As placas de Petri foram transferidas para câmara BOD, onde permaneceram por 5 dias. Após a incubação, o micélio de cada fungo foi coletado e sua massa úmida foi determinada.

A extração de cada micélio fúngico ocorreu de acordo com método adaptado de Tian et al. (2012). O micélio fúngico de cada repetição foi transferido para tubo de ensaio, onde foram adicionados: hidróxido de potássio alcoólico a 25% (5 mL), água destilada esterilizada (2 mL) e *n*-heptano (5 mL). As camadas de cada tubo foram separadas em funil de separação e aquela de *n*-heptano foi coletada e analisada por espectrofotometria (Biospectro sp-220) entre 230 e 300 nm. A leitura realizada em 282 nm é caracterizada pela presença de ergosterol, enquanto que a leitura a 230 e 282 nm indica a presença do esterol intermediário 24 (28) dehidroergosterol (TIAN et al., 2012).

A concentração de ergosterol foi calculada por fórmulas adaptadas de Tian et al. (2012): *% 24 (28) dehidroergosterol= (A230/ 518)/ massa úmida micelial*; *% ergosterol + % 24 (28) dehidroergosterol= (A282/ 290)/ massa úmida micelial*. Para as fórmulas mencionadas, 290 e 518 são considerados os valores de E (%/ cm) para o ergosterol cristalino e 24 (28) dehidroergosterol. A redução da quantidade de ergosterol foi calculada através da fórmula descrita por Kedia et al. (2014): *(C-T)/C x 100*, onde C e T são as porcentagens de ergosterol no controle negativo e tratamento, respectivamente. Para a massa micelial, a ANOVA de uma via foi realizada e as médias foram comparadas pelo teste de Tukey. Os valores de absorbância em 282 nm para cada fungo foram submetidos à ANOVA seguida do teste de Tukey. Todas as análises estatísticas foram realizadas através do Microsoft Excel e SigmaPlot 11.0.

2 ARTIGO 1 – STRUCTURAL CHARACTERIZATION OF VEGETATIVE ORGANS OF THE ENDANGERED BRAZILIAN NATIVE SPECIES *Hesperozygis ringens* (Benth.) Epling.

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ABSTRACT

The aim of this study was to describe the structural characterization of *Hesperozygis ringens* (Benth.) Epling. vegetative organs. For this purpose, leaves, stems and roots of the endangered Lamiaceae were collected from a population located in Santo Antão, Santa Maria municipality, Rio Grande do Sul, Brazil. Results demonstrated that the *H. ringens* leaf blade presents glandular and non-glandular trichomes as well as two morphs of diallelocytic stomata, which are usually found above the epidermis level. The petiole is concave on ventral face and convex on its dorsal face, containing glandular and non-glandular trichomes as well as stomata in the epidermis. These types of trichomes were also detected in the stem. In addition, the presence of intercellular spaces within the organ is highlighted. Stomata above epidermis level also occured in the stem. Phenolic idioblasts were found in the cortical region of plant root and deposit of lipophilic substance was observed in phloem cells. Great amount of apparently two different crystals were detected in all organs. Information obtained in this study provides knowledge about the characterization of *H. ringens*, which may be used to distinguish characters of taxa and can help understand the species survival in its occurrence sites.

Keywords: Lamiaceae; leaf blade; petiole; root; stem.

INTRODUCTION

Hesperozygis ringens (Benth.) Epling is a woody herb native from Brazilian Pampa, which is currently on the list of endangered species (GOVERNO DO ESTADO DO RIO GRANDE DO SUL, 2014). Initially called *Glechon ringens* Benth. and commonly known as *espanta-pulga*, the species occurs sparsely in rocky fields of southeastern region of Rio Grande do Sul, Brazil (TROPICOS, [2017]; VON POSER et al., 1996). *Hesperozygis* Epling. is formed by shrubs or sub shrubs, seven species occurring in southern Brazil and one species in Mexico (BRÄUCHLER; MEIMBERG; HEUBL, 2010; HARLEY et al., 2004; PEREIRA; PEREIRA, 1973). The genus belongs to Lamiaceae Martynov (1820) and within this family, it is placed in the Nepetoideae subfamily, Mentheae tribe, and Menthinae subtribe (HARLEY et al., 2004). A study on the molecular phylogeny of this subtribe classified *Hesperozygis* as a

member of the monophyletic group *The New World*, along with other 21 genera (BRÄUCHLER; MEIMBERG; HEUBL, 2010).

Lamiaceae is composed of 236 genera and about 7173 species, which are annual, biennial and perennial herbs, shrubs, sub shrubs or trees (HARLEY et al., 2004). Its species may be used in different areas such as medicinal, culinary and perfumery (NAGHIBI et al., 2005). The aromatic species classified in this family are characterized by essential oil production in glandular trichomes located on aerial organs (WERKER, 1993). *H. ringens* has a great potential for essential oil production (PINHEIRO et al., 2016), it stands out among Lamiaceae species. According to Werker (1993), these extractives may present beneficial functions for producer plants as protection against herbivores and pathogens or as attraction of pollinator agents. This way, they often exhibit significant biological effects on different living organisms (BADAWY; ABDELGALEIL, 2014; PINHEIRO et al., 2017). Confirming this premise, a series of biological activities has already been described for *H. ringens* essential oil. In addition, the oxygenated monoterpenoid pulegone has been reported as its major compound and different authors attributed the activities detected for this extractive especially to this molecule (PINHEIRO et al., 2016; PINHEIRO et al., 2017; RIBEIRO et al., 2010; SILVA et al., 2014; TONI et al., 2014; VON POSER et al., 1996). Studies regarding this species as well as other *Hesperozygis* representatives usually focus on essential oil production, describing their chemical characteristics and possible activities for the plant extractives (CASTILHO et al., 2016; GONZÁLES-CHAVEZ et al., 2011; MARTINI et al., 2011; VON POSER et al., 1996). Despite the chemical importance of the genus, little information is known on the structural characterization of its species.

Although *H. ringens* essential oil has been studied under different aspects in the past years, there is a lack of information about the structural characterization of vegetative organs of this species in literature. Additionally, there are no reports on the production and storage structures of the essential oil in *Hesperozygis* representatives. Since such information could contribute to provide knowledge to be used to distinguish taxa characters and help understand the survival of *H. ringens* in its occurrence sites, the purpose of this study was to perform structural characterization of its leaf, stem and root.

MATERIALS AND METHODS

Plant Material

Hesperozygis ringens has been threatened with extinction (GOVERNO DO ESTADO DO RIO GRANDE DO SUL, 2014) thus a legal authorization for scientific activities was

obtained through *Sistema de Autorização e Informação em Biodiversidade* (SISBIO, number 44197-2). *Hesperozygis ringens* leaf blade, petioles, stems and roots were randomly gathered from a single population located in Santo Antão (S 29° 37'; W 53° 52'), Santa Maria municipality, Rio Grande do Sul, Brazil, in April 2016. Leaf blades, petioles and stem samples were gathered from 10 individuals, while root samples were gathered from 3 individuals. A voucher specimen was deposited at the Herbarium of the Forest Science Department (HDCF 6720), UFSM, Brazil. Plant material was analyzed in the Structural Botany Laboratory (Federal University of Santa Maria).

Procedures for histology and histochemistry

Hesperozygis ringens vegetative organs were analyzed and dissected under stereomicroscope. Then, the materials were fixed in 1% glutaraldehyde and 4% formaldehyde in sodium phosphate 0.1M pH 7.2 buffer (GABRIEL, 1982; MCDOWELL; TRUMP 1976). Thereafter, fixed materials were submitted to vacuum, followed by the procedure of washing in sodium phosphate buffer 0.1M pH 7.2 for 15 min, according to method adapted from Gabriel (1982). Afterwards, materials were washed in distilled water for 15 min, immersed in Tween 20 2 mL/L (adapted from FREUDENSTEIN; HARRIS; RASMUSSEN, 2002), and submitted to rotation for 15 days to remove epicuticular waxes. In the next procedure, the dehydration of plant materials was performed in ascending ethylic series (10, 30, 50, 70, 90, 100%) for 15 min in each concentration, followed by pre-infiltration into (2-hydroxyethyl) methacrylate (HEMA) and absolute ethanol, finalizing by a process of infiltration in HEMA. Materials were put in embedding moulds filled with HEMA until polymerization, according to Gerrits and Smid (1983). The procedures for histology occurred at least 3 times for each plant material.

Crystal characterization was performed by tests in fresh and included material. Samples were submitted to alcoholic phloroglucin for two minutes followed by mounting in conc. HCl for inulin identification, based on the formation of stained precipitate (GAHAN, 1984). Inulin was also identified by fresh material dehydration in ethanol and reaction of phenol crystals and H_2SO_4 (ZARLAVSKY, 2014). HCl at 7 and 25% was used for crystal solubilization and eventual gas release, allowing to identify calcium oxalate or calcium carbonate (ZARLAVSKY, 2014).

Microscopic analysis and photographic record

From the embedded leaves, petioles, stems and roots, 4 μm thick sections were obtained by a Leica RM2245 rotary microtome. The sections were stained by Toluidine Blue O (FEDER; O'BRIEN, 1968) at 0.05% pH 4.4 in sodium benzoate buffer (SIDMAN; MOTTLA; FEDER, 1961). Sections of leaf blade containing glandular trichomes were stained by Sudan Red 7B (BRUNDRETT; KENDRICK; PETERSON, 1991). Sudan Black B was also applied on stem sections for detecting lipophilic substances in the endoderm (PEARSE, 1972). Iodine dissolved in potassium iodide (lugol's reagent) was applied for starch (JOHANSEN, 1940). Slide analyses were performed under a Leica DM2000 light microscope in bright field and polarized light for crystals. The plant material dissection and photographic records were performed by a Leica DM80 stereomicroscope. Photographic records for this study were obtained using a Leica DFC 295 digital capture system and software LAS (Leica^{TM}), as well as Zeiss Axio Imager A2 microscope, Zeiss MCr digital capture system and ZEN (Zeiss^{TM}) software.

RESULTS

Leaf blade

Leaf epidermis on abaxial and adaxial surfaces presents one cell layer which is amphistomatic (Figure 1a) with two different types of diallelocytic stomata (Figures 1b-c). The guard cells are commonly observed above the level of the epidermis, a structure that is promoted by the growth of the palisade cells surrounding the substomatic chamber (Figure 1a). The vascular system consists of collateral vascular bundles (Figure 1d). Non-glandular and glandular trichomes occur in the organ (Figures 1e-i). The set of trichomes does not form a dense indument (Figure 1e). Secretory cells of the glandular trichomes vary in a natural orange coloration (Figure 1f). Usually secretion accumulates between the cell wall and the cuticule of the glandular trichome, accompanied by cuticule distention (Figures 1f-g). The glandular ones are characterized by the presence of a single basal cell, a single stalk cell and eight secretory head cells arranged in circular form (Figures 1g-h). Cutinized outer portion of epidermal cells can also be observed (Figure 1g). Glandular trichomes occur below the epidermis level since the cells of the underlying palisade parenchyma do not grow, unlike the

surrounding cells. This generates a depression where the glandular trichomes are lodged (Figures 1f-g). Non-glandular trichomes have three cells in a single cell series (Figure 1i).

Mesophyll is isobilateral (Figure 1a). The palisade parenchyma on both faces presents two to three thick cell layers. It also has wide intercellular spaces which are well developed in the substomatic chambers (Figure 1a). In the central portion, the parenchyma tends to be compact with isodiametric or prismatic cells, but it is perpendicular to the palisade cells (Figure 1a).

Crystals are widely distributed in epidermis and mesophyll (Figures 1a, g, i and j). They may also be found in extracellular spaces as substomatic chambers, where they usually obliterate the space (Figure 1j), and in non-glandular trichomes (Figure 1i). These crystals, under polarization, show the typical maltese cross (Figure 1l) in fresh and included material, and streaks after a long dehydration period (Figure 1m). In dried material, precipitated was not detected in crystal areas. In fresh material, the crystals appear structured in a spherical contour, except for those found in intercellular spaces where they are molded according to the space (Figure 1j). The phloroglucin reaction generates reddish precipitate in this organ (Figure 1n). After the leaf imbibition in HCl, no crystal dissolution or gas release was detected. The set of results indicate the presence of inulin crystals in *H. ringens* tissues.

Petiole

The petiole is concave on its ventral face and convex on its dorsal one (Figure 2a). Epidermis is composed of a single cell layer with glandular and non-glandular trichomes and stomata (Figures 2a-b). The fundamental tissue is formed by superficial collenchyma and more internal parenchyma; intercellular spaces are developed in this region (Figure 2a). Although collenchyma is formed in both faces, it presents greater number of cell layers in ventral face, showing to be intermediate between angular and lamellar (Figure 2a-b). Collenchyma presents typical structure with elongated cells and irregular parietal thickenings (Figure 2c). Crystals occur in intercellular spaces and intracellularly including trichomes, mainly the non-glandular ones (Figure 2b). The endodermis appears as amiliferous sheath (Figure 2d). The vascular tissue occurs as a collateral bundle with a shallow arc contour, presenting secondary growth.

Stem

Primary stems are quadrangular in section, keeping this form during the beginning of the secondary growth (Figure 3a). The epidermis is composed of a single cell layer, which presents stomata, uniseriate non glandular trichomes (Figure 3a), and glandular trichomes similar to those found in leaves (Figure 3b). The stomata occur above epidermis level with guard cells elevated by the subsidiary cells (Figure 3c). The non-glandular trichomes present a series of 3 to 4 cells (Figure 3a). Crystals occur in epidermal cells and in the non-glandular trichomes basal cells, in addition to the intercellular spaces (Figures 3a). The phloroglucin and HCl test generates reddish and brownish precipitate in the stem (Figures 3e-g). After the organ imbibition in HCl, no crystal dissolution or gas release was observed. As occurred in leaf blade, the set of results indicate the presence of inulin crystals in stem tissues.

Cortical region has lacunar subepidermal collenchyma in the angles, composed of 1 to 3 layers of tipical elongated cells, and internal fiber bundles (Figure 3a-b). In general, the fundamental tissue in this region presents large intercellular spaces (Figure 3a). In the stem, the superficial layers are chlorenchymatics (Figure 3a). Parenchymal tissue occurs internally, until the endodermis (Figure 3a). The endodermis is formed by a single layer of juxtaposed cells showing plasts with starch and Casparian strips, as well as alternating cells with lipophilic substance in the cell wall composition (Figure 3d). Such cells are relatively bulkier and demonstrate thickened walls.

The primary phloem shows conductive cells, parenchyma, and rare isolated fibers (Figure 3a). The formed secondary phloem also demonstrated conductive elements and parenchyma, in addition to companion cells soon after the beginning of the cambial activity (Figure 3a). The primary and secondary xylems show vessel elements, fibers and parenchyma. The vascular cambium differentiation is observed in very young stems, in addition to continuous xylem and phloem (Figure 3a). Crystals are observed in both primary and secondary xylem and phloem (Figure 3a).

The pith has a peripheral region composed of sclerenchymatous tissue, which is derived from typical parenchyma when younger internodes are analyzed (Figure 3a). The central portion presents parenchymatous tissue with bulky cells and disaggregation from the middle lamella, generating large intercellular spaces (Figure 3a). Crystals are commonly found in this region, especially intracellularly.

Secondary stem

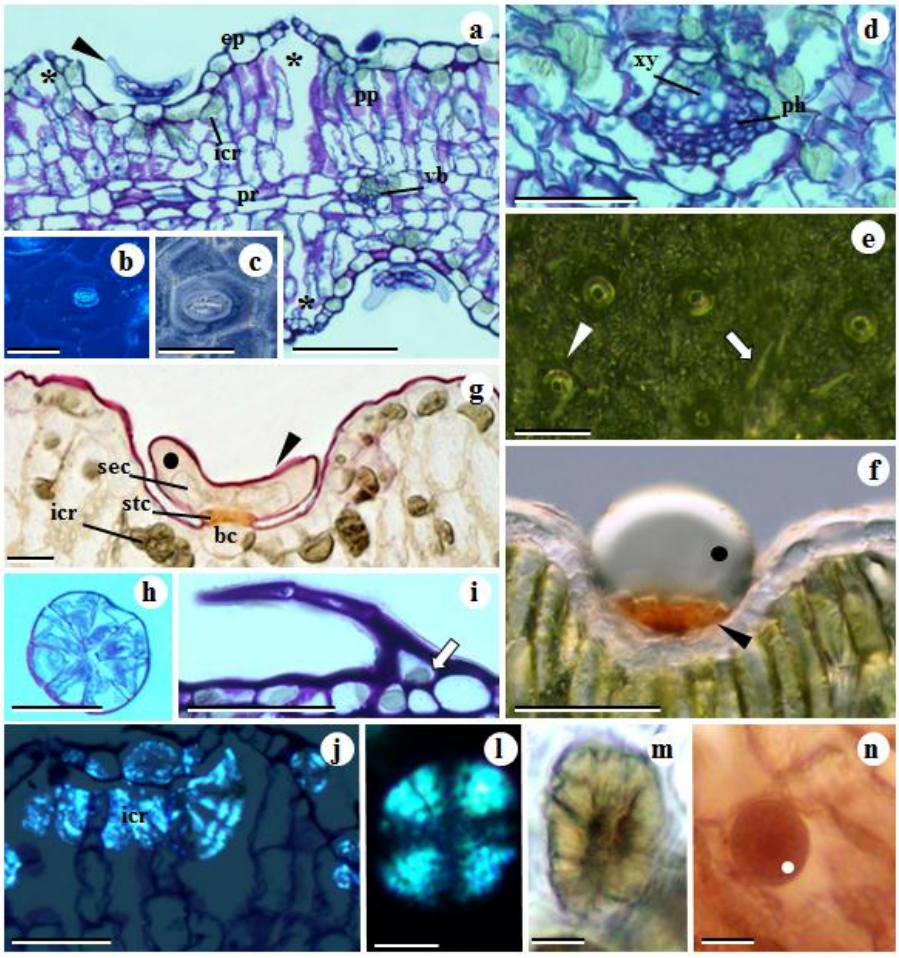
The secondary xylem frequently presents isolated vessel elements in pairs and rarely in trios (Figure 3h). Interfascicular cambium produces only ray cells for both sides (Figure 3h). Crystals are found on the side of the newly formed xylem and phloem (Figure 3h). A great number of cells with phenolic compounds occur in the secondary phloem and xylem (Figure 3h). The pith is partially sclerified and fistulous (Figure 3h). In mature stems, the cells of the xylem ray are not sclerified, generating a contrast between the rays and the axial elements of the xylem tissue (Figure 3h). There are rays with different widths (Figure 3h).

Root

In young root the epidermis has isodiametric cells in transverse section (Figure 4a). The region demonstrates more thickened radial and external tangential walls as well as deposit of lipophilic substances (Figure 4b). In mature root, outer and inner portions of the bark accumulate a great quantity of secondary metabolites (Figure 4b). The cells of xylematic ray present a lignification gradient where the most recent ones are non lignified. The presence and absence of lignin in sclerified radial tissues generate a contrast between non lignified and lignified portions (Figure 4b). Secondary phloem is not associated to sclerified tissue. Within the vessel elements, crystals occur in great quantity occasionally obliterating the vessel elements. Such crystals also occur in newly formed vessel elements and sclerenchyma. The crystals of xylem differ from those found in other tissues. These crystals are acicular and present arrangement in rosette usually connected to the walls of the vessel elements (Figure 4c). Phloroglucin and HCl test produced no reaction in the crystals, but the structures were dissolved by H_2SO_4 and phenol (Figure 4d), indicating the presence of hesperidin crystals.

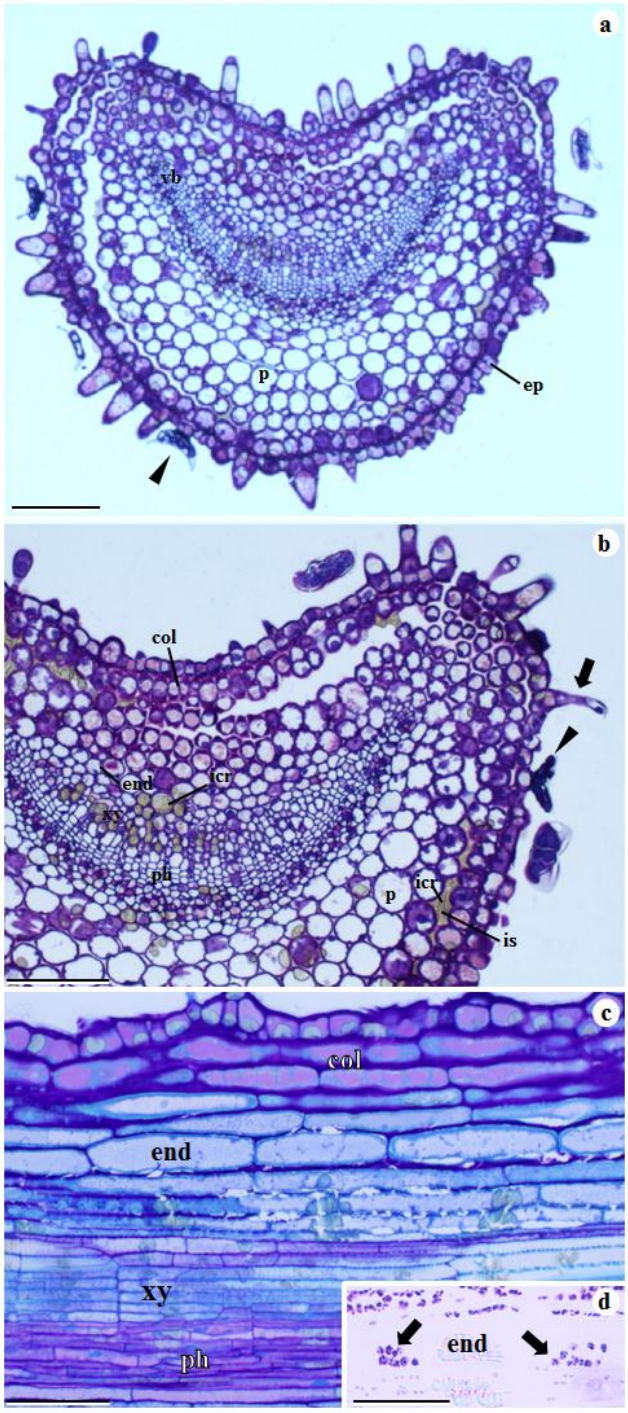
In xylem, the vessel elements are predominantly isolated; they are frequently found in pairs and rarely in trios (Figure 4b). Phellogen appears internally (Figure 4a). The cortical region has phenolic idioblasts and phloem cells presenting deposit of lipophilic substances (Figures 4a-b). The occurrence of fungal hyphae in this region is high. Some cells in cortex and phloem are compressed and others grow very large (Figure 4a).

Figure 1 - Structural characterization of *Hesperozygis ringens* leaf blade. Figures 1a, d, f-g, i-n in transversal sections. Figures 1b-c and e in frontal view. Figure 1h in paradermal section. **(a)** Epidermis (ep), inulin crystal (icr), palisade parenchyma (pp), perpendicular parenchyma (pr), vascular bundle (vb), glandular trichome (arrow head), substomatic chambers (asterisks). **(b)** Detail of diallelocytic stomata. **(c)** Detail of diacytic stomata. **(d)** Detail of the vascular bundle, highlighting xylem (xy) and phloem (ph). **(e)** Leaf blade indumentum presenting glandular trichome (arrow head) and non-glandular trichome (arrow). **(f)** Detail of glandular trichome with a natural orange coloration (arrow head) and distended cuticle (black circle). **(g)** Detail of glandular trichome (arrow head) stained by Sudan Red 7B, highlighting inulin crystal (icr), basal cell (bc), stalk cell (stc), secretory cell (sec) and distended cuticle (black circle). **(h)** Detail of glandular trichome presenting eight secretory cells. **(i)** Detail of non-glandular trichome and inulin crystal (arrow).**(j)** Inulin crystal (icr) under polarization. **(l)** Detail of inulin crystal under polarization presenting a shape of maltese cross. **(m)** Detail of inulin crystal presenting streaks after dehydration period. **(n)** Detail of reddish precipitate (circle) after floroglucine reaction. Scale bars: 20 µm **(l, m and n)**; 50 µm **(b, c, d, h, i and j)**; 100 µm **(a, f and g)**; 500 µm **(e)**.



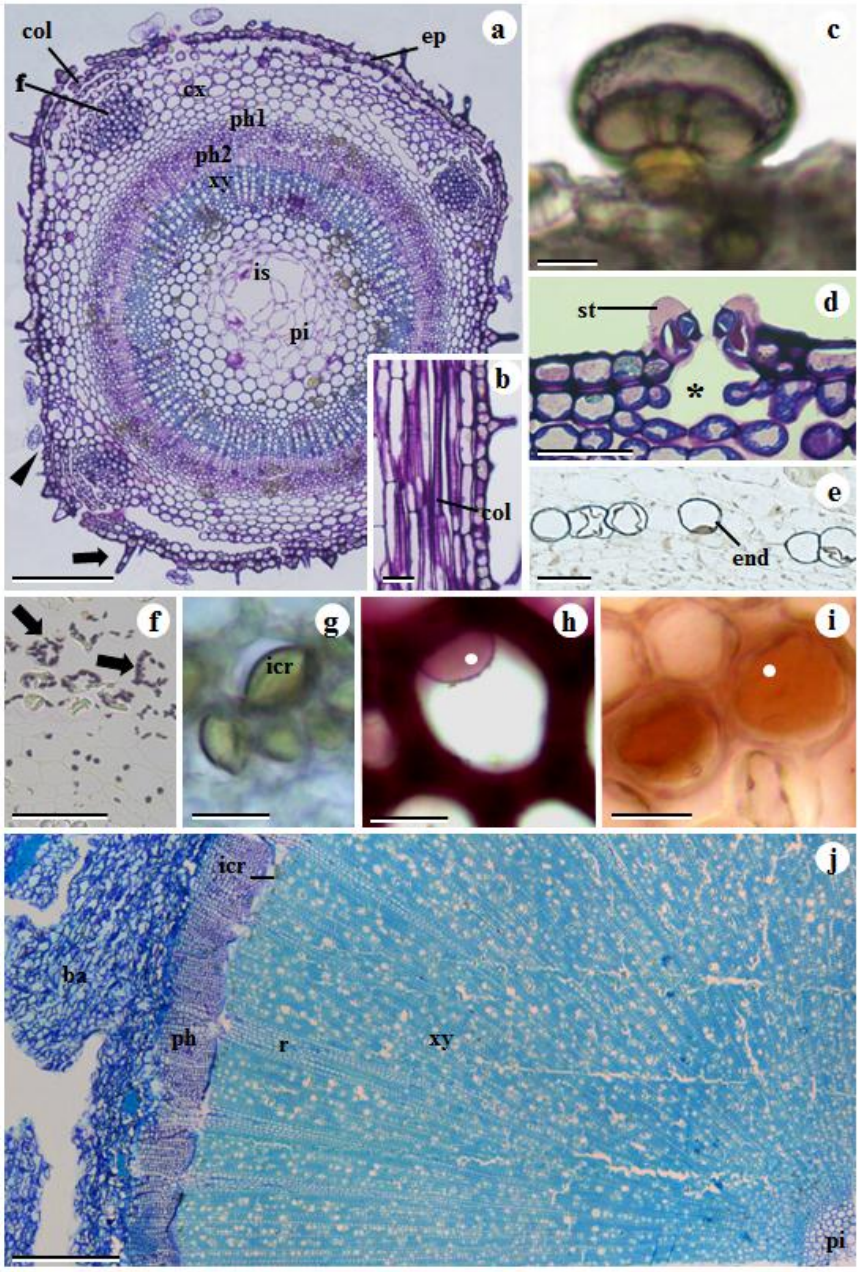
Source: The authors.

Figure 2 - Structural characterization of *Hesperozygis ringens* leaf petiole. Figures 2a-b in transversal sections. Figures 2c-d in longitudinal section. **(a)** Epidermis (ep), parenchyma (p) and vascular bundle (vb). **(b)** Non-glandular trichome (arrow), glandular trichome (arrow head), collenchyma (col), endodermis (end), inulin crystals (icr), xylem (xy), phloem (ph), parenchyma (p) and intercellular space (is). **(c)** Detail of collenchyma (col), endodermis (end), xylem (xy) and phloem (ph). **(d)** Detail of starch (black arrow) in endodermis after reaction with lugol. Scale bars: 50 µm **(d)**; 100 µm **(a, b and c)**.



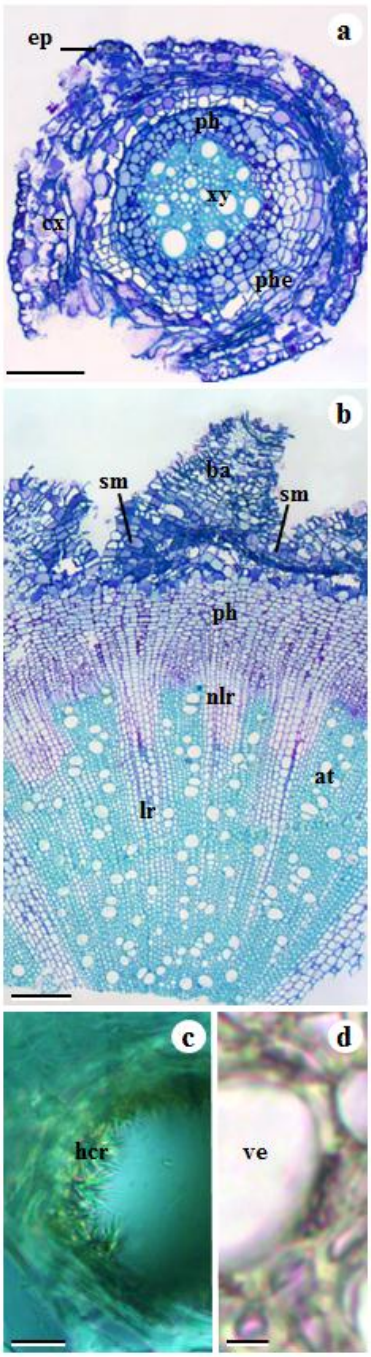
Source: The authors.

Figure 3 - Structural characterization of *Hesperozygis ringens* stem. Figures 1a, c-j in transversal sections. Figure 1b in longitudinal section. **(a)** Glandular trichome (arrow head), non-glandular trichome (arrow), epidermis (ep), collenchyma (col), fiber (f), cortex (cx), primary phloem (ph1), secondary phloem (ph2), xylem (xy), intercellular space (is), pith (pi). **(b)** Detail of collenchyma (col). **(c)** Detail of glandular trichome. **(d)** Detail of stomata (st) and substomatic chamber (asterisk). **(e)** Detail of endodermis (end) stained by Sudan Black B. **(f)** Detail of starch (black arrow) in endodermis after reaction with lugol. **(g)** Detail of inulin crystals (icr). **(h)** Detail of reddish precipitate (circle) after phloroglucin reaction. **(i)** Detail of orange precipitate (circle) after phloroglucin reaction. **(j)** Bark (ba), inulin crystal (cr), phloem (ph), xylem (xy), rays (r) and pith (pi). Scale bars: 20 µm **(c, g, h, i and j)**; 50 µm **(b, d and f)**; 100 µm **(a)**; 500 µm **(e)**.



Source: The authors.

Figure 4 - Structural characterization of *Hesperozygis ringens* root in transversal sections. **(a)** Epidermis (ep), cortex (cx), phellogen (phe), phloem (ph), xylem (xy). **(b)** Bark (ba), secondary metabolites (sm), phloem (ph), non lignified rays (nlr), lignified rays (lr), axial tissue (at). **(c)** Detail of vessel element highlighting hesperidin crystals (hcr). **(d)** Detail of vessel element (ve). Scale bars: 10 µm **(c and d)**, 200 µm **(a and b)**.



Source: The authors.

DISCUSSION

Leaf blade

Leaves of species classified in the subtribe Menthinae present indumentum characterized by the presence of glandular and non-glandular trichomes in both faces (NOVOA; MONTI; VIZCAÍNO, 2005; SATIL et al., 2002; TOLEDO; ALQUINI; NAKASHIMA, 2004). In *H. ringens* diallelocytic, stomata were detected in both leaf faces in the blade and petiole. Amphistomatic leaves and stomata, predominantly diacitic, are commonly found in species of the same subtribe, as occurred in *Cunila microcephala* Benth., *Hedeoma multiflora* Benth. and *Satureja* L. genus. In the latter, stomata occur above epidermis level (NOVOA; MONTI; VIZCAÍNO, 2005; SATIL et al., 2002; SATIL; KAYA, 2007; TOLEDO; ALQUINI; NAKASHIMA, 2004). Amphistomatic leaves are also found in *Salvia nutans* L. (GÜRCAN; ERKARA; ÖZTÜRK, 2016). Anisocytic stomata have also been detected in Lamiaceae, as occurred in *Stachys iberica* Bieb. subsp. *iberica* var. *densipilosa* Bhattacharjee (ERKARA et al., 2010). Diallelocytic stomata are characterized by the presence of subsidiary cells positioned perpendicularly to the guard cells and with common walls obliquely positioned described as C-shape (CANTINO, 1990). Those found in *H. ringens* demonstrate two and three subsidiary cells. Diallelocytic stomata have already been identified in Nepetoideae representatives, including some of *The New World* genus such as *Cunila* D. Royen ex L., *Rhododon* Epling, *Pogogyne* Benth. and *Monardella* Benth. They were also described in other Menthinae genus, such as *Mentha* L., *Hedeoma* Pers., *Micromeria* Benth., *Satureja* L. and *Thymus* L. (CANTINO, 1990). This type of stomata was also detected in *Wenchengia* C. Y. Wu & S. Chow, another genus belonging to Lamiaceae (CANTINO; ABU-ASAD, 1993). Attention should be given to the palisade parenchyma and its intercellular spaces. It apparently assumes the role of the spongy parenchyma that is poorly developed and tends to be compact and reduced to a few cells beyond the endodermis. Although the central tissue of the mesophyll is not typically spongy, it presents a distinct basic structure when compared to the palisade tissue. This allows to interpret the mesophyll as isobilateral and heterogeneous.

Petiole

Similarly to *H. ringens*, petiole contour was also described for *Lamium* L. and *Salvia* L. species (ATALAY et al., 2016; BAGHERPOUR et al., 2010; BERCU; BAVARU; BROASCĂ, 2011; CELEP et al., 2014; ERBANO et al., 2012; ÖZDEMIR; ÖZDEMIR; YETISEN, 2016). Petiole contour is a mandatory state in morphological studies, being taxonomically useful in Lamiaceae, as observed for *Lamium* (ATALAY et al., 2016) and *Salvia* (ÖZDEMIR; ÖZDEMIR; YETISEN, 2016). However, this feature was not considered a diagnostic character for *Hesperozygis* species (PEREIRA; PEREIRA, 1973). Given the great similarities between species of *Hesperozygis* and *Glechon* and the obvious basis for support in taxonomic difficulties, it is noteworthy that the petiole form found in this study is different from that of *G. spathulata* (BANDERÓ FILHO et al., 2010). According to the authors, the plant presents mainly circular form in cross section analysis. The presence of glandular and non-glandular trichomes in Lamiaceae petioles was already described (AKÇIN; ÖZYURT; ŞENEL, 2011). The report of these structures on the petiole could be of interest considering *Hesperozygis* representatives, since *Hesperozygis nitida* (Benth.) Epling is the only one presenting glabrous leaves on its adaxial surfaces (PEREIRA; PEREIRA, 1973). The vascular structure is considered a very important feature of petiole (METCALFE; CHALK, 1972), mainly among species of the same genus. A great overlap of characteristics is found in Lamiaceae genera, as mentioned for *Salvia* and *Lamium* (ATALAY et al., 2016; BAGHERPOUR et al., 2010; BERCU; BAVARU; BROASCĂ, 2011; CELEP et al., 2014; ERBANO et al., 2012; ÖZDEMIR; ÖZDEMIR; YETISEN, 2016).

Stem

Other Menthinae representatives have shown quadrangular stems in cross section (NOVOA; MONTI; VIZCAÍNO, 2005; OZCAN; EMINAGAOGLU, 2014), as detected in *H. ringens*. This is considered a usual Lamiaceae characteristic (HARLEY et al., 2004). The subtribe stem indumentums present glandular and non-glandular trichomes, as already described for *Hedeoma multiflora* Benth., *Origanum rotundifolium* Boiss. and *O. vulgare* L. ssp. *viride* (Boiss.) Hayek (NOVOA; MONTI; VIZCAÍNO, 2005; OZCAN; EMINAGAOGLU, 2014). Stomata were also found above epidermal level in stems of *Salvia nutans* L., a species belonging to another subtribe of the same family (BERCU; BAVARU; BROASCĂ, 2011). In *H. ringens* stem, the endodermis has remarkable features such as

impregnation of some cell walls with lipophilic substances. This characteristic has not been described for other Lamiaceae species yet. The stem pith with parenchymatous tissue including intercellular spaces was found in *Lamium moschatum* Miller var. *rhodium* (Gand.) R. Mill; however, it presented roundish cells (BARAN; ÖZDEMIR, 2011). Apparently, the disaggregation described for *H. ringens* pith culminates in typical fistulous internodes of some Lamiaceae genus (HARLEY et al., 2004). The pith of the *Satureja parnassica* Heldr. et Sart. subsp. *sipylea* P.H. Davis (Menthinae) demonstrates parenchymatous cells as well as the central region frequently broken in pieces (SATIL et al., 2002). *Salvia divinorum* Epling & Játiva stem presents degraded cells in pith centre forming a large cavity including internodes and being visible to the naked eye in adult organs (KOWALCZUK et al., 2014). In some semi-aquatic plants, the description of large intercellular spaces was also reported (HARLEY et al., 2004). Their places of occurrence clearly differ from *H. ringens* habitat, which occurs in drained soil, both sandy and rocky ones. Thus, the formed spaces can be either considered constitutive or associated with some other functionality.

Root

In this study, fungal hyphae were detected in *H. ringens* roots. In general, the soil presents a wide diversity of microorganisms in the plant rhizosphere due to the nutrients secreted by the organ (SALA; FREITAS; DA SILVEIRA, 2007). On the other hand, secondary metabolites exuded by roots are often sources of chemotaxis, favoring symbiotic interactions of plant organs with microorganisms (CHEYNIER et al., 2013). Associations with mycorrhizal fungi may confer benefits to different plant species, which were also described for Lamiaceae representatives. According to Tarraf et al. (2015), arbuscular mycorrhizae have already provided increase in essential oil and biomass productions of aerial parts of *Salvia officinalis* L., *Origanum vulgare* L. and *Thymus vulgaris* L. In addition, *Satureja macrostema* (Benth.) Briq. was benefited by the mycorrhizae colonization, presenting increase in contents of the essential oil major compounds (CARREÓN-ABUD et al., 2015).

Glandular trichomes

Lamiaceae species usually produce chemical substances in different types of glandular trichomes with distinct function, aiming at the survival and perpetuation of the producer plant

(WERKER, 1993, 2000). In this study, glandular trichomes, classified as peltate containing eight secretory head cells, were found in *H. ringens* leaves and stems, a similar type described for *Cunila microcephala* (TOLEDO; ALQUINI; NAKASHIMA, 2004). Peltate trichomes with multicellular heads are commonly found in representatives of Menthinae subtribe, as observed in studies conducted with *Lamium*, *Thymus quinquecostatus* Celak, *Satureja horvatii* Silic and *Micromeria thymifolia* (Scop.) Fritsch (BARAN; ÖZDEMIR, 2009, 2011; JING et al., 2014; MARIN; ASCENSAO; LAKUŠIĆ, 2012; MARIN; JASNIĆ; ASCENSÃO, 2013).

Non-glandular trichomes

The function of non-glandular trichomes depends on the organ location, morphology and orientation (WERKER, 2000). Results indicate that these structures are distributed in abaxial and adaxial surfaces of leaf blade, petiole and stem in *H. ringens*. This distribution may be related with a defense mechanism, since these structures have the function of providing protection to glandular trichomes, when the indumentum is dense (WERKER, 2000). The municipality of Santa Maria presents temperatures over 30°C in the hottest months of its summer (MORENO, 1961), thus we hypothesized that non-glandular trichomes may favor the *H. ringens* survival in months with less rain and higher temperatures. The referred structures may serve as a mechanical barrier against extreme temperatures, extensive light and water loss (WERKER, 2000).

Taxonomic considerations

Characters used to differentiate *Hesperozygis spathulata* Epling, *H. nitida* and *H. rhododon* Epling consider calyx tube length, leaf type, glabrous or hairy upper page, as well as sessile or pedunculated summits (PEREIRA; PEREIRA, 1973). However, *H. ringens* characteristics are not described in the referred work. No information on the structural aspects of *H. ringens* such as trichome distribution and its classification or vegetative organ characteristics has been found in literature. For this reason, this study is necessary in order to provide information about the diagnostic characters of the species.

Studies have been conducted with other Lamiaceae representatives searching for description and classification of structural aspects of taxonomic importance. Characters such as glandular and non-glandular trichomes, stem form in cross-section, epidermal cell form, cortex pith, vascular bundles, leaf form in cross section and mesophyll characteristics have

been shown to be useful for species identification (KALICHARAN et al., 2015; KHALIK; KARAKISH, 2016; SATIL; KAYA, 2007; SEYEDI; SALMAKI, 2015).

Crystal inclusions

The anatomical characterization of 39 Lamiaceae representatives (ABU-ASAB; CANTINO, 1987) and morphological review of the family (HARLEY et al., 2004) indicated that crystal inclusions are common. However, information on their chemical compositions were not found. In our study, the great number of crystals in *H. ringens* leaf, stem and root is highlighted. They can be found even in apoplast, including inside vessel elements. This morphological feature resembles the one described for stems and roots of other taxonomic groups of Lamiaceae (ROMBERGER; HEJNOWICZ; HILL, 1993). Crystals presenting similar structures were found in the adaxial epidermis of *Teucrium sandrasicum* O. Schwarz (DINÇ et al., 2008). Morphologically differing from the crystals visualized in the aerial organs, *H. ringens* roots demonstrate crystalline structures in vessel elements. Similar crystals composed of hesperidin were described inside vessel elements of *Citrus sinensis* (L.) Osbeck as a response to infections caused by *Xylella fastidiosa* (ALVES et al., 2009) and by *Phytophthora citrophthora* (DEL RIO et al., 2004). In *H. ringens* roots, crystals were dissolved by H_2SO_4. This diluted acid was already used as catalyst for hesperidin hydrolysis in different temperatures (GROHMANN; MANTHEY; CAMERON, 2000), suggesting the characterization of hesperidin crystals in the *H. ringens* roots. Hesperidin was already found in Lamiaceae representatives (METCALFE; CHALKE, 1972), as described in *Clinopodium gracile* (Benth.) Matsum, where the substance was considered part of the defense response to the attack of *Aedes albopictus* Skuser mosquito (CHEN et al., 2013).

Histochemical tests were performed for detection of inulin, calcium oxalate and calcium carbonate. Calcium oxalate and calcium carbonate were disregarded due to the absence of solubilization. Inulin is one of the possible crystals occurring in *H. ringens* leaf and stem based on the presence of maltese cross and streaks after the dehydration period. In this study, phloroglucin reaction promoted disappearance of crystals and formation of a red-brown precipitate, a similar reaction described by Gahan (1984), apart from crystals in vessel elements. However, some results do not corroborate with this hypothesis due to the absence of coloration after phenol and H_2SO_4 test. Inulin crystals were already described in the Menthinae species *Cunila microcephala* (TOLEDO; ALQUINI; NAKASHIMA, 2004). The authors reported the presence of polysaccharide crystals, with similar morphology to those

described in our study, demonstrating that a wide distribution of such structures may occur in leaves of *The New World* group representatives. Although *Hesperozygis* and *Glechon* were considered synonymous, in an anatomical study performed with *G. spathulata*, no crystals were detected (BANDERÓ FILHO et al., 2010). This suggests that the presence of these structures can be considered a differentiation characteristic between the genera. Inulin is considered a reserve of sugar, especially of fructose (TOLEDO; ALQUINI; NAKASHIMA, 2004), and is explored commercially and medicinally for different purposes (FUCHS, 1987; KIERSTAN, 1978). For plants in general, fructans originating from inulin generate cold and drought tolerances (RITSEMA; SMEEKENS, 2003), which are common environmental situations to *H. ringens* individuals at the occurrence site. In addition to hesperidin and inulin, the Lamiaceae representatives may also present other crystals with distinct features in relation to those found in *H. ringens*, such as calcium oxalate, as occurred in *Salvia divinorum* Epling & Játiva (KOWALCZUK et al., 2014).

Ecological considerations

As detected for *H. ringens*, amphistomatic leaves, stomata above epidermis level and intercellular spaces are related to mesic environments. In a study of *Aegiphila sellowiana* Cham. juveniles submitted to flooding, amphistomatic leaves and the position of the stomata above epidermis level were reported (MEDRI et al., 2011). However, *H. ringens* shows small leaves and developed indumentum related to xeric environments. These structural aspects may mean part of the complexity of *H. ringens* adaptations, since Santa Maria may present rainy months along the year, rainless periods with high temperatures or cold and dry periods (DA SILVA et al., 2007). These phenomena may even alternate in the same month. The referred structural features can also serve as a defense mechanism of the plant against possible biotic factors. The production of the two types of crystals may be associated with the conditions at which the species is exposed to in its habitat. No information regarding the concomitant production of these crystals in Lamiaceae representatives has been found in literature. For a better understanding of composition and functions of the crystals, further studies are suggested, considering the different plant organs.

Conclusions

Hesperozygis ringens presents remarkable characteristics such as the presence of glandular and non-glandular trichomes in leaf and stem, two morphs of diallelocytic stomata above epidermis, intercellular spaces in stem, presence of lipids in cell walls of stem endodermis, phenolic idioblasts in roots, and wide amount of crystals spread in all vegetative organs. Results found in this study may help understand the plant survival in its sites and encourage further studies aiming to preserve and reproduce this species.

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3 ARTIGO 2 – HISTOLOGICAL AND HISTOCHEMICAL CHARACTERIZATION OF LEAVES AND PETALS OF THE ENDANGERED NATIVE BRAZILIAN SPECIES *Hesperozygis ringens* (Benth.) Epling.

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Histological and histochemical characterization of leaves and petals of the endangered native Brazilian species *Hesperozygis ringens* (Benth.) Epling

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ABSTRACT

Hesperozygis ringens (Benth.) Epling is an endangered Lamiaceae woody herb characterized by the abundance of essential oil (EO) in its leaves. However, no information has been found in literature about the plant tissues where this extractive is biosynthesized, and what other constituents are produced by the organ. In addition, the classes of compounds biosynthesized by its petals and the composition of EO produced by the inflorescences have not been reported either. Because this information may help the *H. ringens* identification, the aim of this study was to characterize the compound classes in the plant leaf and petal by histochemical tests. The leaves and flowers of this species were collected from a population in Santo Antão, municipality of Santa Maria, Rio Grande do Sul, Brazil. Fresh plant material was analyzed to detect total lipids; acid and neutral lipids; essential oils (EOs), resins and oil-resins; proteins; starch; phenolic compounds; tannins; flavonoids; and alkaloids. The EOs of leaves and inflorescences were extracted by hydrodistillation and their chemical compositions were analyzed by gas chromatography coupled to mass spectrometry (GC–MS) and GC with flame ionization detector.

Results obtained in histochemical tests detected total, neutral and acid lipids, EOs, oil-resins, protein, phenolic compounds, flavonoids and alkaloids in the leaves and petals of *H. ringens*. Total lipids were found mainly in leaf mesophyll and glandular trichomes of both plant organs. Essential oils were marked in leaf vascular bundle, petal surface and glandular trichomes of both fresh organs. Oil-resins were detected mainly in leaf mesophylls and petal glandular trichomes. The chemical characterization of the EOs produced by leaves and inflorescences detected the oxygenated monoterpenoid pulegone as their major compound. The species presented histochemical characteristics which have not been found for other Lamiaceae representatives yet, and may serve to identify *H. ringens*.

1. Introduction

The Lamiaceae family is characterized by several species containing aromatic substances, and they are used as herbs in folk medicine or as flavoring due to their essential oils (EOs). The family species are characterized by essential oil (EO) production and storage in glandular trichomes (Werker, 1993). Among the Lamiaceae genus, *Hesperozygis* Epling stands out with several representatives occurring in Brazil and Mexico, which issued a number of studies aiming to investigate the EO characteristics of its species (González-Chávez et al., 2011; Martini et al., 2011; Pinheiro et al., 2016; Pinheiro et al., 2017; Von Poser et al., 1996). According to these authors, the analyzed species demonstrate high potential to produce EO and studies have also shown that some of their biological activities are allelophatic, antimicrobial and fungicidal.

Hesperozygis ringens is also known as *espanta-pulga*, which is equivalent to *keeping fleas away*. It is an endangered woody herb that occurs in the rocky fields of Rio Grande do Sul State, Brazil (Von Poser et al., 1996; Secretaria Estadual do Meio Ambiente, 2014). In the last two decades, chemical studies on this species have revealed high EO yields, with monoterpenoid pulegone as the major constituent (Pinheiro

Abbreviations: EO, essential oil; D, Dermal; F, Fundamental; V, Vascular

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et al., 2016; Pinheiro et al., 2017; Ribeiro et al., 2010; Silva et al., 2014; Toni et al., 2014; Von Poser et al., 1996). Additional studies have shown that biological activities of this extractive can also be acaricidal, anesthetic, allelophatic and larvicidal (Pinheiro et al., 2017; Ribeiro et al., 2010; Silva et al., 2014; Toni et al., 2014; Von Poser et al., 1996).

Despite the importance of the chemical knowledge of the EO, previous studies have not informed the details on the location or which other compound classes are produced by *H. ringens*, since the histochemistry of the secretory organs is unknown. Nonetheless, the literature reports information about other Lamiaceae species, addressing the aspects mentioned above. A large number of Lamiaceae representatives produce lipids, including EOs, through glandular trichomes distributed in the aerial organs (Boix et al., 2013; Marin et al., 2012; Mota et al., 2013). In general, extractives produced by trichomes apparently present beneficial functions to plants, such as protection against herbivores and pathogens (Werker, 1993). They are closely related to environmental conditions and adaptive characteristics of the Lamiaceae species (Tomás-Barberán and Wollenweber, 1990). Giuliani and Bini (2008) emphasized the need for structural and chemical studies on glandular trichomes in Lamiaceae, while aiming to distinguish characters of taxa as subfamilies or genus. *H. ringens* presents EO in abundance thus it can produce the extractive in glandular trichomes as well as in other plant tissues. In addition to leaves, the species can show high amounts of EO in its inflorescences. It is known that the plant may also biosynthesize other classes of constituents. The discovery of these compounds and their producing tissues may help the *H. ringens* identification.

A great number of histochemical methods have been used in previous studies with plant tissues to detect not only EOs and lipids in general, but also phenols, alkaloids, and other important plant constituent classes (Maggi et al., 2010; Naidoo et al., 2013). Since the literature does not present information about histochemical tests on *H. ringens*, the purpose of this study is to provide knowledge about the histochemical characterization of compound classes and their locations in leaves and petals. The yields and chemical compositions of the EOs produced by leaves and inflorescences are also described.

2. Materials and methods

2.1. Legal authorization

Legal authorization for scientific activities was obtained through Biodiversity Information and Authorization System (*Sistema de Autorização e Informação em Biodiversidade*, SISBIO, N. 44197-2) because *H. ringens* is an endangered species (Secretaria Estadual do Meio Ambiente, 2014). An authorization to the genetic heritage was also requested to National Council of Research and Scientific Development (*Conselho Nacional de Pesquisa e Desenvolvimento Científico*, CNPq, N. 010191/2014-3).

2.2. Study site

Hesperozygis ringens leaves and flowers during anthesis were randomly gathered from a single population located in Santo Antão (S 29° 37′; W 53° 52′), municipality of Santa Maria, Rio Grande do Sul, Brazil, between August and December, 2014. A voucher specimen was deposited at the Herbarium of the Forest Science Department (HDCF 6720), UFSM, Brazil.

2.3. Histochemical tests

Transverse hand sections in middle portions of the leaves and petals were made to investigate the main compound classes (Table 1) and their respective location in material (Table 2). The classes observed in plant material were investigated following histochemical tests described in the corresponding references, considering negative and positive controls: Sudan Black B, Sudan III, Neutral Red and Sudan Red 7B for total lipids (Brundrett et al., 1991; Kirk 1940; Pearse 1972), Nile Blue A for acid/neutral lipids (Cain 1947), NADI reagent for EO and oil-resin (David and Carde 1964), Comassie Blue for protein (O'Brien and McCully 1981), lugol for starch (Gabe 1968), potassium dichromate and ferric chloride for phenolic compounds (Gabe 1968; Johansen 1940), vanillin hydrochloric for tannins (Mace and Howell, 1974), aluminum chloride for flavonoids (Charriére-Ladreix, 1976) and Wagner reagent for alkaloids (Furr and Mahlberg 1981). Testimonial materials of leaves and petals were also analyzed.

Table 1
Histochemical characterization of the main classes of compounds present in fresh leaves and petals of *Hesperozygis ringens*.

Target	Test	Color	Leaves	Petals
Total lipids	Sudan black B[1]	Black	+ +	+ +
	Sudan III[1]	Orange	+ +	+ +
	Neutral red[2]	Green	+ +	+ +
	Sudan red 7B[3]	Red	+ +	+ +
Acid/Neutral lipids	Nile blue A[4]	Blue/Red	+	+
EOs and resins	NADI reagent[5]	Blue (EOs)/Red (Resins)/Purple (oil-resin)	+ +	+ +
Protein	Comassie blue[6]	Blue	+ +	+ +
Starch	Lugol[7]	Brown	–	–
Phenolic compounds	Potassium dichromate[8]	Brown-orange	+ +	+ +
	Ferric chloride[7]	Brown-orange	+	+ +
Tannins	Vanillin hydrochloric[9]	Red	–	–
Flavonoids	Aluminum chloride[10]	Green-yellowish	+	+
Alkaloids	Wagner reagent[11]	Reddish brown	+ +	+

Abbreviations: EOs = Essential oils; [1] = ; Pearse, 1972; [2] = Kirk, 1940; [3] = ; Brundrett et al., 1991; [4] = ; Cain, 1947; [5] = ; David and Carde, 1964; [6] = ; O'Brien and Mccully, 1981; [7] = ; Gabe, 1968; [8] = ; Johansen, 1940; [9] = ; Mace and Howell, 1974; [10] = Charriére-Ladreix, 1976; [11] = ; Furr and Mahlberg, 1981. + + = strong reaction; + = weak reaction; – = negative reaction.

2.4. Microscopic analysis and photographic record

Observation of the histological slides and photomicrographs was performed under a Leica DM2000 light microscope in bright field and polarized light. Analysis, dissection, and photographic record were also performed under a Leica M80 stereomicroscope. For both microscopes mentioned, the photographic records were performed through the Leica DFC 295 digital capture system and software LAS 4.0 (Leica™). In addition, slides were also observed under a Zeiss AxioImager A2 microscope, and photographic records were performed through the Zeiss MCr digital capture system and software ZEN (Zeiss™).

2.5. Essential oil extraction and chemical characterization

The EOs from fresh leaves and inflorescences were extracted in triplicate by hydrodistillation using a Clevenger-type apparatus for 2 h, and their yields were determined as % w/w on fresh weight basis (Pinheiro et al., 2016, 2017). The chemical composition of the extractives was performed using an Agilent 7890 gas chromatograph coupled to an Agilent 5975C mass selective detector. The EOs were quantitatively characterized by gas chromatography with flame ionization detector (GC-FID), and qualitatively by gas chromatography coupled to mass spectrometry (GC–MS), using the same equipment conditions reported by Pinheiro et al. (2016). The chemical constituents detected in the EOs were identified by comparison of the obtained retention indices and of the fragmentation pattern of the mass spectra with the literature data (Adams, 2009; NIST, 2010).

Table 2
Specific localization of the main classes of compounds present in fresh leaves and petals of *Hesperozygis ringens*.

Target	Test	Leaves						Petals					
		D		F		V		D		F		V	
		1	2	1	2	1	2	1	2	1	2	1	2
Total lipids	Sudan black B	+ +	+ +	+ +	+ +	−	−	+ +	+ +	−	−	−	−
	Sudan III	+ +	+ +	+ +	+ +	−	−	+ +	+ +	−	−	−	−
	Neutral red	+ +	+ +	+ +	+ +	−	−	+ +	+ +	−	−	−	−
	Sudan red 7B	+ +	+ +	+ +	+ +	−	−	+ +	+ +	−	−	−	−
Acid/Neutral lipids	Nile blue A	+ +	+ +	+ +	+ +	−	−	+ +	+ +	−	−	−	−
EOs and resins	NADI reagent	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	−	−
Protein	Comassie blue	+	−	+	−	+	−	+	+	−	−	−	−
Starch	Lugol	−	−	−	−	−	−	−	−	−	−	−	−
Phenolic compounds	Potassium dichromate	+ +	−	+ +	−	−	−	+ +	−	−	−	−	−
	Ferric chloride	+ +	−	+	−	−	−	+ +	−	+	−	−	−
Tannins	Vanillin hydrochloric	−	−	−	−	−	−	−	−	−	−	−	−
Flavonoids	Aluminum chloride	+	−	+	−	−	−	+	−	−	−	−	−
Alkaloids	Wagner reagent	+ +	+ +	+ +	−	−	−	+	−	+	−	−	−

Abbreviations: D = Dermal; F = Fundamental; V = Vascular; EOs = Essential oils; 1 = Structural; 2 = Secretion; + + = strong reaction; + = weak reaction; − = negative reaction.

3. Results

Places of *H. ringens* occurrence are usually characterized by sandy or rocky soil in rock elevations (Fig. 1a). The species is a woody herb, and its individuals may vary in size and reach a height of 50 cm (Fig. 1b).

3.1. General aspects of leaves and petals

The leaves of *H. ringens* are amphistomatic and present the epidermis with a single cell layer in both surfaces. The leaf surface presents glandular and non-glandular trichomes. The glandular ones are characterized by the presence of a single basal cell, a single stalk cell, and eight secretory cells radially arranged. These structures occur below epidermis level due to its modification and nearby mesophyll (Fig. 2a).

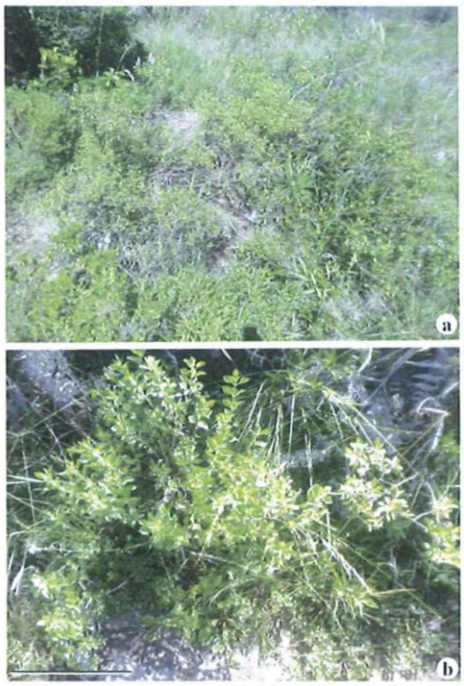

Fig. 1. Characterization of *Hesperozygis ringens* studied population, in Santa Maria, Rio Grande do Sul. A, Occurrence site of the species. B, An individual of *H. ringens*. Scale bar: (B) 25 cm.

3.2. Distribution and identification of chemical classes

3.2.1. Leaves

Results of histochemical tests performed for fresh leaves and petals of *H. ringens* are presented in Table 1. Results of specific localization of the main classes of compounds in leaves are shown in Table 2. Sudan III detected structural lipids in periclinal cell walls of the epidermis, without distinction between cellulosic cell wall, middle lamella, cuticle and epicuticular lipophilic substances. In addition, storage lipids were detected in the mesophyll and inside secretory cells of glandular trichomes (Fig. 2a). The lipid droplets vary between 2 and 15 μm in diameter. There are usually several droplets of different sizes, which may be aggregated or dispersed in the palisade and spongy parenchyma. Using Sudan III reagent, two different colors can be visualized. Lipid drops in glandular trichome cytoplasm had red color, while the mesophyll ones had orange (Fig. 2a). Sudan red 7B also stained structural and storage lipids in the mesophyll (Fig. 2b) and inside secretory cells of glandular trichomes (Fig. 2c). Storage and structural lipids were also marked in the same structures by Sudan Black B (Fig. 2d and e) and Neutral Red (Fig. 2f).

After Nile blue A reaction, neutral (pink) and acid (blue) lipids were detected in mesophyll (Fig. 2g) and stained predominantly neutral ones in the subcuticular space of glandular trichomes (Fig. 2h). NADI reagent positively stained for oil-resin in the mesophyll and secretion in secretory cell of glandular trichomes (Fig. 2i). A similar color for EO was detected in cuticle (Fig. 2i) in the walls of stalk cells of glandular trichomes (Fig. 2j), as well as in drops in the vascular bundles (Fig. 2l). Drops stained by NADI reagent in the mesophyll presented a thin layer of EO around oil-resin (Fig. 2m).

The indicative of phenolic compounds presence was obtained with potassium dichromate through the detection of brown-orange drops in the mesophyll cells of the leaf and in secretory cells of glandular trichomes. Ferric chloride also positively stained the phenolic compounds in mesophyll, but in apparent smaller abundance than the potassium dichromate. Moreover, it stained the walls of stalk cells of glandular trichomes. Vanillin hydrochloric did not detect tannins. After aluminum chloride, flavonoids were detected only in the walls of epidermal cells, in stalk cells of glandular trichomes, and in mesophyll cells. The Wagner reagent indicated the occurrence of alkaloids in cuticle, including secretory cells of the glandular trichomes and their secretion

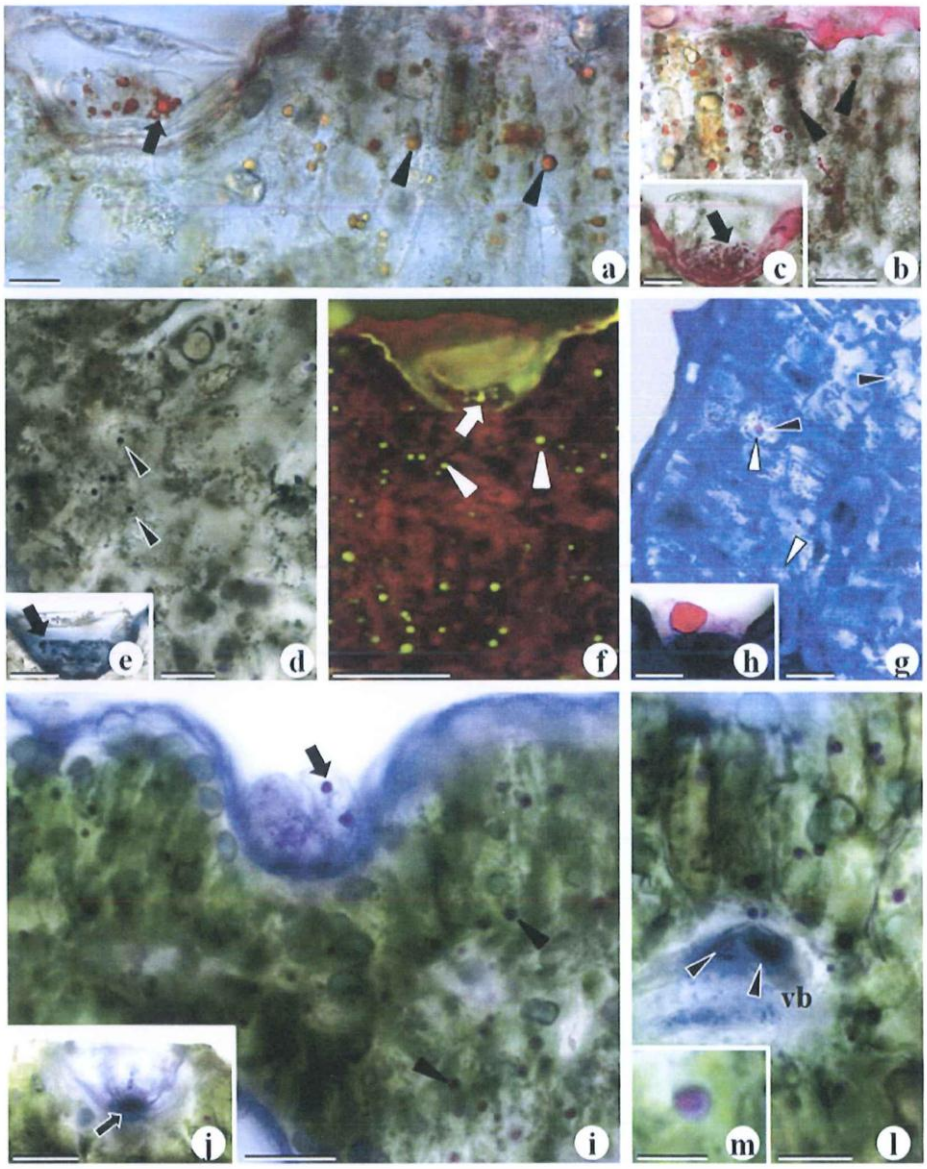

Fig. 2. Total lipid histochemical characterization in *Hesperozygis ringens* fresh leaf. (a) Staining of Sudan III for lipids in mesophyll (arrow heads) and glandular trichomes (arrow). (b, c) Staining of Sudan Red 7B for lipids in mesophyll (arrow heads) and glandular trichomes (arrow), respectively. (d, e) Positive staining of Sudan Black B for lipids in mesophyll (arrow heads) and glandular trichome (arrow), respectively. (f) Positive staining of Neutral Red for total lipids in mesophyll (arrow heads) and glandular trichome (arrow). (g, h) Positive staining of Nile Blue A for neutral (black arrow heads) and acid (white arrow heads) lipids in mesophyll and glandular trichome, respectively. (i, j, l, m) Positive staining of NADI reagent for essential oil and oil-resin in mesophyll (arrow heads), glandular trichomes (arrow) and vascular bundles (vb). Scale bars: (m) 40 μm, (a, f and i) 100 μm, (c, d, e, and l) 150 μm, (b, h, g, j) 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

(Table 2).

3.2.2. Petals

The specific localization of the main classes of compounds in petals is presented in Table 2. Sudan III and Sudan Black B stained drops of lipophilic compounds in non-glandular trichomes, in which these classes of substances accumulate (Fig. 3a and e) and positively stained in stalk cells of glandular trichomes (Fig. 3b). Sudans Red 7B and Black B also detected lipids as drops in stalk cells of glandular trichomes (Figs. 3c and e) and cutinized portions in non-glandular trichomes (Fig. 3d). Secretion remains stainable after its release from glandular trichomes (Fig. 3d and f). Cutinized portions of epidermal cells were also marked by Sudan Black B (Fig. 3g). The cuticle in non-glandular trichomes intensively reacted when compared to pavement cells after applying Sudan III, Sudan Red 7B, and Neutral Red (Figs. 3a, c, d, h). A similar reaction was observed in stalk cells of glandular trichomes.

Nile Blue A detected acid (blue) and neutral lipids (pink) as drops in epidermis, including its glandular trichomes and cuticle, whereas only

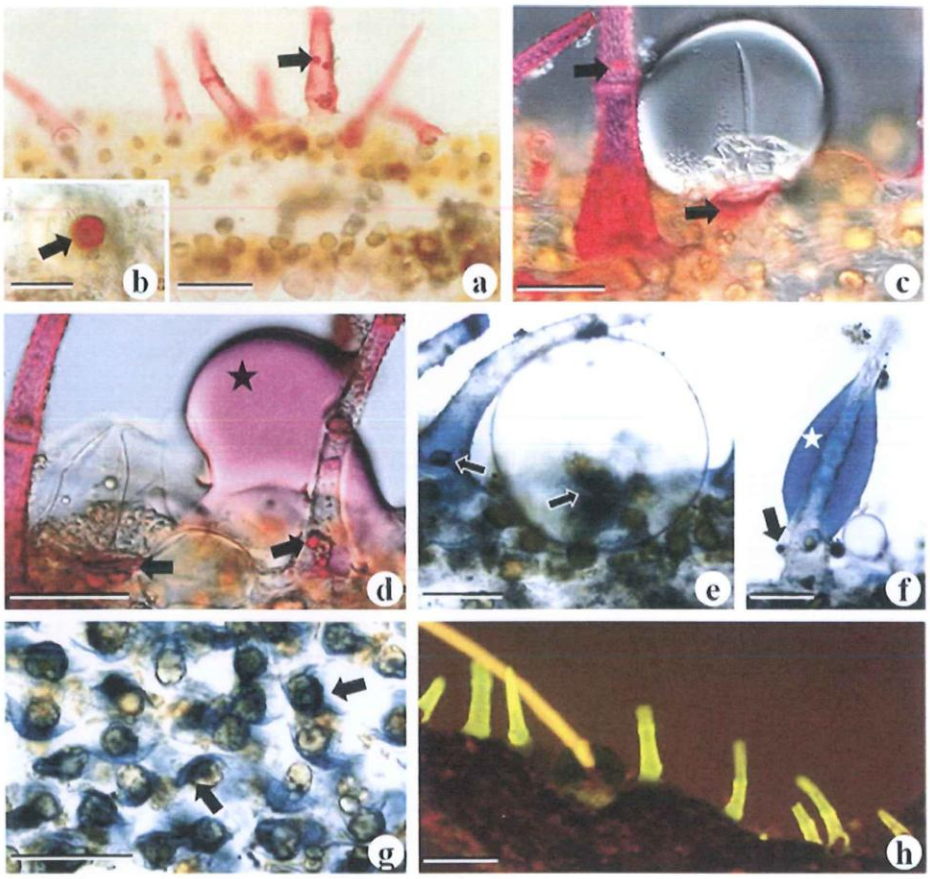

Fig. 3. Total lipid histochemical characterization in *Hesperozygis ringens* fresh petal. (a, b) Positive staining of Sudan III for total lipids (arrows). b = frontal view. (c, d), Positive staining of Sudan Red 7B for total lipids in glandular and non-glandular trichomes (arrows), and secretion (star). c = lateral view. (e-g), Positive staining of Sudan Black B for total lipids in petal (arrows), and secretion (star). (h), Fluorescence. Positive staining of Neutral Red for total lipids (arrows). Scale bars: (h) 100 μm, (a) 150 μm, and (b–g) 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

drops of acid lipids were observed in non-glandular trichomes (Fig. 4a–d). Acid lipids typically accumulate in secretory cells of glandular trichomes and take part of the chemical composition of its walls.

After NADI reaction, a high number of small EO drops were observed in epidermal cells, non-glandular trichomes and secretory cells of glandular trichomes. The stalk cells of these structures were also stained (Fig. 4e–j). The coloring pattern also indicates the presence of mixtures composed of EO and oil-resins in relatively larger drops accumulated in subcuticular space of glandular trichomes (Fig. 4h). Furthermore, the test indicated that the chemical composition of secretory cell walls is variable. There may be both mixtures of substances (Fig. 4i) and only EO (Fig. 4j), while in non-glandular trichomes and other epidermal cells, only EO are possible to verify (Fig. 4e–g).

Tests for starch and protein reserves were negative (Table 2). Phenolic compounds stained positively in pavement epidermal cells and stalk cells of its glandular trichomes. On the other hand, epidermal cells in petals did not react for tannins after reaction with vanillin hydrochloric. Aluminum chloride detected flavonoids in cuticle of both non-glandular and glandular trichomes. Alkaloids were detected as drops in petal epidermal cells and in the stalk cells of glandular trichomes (Table 2).

3.3. Essential oils characteristics

The EO obtained from fresh leaves presented a yield of 3.08%, while the one obtained from inflorescences was 4.45%. The densities presented by leaves and inflorescences extractives were 0.94 and 0.90 gmL^{-1}, respectively. The chemical characterizations of both EOs showed pulegone as the major compound (Table 3). In leaf extractive, the oxygenated monoterpenoid represented 79.94% of the total composition. In the EO obtained from inflorescences, pulegone presented a percentual of 63.86%. For both samples, the major chemical class was the oxygenated monoterpenoids.

4. Discussion

This study demonstrated the wide distribution and occurrence of lipids in the leaves and petals of *H. ringens*. Sugars in starch form or reserve proteins were not detected. The protein detected in the tests indicate only the ones common to cells, with structural or enzymatic functions, but not as protein grains with metabolic reserve. Lipids were found in glandular and non-glandular trichomes. However, they occur in greater quantity in the mesophyll, since an apparently higher volume in this plant tissue was observed when compared to epidermis and vascular bundles. In addition, almost all mesophyll cells accumulate

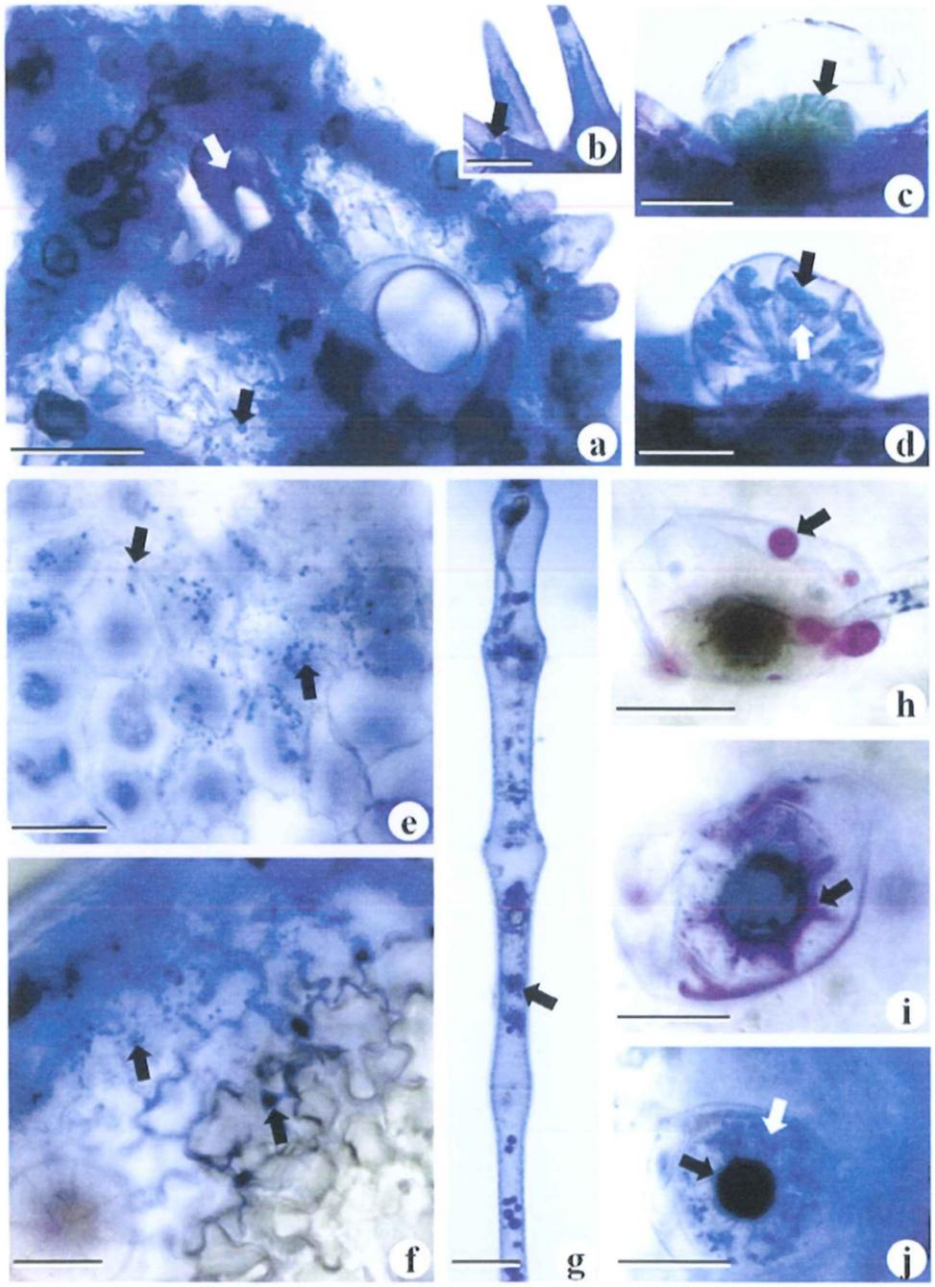

Fig. 4. Total lipid histochemical characterization in *Hesperozygis ringens* fresh petal. (a–d) Positive staining of Nile Blue A for neutral (white arrows) and acid (black arrows) lipids. c and d = lateral views. (e–j), Positive staining of NADI reagent (arrows) for essential oil and oil-resin. h, i and j = frontal views. Scale bars: (d) 100 μm, (c, e–i) 200 μm, and (j) 260 μm, and (a, b) 300 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

this class of substances. Glandular trichomes in the leaves and petals are identical in structure and similar in chemical composition, in both the cell walls and cytoplasmic content. The same occurred for non-glandular trichomes. These structures also showed EOs and oil-resins in their cells. The main differences between the leaves and petals are the absence of total, acid and neutral lipids, both storage and structural ones, in the petal mesophyll and the predominant presence of lipid drops in petal epidermal cells.

Lipids and lipophilic substances are often found in different structures of Lamiaceae species. However, they have always been considered predominantly superficial in glandular trichomes, as previously described for *Rosmarinus officinalis* L. sepals (Bottega and Corsi, 2000), leaves of *Satureja horvatii* Silic (Marin et al., 2012), *Micromeria thymifolia* Fritsch (Marin et al., 2013) and *Ocimum obovatum* E. Mey. ex

Table 3
Chemical characterization of essential oils extracted from leaves and inflorescences of *Hesperozygis ringens*.

Compound	Retention Index			Percentual		
	Leaves	Inflorescences	Literature[a,b]	Leaves	Inflorescences	Classes
α-Pinene	932	931	930[a]	0.45	0.23	MH[c]
Sabinene	971	–	970[a]	0.30	–	MH
β-Pinene	974	–	968[a]	0.47	–	MH
β-Myrcene	989	–	988[a]	0.43	–	MH
Limonene	1027	1027	1022[a]	2.71	1.62	MH
β-Z-ocimene	1047	1048	1041[a]	0.89	3.20	MH
Linalool	1099	1099	1099[a]	1.60	0.74	OM[d]
Isopulegol	1165	–	1165[a]	1.72	–	OM
Isopulegone	1177	1173	1177[a]	1.62	2.62	OM
α-Terpineol	1195	–	1197[a]	0.30	–	OM
Verbenone	1211	1209	1207[a]	0.32	2.67	OM
2.6-Dimethyl-3.5.7-octatriene-2-ol. *.E.E-*	–	1217	1209[a]	–	3.56	OM
Pulegone	1251	1246	1244[a]	79.94	63.86	OM
α-Terpineolacetate	–	1347	1351[a]	–	1.80	SH[e]
Phenyl ethyl isobutanoate	–	1384	1394[b]	–	0.94	P[f]
β-Caryophyllene	1423	1424	1418[a]	0.72	0.71	SH
α-Caryophyllene	–	1432	1438[a]	–	0.50	SH
t-Elemene	–	1493	1484[a]	–	2.42	SH
Germacrene D	1497	–	1496[a]	0.38	–	SH
Spathulenol	1583	1579	1578[a]	0.70	0.49	OS[g]
Caryophyllene oxide	1586	–	1581[a]	0.23	–	OS
Viridiflorol	1589	–	1590[a]	1.07	–	OS
Identified components				**93.95**	**85.42**	

References: [a]NIST; [b]Adams; [c]MH = Monoterpene hydrocarbon; [d]OM = Oxygenated monoterpenoid; [e]SH = Sesquiterpene hydrocarbon; [f]P = Phenylpropanoid; [g]OS = Oxygenated sesquiterpenoid.

Benth. (Naidoo et al., 2013), in addition to the leaves, bracts, and perianth of *Plectranthus grandidentatus* Gurke (Mota et al., 2013), and *Stachys tymphaea* L. inflorescences (Venditti et al., 2014). Considering the diversity of protective functions attributed to glandular trichomes and their secreted substances, their superficial occurrence seems to be enough for the aforementioned species, in contrast to the *H. ringens* case, which also demonstrates a great accumulation of substances in the mesophyll. However, the presence of lipid drops in the leaf mesophyll was also described for different Lamiaceae representatives, including *Physostegia virginiana* (L.) Benth. (Lersten and Curtis, 1998; Lersten et al., 2006). In this context, an investigation encompassing all tissues in the Lamiaceae leaves is recommended, since Lersten et al. (2006) indicated that lipid analysis in the leaf mesophyll is usually neglected.

In this study, total lipids were detected in structural and storage forms in both leaf and petal. Together with EOs, other lipophilic substances were produced by glandular trichomes of Lamiaceae species, such as flavonoid aglycones (Mota et al., 2013) as well as waxes, and fats (Evert, 2006) which may also play roles in chemical protection against herbivores and pathogens (Werker, 2000). Among lipophilic compounds, EOs and oil-resins were found in both plant organs analyzed in our study. While EOs contain volatile low-molecular-weight terpenoids, oil-resin is characterized by mixtures of volatile and non-volatile terpenoids (Fahn, 2000). According to this author, peltate glandular trichomes are considered typical EO producer in Lamiaceae representatives. Although these substances were found in secretory cells of peltate trichomes in *H. ringens*, they were also detected in non-glandular trichome and mesophyll cells of vegetative and reproductive organs.

Acid and neutral lipids were also detected in *H. ringens* leaves and petals. The group of neutral lipids is composed by triacylglycerols, steryl esters and wax esters (Athenstaedt and Daum, 2006; Kunst and Samuels, 2003). Triacylglycerols and steryl esters are unable to integrate phospholipid bilayers. For this reason, these substances are grouped together and form a hydrophobic core of specialized cellular compartments for lipid storage (Athenstaedt and Daum, 2006). According to the authors, these compartments are known as lipid particles, droplets or bodies, oil bodies or oleosomes. Lipid droplets are involved in a great number of processes such as hormone metabolism, stress response and pathogen resistance (Chapman et al., 2012). Lipid bodies may occupy much space in the leaf cell cytoplasm and they usually become apparent when they accumulate at high levels (Murphy, 2001). Chapman et al. (2012) mention that these droplets are frequently associated in seeds with proteins such as oleosins, caleosins, and steroleosins.

The function of glandular trichomes varies according to the secreted substance (Werker, 2000). In Lamiaceae, glandular trichomes are considered the site of synthesis and accumulation of monoterpenoids rich EOs (Hallahan, 2000) and these structures are among the most analyzed ones (Giuliani and Bini, 2008). Nonetheless, despite studies about the biological potential of *H. ringens* EO, structural analysis about its glandular trichomes and their produced substances has not been found. Non-glandular trichomes distributed on *H. ringens* leaves and petals may also present defense mechanisms and their functions, as already described for glandular trichomes, depend on some factors, such as structure location (Werker, 2000).

Peltate trichomes are characterized by the presence of a single basal cell, a single stalk cell, and four or more head cells (Hallahan, 2000). These characteristics are in accordance to morphological features of glandular trichomes found in our study, which presented eight head cells. According to Fahn (2000), anticlinal cell walls of the stalk cells in this kind of trichomes are commonly completely cutinized. Acid lipids as drops in the cytoplasm, alkaloids, phenolic compounds, terpenoids and flavonoids were detected in vacuoles of *H. ringens* cells. In addition, these classes of compounds are observed in apparent higher abundance when compared to other portions of the same structure. In the testimonial material, some trichomes of brown color were observed, while others were translucent. However, both types presented the same classes of accumulated compounds. A natural orange color in peltate glandular trichomes was found in the Lamiaceae species (Mota et al., 2013). In the representatives of this family, peltate glandular trichomes figure as production and accumulation sites of the main monoterpenoids that compose their EOs (Hallahan, 2000). This type of trichome is frequently described in different organs of Lamiaceae species (Mota et al., 2013; Turner et al., 2000; Werker, 2000), such as in the

leaves of *P. grandidentatus* (Mota et al., 2013) and flowers of *Salvia farinacea* Benth. (Zhang et al., 2014). In peltate glandular trichomes, the produced substances are gradually secreted from the head cells into the subcuticular space (Werker, 2000). If touched, the cuticle may rupture, releasing the produced substances with a repellent effect (Werker, 1993). Bottega and Corsi (2000) also suggested mechanical and chemical defense functions against pathogens and herbivores for glandular trichomes in *R. officinallis*.

NADI reagent identified the presence of EOs and oil-resins in glandular trichomes and in the leaf mesophyll of both *H. ringens* fresh materials. The abundance of EOs detected by histochemical analysis and extracted by hydrodistillation in present study is in agreement with literature data, considering that *H. ringens* leaves are notable for their oil production, since they present yields above 1.15% (Pinheiro et al., 2016) and reaching 4.0% (Von Poser et al., 1996). Although no reports have been found in literature on inflorescence EO, the results described in the present study demonstrate that this plant organ also produces EO in abundance. The high yields found for the extractives obtained from leaves and inflorescences can be explained by the apparent large amount of EOs not only in glandular trichomes, but also in mesophyll. Pulegone was detected as the major compound in leaves and inflorescence EOs. This finding is in agreement with the literature since this oxygenated monoterpenoid represents more than 53.93% of the EO chemical composition of leaves (Pinheiro et al., 2016). However, the chemical composition of inflorescence EO had not been described so far. Pulegone is known for its biological activities, such as allelopathic (Mucciarelli et al., 2001), insecticide (Rossi et al., 2012), fungicidal (Gata-Gonçalves et al., 2003), and acaricidal (Ribeiro et al., 2010). Despite the large amount of oil-resin detected in this species, its composition is still unknown. The abundance of EO and oil-resin found in mesophyll of leaves and petals may serve as a characteristic for *H. ringens* identification, since similar results have not been found in the literature for any other Lamiaceae species. Moreover, representatives belonging to this family are renowned for EOs production in glandular trichomes on leaves and inflorescences (Harley et al., 2004).

Phenols were already detected by histochemical tests in the Lamiaceae species (Bottega and Corsi, 2000; Marin et al., 2012; Mota et al., 2013; Jing et al., 2014), including in peltate glandular trichomes (Marin et al., 2013). For *H. ringens*, the presence of phenolic compounds was also identified in both plant materials analyzed. Extracts of Lamiaceae species rich in phenolic compounds have already demonstrated different biological activities, such as antioxidant (Nicolai et al., 2016), fungicidal and insecticidal (Boulogne et al., 2012).

Within the phenolic group, flavonoids were also reported in our study, in both leaf and petal. The class may also present bioactivities providing a defense mechanism for *H. ringens*. Flavonoids isolated from Lamiaceae representatives presented antiprotozoal activities against *Leishmania donovani*, *Trypanosoma brucei rhodesiense* and *T. cruzi* (Tasdemir et al., 2006). Aerial parts of *Salvia chloroleuca* Rech. F. & Allen extracts rich in flavonoids are known for their antioxidant activity (Salimikia et al., 2016). According to Asghari et al. (2015), this class of compounds demonstrated α-amylase and α-glucosidase inhibitory activities. The enzymatic inhibition, especially because it affects the enzymes responsible for the degradation of starch in the digestive tract of insects also serves as part of plant defense system (Franco et al., 2002) by reducing the animal digestive capacity (Mello and Silva-Filho, 2002). Flavonoids are associated to UV-B light absorption and play a protective function against radiation excess (Dolzhenko et al., 2010; Logemann et al., 2000) adding another function to this class of compounds. Lamiaceae species from arid and semi-arid habitats tend to accumulate external flavonoid aglycones, unlike species from humid or mesic habitats, which suggests the class role in adaptation of species to stress (Tomás-Barberán and Wollenweber, 1990). The abundance in the species from other families occurring in similar habitats reinforces the idea of an adaptive function of the compound class (Wollenweber et al., 1987; Wollenweber and Dörr, 2008). Moreover, some flavonoids such as flavones and flavonols may attract insects, including pollinating bees (Ferreira et al., 2008).

Regarding the alkaloids detected in *H. ringens*, this class of compounds was previously described in Lamiaceae, highlighting detection in peltate trichomes (Bottega and Corsi, 2000; Naidoo et al., 2013; Jing et al., 2014), as identified in our study. According to Jing et al. (2014), a number of toxic alkaloids are used by plants as protection against other organisms, representing an ecological role. Lamiaceae species produce different kinds of alkaloids such as phenolic and diterpenoid ones (Dai et al., 2007; Wang et al., 2012).

In general, the literature describes functional specificities in the epidermis for the different substances, usually associated to stress factors. Plant cuticular waxes may present lipids such as terpenoids and minor secondary metabolites (Kunst and Samuels, 2003), and their composition and quantity may vary according to different aspects such as UV-B radiation intensity (Barnes et al., 1996) and species location (Jeffree, 2006).

No diversity of trichomes was observed when comparing leaves and petals in both structural and glandular aspects. Histochemical tests detected total, neutral and acid lipids, EOs, oil-resins, phenolic compounds, flavonoids and alkaloids in plant materials. Results obtained in this investigation showed the diversity of chemical classes of compounds produced by the leaves and petals of *H. ringens* as both structural components and secretion. The large accumulation of lipids in mesophyll cells was peculiar to the species. Although in less quantity, they were also found in cells of vascular tissues. Factors such as temperature and ultraviolet radiation may stimulate the production of secondary metabolites (Gobbo-Neto and Lopes, 2007), the apparent lower amount of metabolites in petals is probably associated with the shorter period of exposure to the environment. This is a typical characteristic of the ephemerality in the flowering period in general, and may be an accessory to the reproductive process. In this context, the functionality of compounds associated with the respective amount is suggested when comparing leaves and petals. No details regarding the interaction of the plant with biotic and abiotic factors were analyzed in this work. However, since *H. ringens* is on the list of endangered species, the discovery of the produced phytochemical compounds may stimulate new research for its conservation. Remarkable characteristics such as the abundant presence of EOs and oil-resins in mesophylls of leaves and petals may help in the species identification with the objective of avoiding losses of individuals due to cuts, burnings, and other factors. The results confirmed that the species inflorescences produce high amounts of EO rich in pulegone, as well as already described for the leaves. Additionally, this study adds information about classes of compounds produced by the genus, since no reports of histochemical tests have been found for other *Hesperozygis* representatives.

5. Conclusion

Results obtained demonstrate the high diversity of compounds produced by *H. ringens* leaves and petals. Among the detected classes, lipids, EO, and oil-resin stand out not only in glandular trichomes, but also in plant mesophyll. The EOs extracted from leaves and petals demonstrated the oxygenated monoterpenoid pulegone as the major compound. Histochemical results present characteristics that have not been found in literature for other Lamiaceae species, and may serve as identification characters of *H. ringens*. Considering the diversity of metabolites, their location sites, and literature data for Lamiaceae, this study allows us to postulate that the detected metabolites present functions related to the daily interaction of the individuals with the environment, usually related to overcoming of different stressors.

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4 ARTIGO 3 – METHOD FOR EVALUATION OF THE ERGOSTEROL CONTENTS IN WOOD-DECAY FUNGI

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ABSTRACT

The aim of this study was to optimize a method for ergosterol evaluation in wood decay fungi. The white-rot species selected were *Ganoderma applanatum* and *Trametes versicolor*, while the brown-rot ones were *Gloeophyllum trabeum* and *Lentinus lepideus*. For the major sterol access, discs of mycelia culture of each species were transferred to Petri dishes containing cellophane membrane-covered medium. Mycelia of each fungus were collected, their wet wt were obtained, and they were transferred to test tubes with 5 mL 25% alcoholic potassium hydroxide. The tubes content were vortex mixed for 5 min and submitted to ultrasound for 5 min. The tubes were incubated at 85ºC for 4 h, and afterwards, 2 mL of sterile distilled water and 5 mL of *n*-heptane were added, following ultrasound shaking for 2 min. The *n*-heptane layer was analyzed by UV spectrophotometry. Control group containing only *n*-heptane was also analyzed. The mycelia wet wt of the fungi ranged between 0.061 and 0.296 g. *Lentinus lepideus* showed an ergosterol content of 0.007%, while the other species presented 0.004% of this compound. For all fungi the absorbance values were higher than the ones observed in control group. The adapted method was efficient for ergosterol extraction.

Keywords: White-rot; brown-rot; major sterol; cellophane membrane; mycelia wet wt; UV spectrophotometry.

INTRODUCTION

Wood-decay fungi can cause serious damage in wooden structures and it may result in considerable economic and resources losses (CHENG et al., 2008). These fungi belong to Basidiomycetes and may be classified as white and brown-rot (OLIVEIRA et al., 2005). The first group is characterized by the capacity to degrade lignin, hemicellulose and cellulose (MARTÍNEZ et al., 2005), and after the fungi attack, the decayed wood becomes white (MCCARTHY; COOKSON; SCOWN, 2009). The second group gives to the attacked wood brown coloration (OLIVEIRA et al., 2005), and these fungal species are characterized by the degradation of wood polysaccharides (MARTÍNEZ et al., 2005).

Ergosterol is considered the major sterol in the fungal membrane (HU et al., 2017) and presents important functions for its growth (DE LIRA MOTA et al., 2012). Among these, stand out the contribution to membrane fluidity and integrity, and the support to the normal operation of membrane-bound enzymes (LUPETTI et al., 2002). The effect of a product on ergosterol content in plasma membrane is a known mechanism of antifungal action (AVANÇO et al., 2017; KEDIA et al., 2014; TIAN et al., 2012).

The aim of this study was to optimize a method for ergosterol evaluation in wood rotting fungi. For method development, the white-rot fungi *Ganoderma applanatum* and *Trametes versicolor*, in addition to the brown-rot fungi *Gloeophyllum trabeum* and *Lentinus lepideus* were selected.

MATERIALS AND METHODS

Fungal growth in PDA with and without cellophane membrane

The two white-rot fungi *Ganoderma applanatum* and *Trametes versicolor*, and the two brown-rot ones *Gloeophyllum trabeum* and *Lentinus lepideus* were provided by the Forest Products Laboratory (Laboratório de Produtos Florestais), situated at Universidade de Brasília, DF, Brazil. The species were initially cultivated in Petri plates (9 cm of diameter) containing potato-dextrose-agar (PDA) covered with a cellophane membrane (BENTO et al., 2014) and also without it, in order to verify if this membrane would influence the mycelia growth. Discs of mycelial culture (11 mm of diameter) of each fungi species were transferred to the Petri dishes (in three repetitions). Then, these were sealed with parafilm and remained in Biochemical Oxygen Demand (BOD) chamber at 25°C (±1), in light/dark (12h/12h). The first evaluation occurred on the seventh day after the beginning of the experiment, through two measurements diametrically opposed of the fungal colony. The test was finished when the fungal growth of each dish completely covered the medium (BADAWY; ABDELGALEIL, 2014). The data normality and variances homogeneity were verified, and then the ANOVA one-way test was performed and the means were compared by Tukey test using SigmaPlot 11.0 software.

Analysis of ergosterol content in plasma membrane of the wood-decay fungi

The white-rot fungi *G. applanatum* and *T. versicolor*, and the brown-rot ones *G. trabeum* and *L. lepideus* were firstly cultivated in Petri dishes containing PDA, for a period of fifteen days, in BOD chamber at 25°C (±1) in light/dark (12h/12h). Discs of mycelia culture (11 mm of diameter) of each fungal species were transferred to Petri dishes containing cellophane membrane-covered PDA (BENTO et al., 2014). After, the dishes were sealed and moved to BOD chamber where they remained for 5 days. All treatments were realized in three repetitions.

For mycelia ergosterol extraction, an adaptation of Arthington-Skaggs et al. (1999) and Tian et al. (2012) method was developed. After the incubation period, mycelia of each wood-decay fungi were harvested and their wet wt were verified. Afterwards, the fungal mycelia, with 5 mL of 25% alcoholic potassium hydroxide was transferred to test tubes. They were vortex mixed (Velp Scietifica, Vortex Mixer, Wizard X) for 5 min, and then, transferred to ultrasound (Ultra Cleaner 1450A with heating. Unique Ultrasonic Clean), where the tubes remained for 5 min. After, they were incubated at 85ºC for 4 h and then, 2 mL of sterile distilled water and 5 mL of *n*-heptane were added to the test tubes, which were stay in ultrasound bath for 2 min. The solution layers corresponding to each fungus were separated in funnels for 1 h at room temperature. The *n*-heptane layer of each tube was collected and analyzed by UV spectrophotometry (Biospetro sp-220) between 230 and 300 nm. The absorbance at 282 nm is characterized the presence of ergosterol in *n*-heptane layer, while the reading obtained at 230 and 282 nm corresponded to the late sterol intermediate 24(28) dehydroergosterol (TIAN et al., 2012). Controls containing only *n*-heptane were also analyzed by UV spectrophotometry at the same wavelengths.

The calculation of ergosterol amount was performed based on the fungal mycelia wet wt (g) and absorbance values obtained, considering the formulas adapted from Tian et al. (2012): *% 24(28) dehydroergosterol = (A230/ 518)/ mycelia wet wt*; *% ergosterol + % 24(28) dehydroergosterol = (A282/ 290)/ mycelia wet wt*. For the described formulas, 290 and 518 are considered the E (%/cm) values for crystalline ergosterol and 24(28) dehydroergosterol. Graphics and analysis were obtained using SigmaPlot 11.0 software.

RESULTS AND DISCUSSION

After 15 days, all wood-decay fungi completely covered the Petri dishes containing PDA with and without cellophane membrane. The cellophane membrane was used for growth of wood-decay fungi in the study by Bento et al. (2014), in order to facilitate removal of the mycelium without fragments of the nutrient medium. However, the authors determined the activity of antioxidant enzymes of filamentous fungi in the presence of plant extracts, and they evaluated only the white-rot species *Trametes villosa* and *Pycnoporus sanguineus*. In present study we compared the mycelia growth of *G. applanatum*, *T. versicolor*, *G. trabeum* and *L. lepideus* in PDA with and without the cellophane membrane, and no significant differences were found (Table 1). In some previous described methods, the mycelia are washed in sterile distilled water after the removal from the culture medium (ARTHINGTON-SKAGGS et al., 1999; KEDIA et al., 2014; TIAN et al., 2012). However, the use of cellophane membrane makes removal of the fungal mycelia possible without the need for washing them. This alternative avoids the washing water to influence the mycelia wet wt.

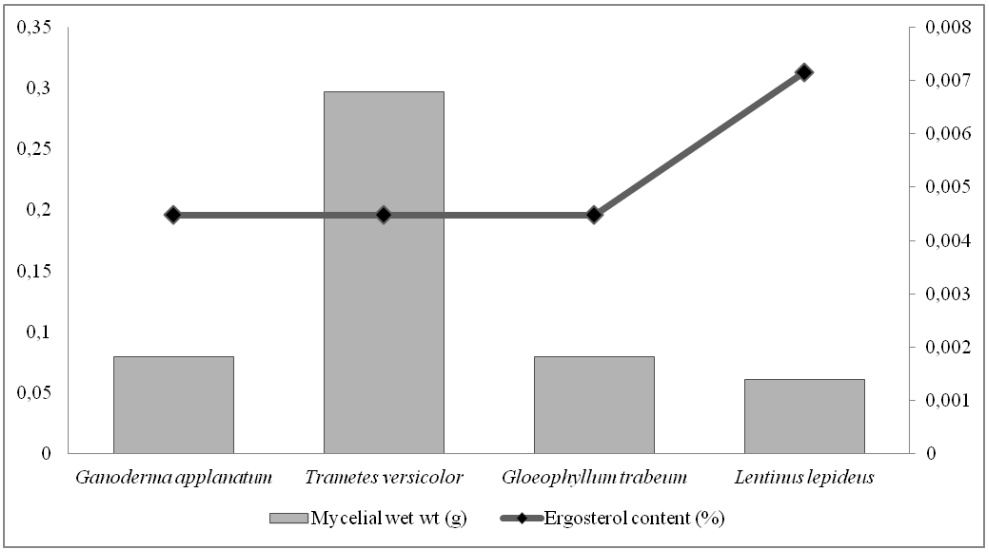
The procedure tested in present study provided mycelial wet weights of 0.079±0.225, 0.296±0.0267, 0.079±0.0225 and 0.061±0.0044 g for *G. applanatum*, *T. versicolor*, *G. trabeum* and *L. lepideus*, respectively (Figure 1). For the first three species, ergosterol content of 0.004±0.0005% was obtained. On the other hand, *Lentinus lepideus* presented an ergosterol amount of 0.007±0.0013% (Figure 1). The percentages of ergosterol varie according to the fungal species (BARAJAS-ACEVES et al., 2002).

Table 1 - Mycelial growth of *Ganoderma applanatum*, *Trametes versicolor*, *Gloeophyllum trabeum* and *Lentinus lepideus* with and without cellophane membrane (N=3).

Cellophane membrane	***Ganoderma applanatum***	***Trametes versicolor***	***Gloeophyllum trabeum***	***Lentinus lepideus***
With	52.242±2.358	44.916±1.062	25.340±0.633	21.628±1.396
Without	54.402±2.165	38.232±2.451	27.363±0.845	16.676±1.697

Source: The authors.

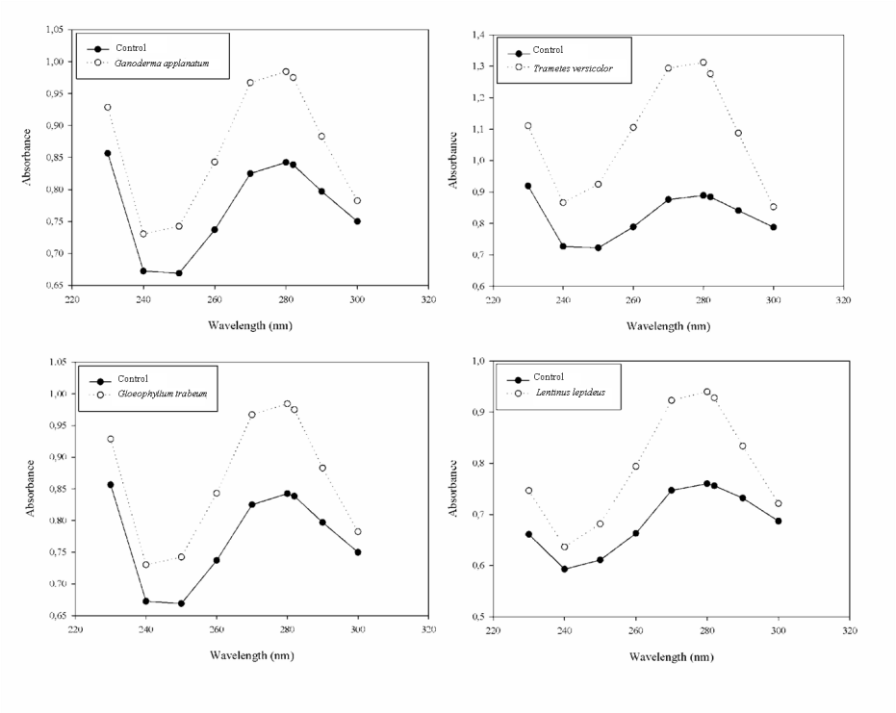
Figure 1 - Mycelial wet wt (primary axis) and ergosterol contents (secondary axis) of different species of wood-decay fungi (N=3).



Source: The authors.

Aiming to demonstrate the efficiency of the ergosterol extraction method, we opted for two ways of results presentation. Not only the percentage of ergosterol achieved for each fungus was demonstrated, but also the UV spectrophotometric profiles of this sterol for each species were presented (Figure 2). For all analyzed treatment samples, the absorbance values read between 230 and 300 nm were higher than the ones verified in controls, which indicates the presence of ergosterol. The readings of absorbance values in this range have been used as indication of ergosterol presence in differet works (BREIVIK; OWADES, 1957; KEDIA et al., 2014; TIAN et al., 2012).

Figure 2 - UV spectrophotometric profile of ergosterol extracted from different wood-decay fungal species (N=3).



Source: The authors.

The literature already presents studies on ergosterol in wood-decay fungi (BARAJAS-ACEVES, 2002; CHEDGY; LIM; BREUIL, 2009; EIKENES et al., 2005; GAO; CHEN; BREUIL, 1993; NIEMENMAA; GALKIN; HATAKKA, 2008; SONG et al., 2012). However, no information was found about the extraction using similar procedure to those used in the present study. However, a similar method have already been used for the extraction of ergosterol from yeasts, as *Saccharomyces cerevisiae* (BREIVIK; OWADES, 1957) and *Candida albicans* (ARTHINGTON-SKAGGS et al., 1999) and also from filamentous fungus *Aspergillus flavus* (KEDIA et al., 2014; TIAN et al., 2012). Since the previously described method was not effective for ergosterol extraction of white and brown-rot fungi considered in present study (data not shown), the optimization of an existing method was necessary.

Besides the use of cellophane membrane, other steps were added and changes were realized in the already described methods (KEDIA et al., 2014; TIAN et al., 2012), in order to extract the ergosterol of the selected species. After the addition of 25% alcoholic potassium hydroxide to the test tubes containing mycelia, the literature recommend a vortex mix for 2 min (KEDIA et al., 2014; TIAN et al., 2012). In our method, the time of vortex mix was extended to 5 min and a period of 5 min in ultrasound was added. After the addition of 2 mL

sterile distilled water and 5 mL *n*-heptane to the test tubes, the known method described a vortex mix for 2 min (KEDIA et al., 2014; TIAN et al., 2012). In present study, we opted to transfer the test tubes to ultrasound for 2 min.

The present study described a method to quantify the ergosterol in plasma membrane of wood-decay fungi. The use of cellophane membrane did not interfere in mycelial growth of the white-rot species *G. applanatum* and *T. versicolor*, and the brown-rot representatives *G. trabeum* and *L. lepideus*. The adaptation and steps addition realized in methods already described in literature improved the technique, which was efficient for ergosterol extraction of wood-decay fungi, aiming its quantitative evaluation.

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5 ARTIGO 4 – *Hesperozygis ringens* (Benth.) Epling ESSENTIAL OIL: ANTIFUNGAL ACTIVITY AND EFFECT ON ERGOSTEROL CONTENT OF WOOD-DECAY FUNGI

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ABSTRACT

This study aimed to evaluate the antifungal potential of the essential oil (EO) from *Hesperozygis ringens* leaves and its major component, pulegone, against the white-rot fungi *Ganoderma applanatum* and *Trametes versicolor*, and the brown-rot fungi *Gloeophyllum trabeum* and *Lentinus lepideus*. The EO was extracted by hydrodistillation and its chemical composition was analyzed by gas chromatography coupled to mass spectrometry and gas chromatography with a flame ionization detector. The oxygenated monoterpenoid pulegone was isolated through column chromatography. The EO and pulegone were diluted in ethanol (1 µL mL^{-1}) prior to the bioassay. The EO was tested at five concentration levels (0.5, 0.75, 1.00, 1.25, and 1.50 µL mL^{-1}), and the pulegone was tested at three concentration levels (0.35, 0.71, and 1.05 µL mL^{-1}). Propiconazole at 1.50 µL mL^{-1} was used as positive control and negative controls were considered for both bioassays. Mycelial growth inhibition and mycelial growth rate were analyzed for the EO bioassay, but only mycelial growth inhibition was analyzed for the pulegone bioassay. The effects of both bioassays on ergosterol content in the fungal plasma membrane were also evaluated. All fungi species were negatively affected by EO and pulegone. The extractive completely inhibited *T. versicolor* growth while pulegone inhibited growth of both white-rot species. Pulegone presented lower IC50 than the EO for white-rot fungi. Both EO and pulegone negatively affected white-rot fungi ergosterol contents. White-rot fungi, in general, were more susceptible to the effects of the EO and pulegone than the brown-rot fungi.

Keywords: Lamiaceae; antifungal; pulegone; white rot; brown rot; ergosterol.

INTRODUCTION

Biodegradation of wood caused by fungi and insects is considered a problem for wood products since the lack of care and the inability to control these organisms may result in losses for wood industry (CHENG et al., 2008; DE ABREU et al., 2002). Even wood species recognized for their natural durability are often not able to indefinitely withstand the deterioration and decomposition caused by microorganisms, mainly by the fungi which are considered the most important wood-decay organisms in the world (OLIVEIRA;

TOMAZELLO; SILVA, 2005). Decay may happen in wood that is damp or exposed to rain (MCCARTHY; COOKSON; SCOWN, 2009). Within the group of wood-decay fungi responsible for wood rot, white and brown fungi, classified as Basidiomycetes, stand out (OLIVEIRA et al., 2005).

Wood-decay fungi play an important part in forest ecosystems (LONSDALE; PAUTASSO; HOLDENRIEDER, 2008). However, sometimes these fungi need to be controlled by wood preservative (CHENG et al., 2008). Several types of lumber, especially the less durable ones require a preservative treatment in case they are exposed to biodegrading conditions (MCCARTHY; COOKSON; SCOWN, 2009). The use of synthetic preservatives to protect biodegradable materials presents negative aspects as these products are often noxious and rarely renewable (SUNDARARAJ et al., 2015). Almost all synthetic pesticides can be considered poisonous and represent a long-term danger to human beings due to their persistence in the material they were applied or in the human body (EL-WAKEIL, 2013). The need to mitigate possible damage caused by synthetic products has increased the importance of alternative solutions to control pests such as fungi.

Botanical pesticides may be developed based on essential oils (EOs) (EL-WAKEIL, 2013). Among plant families, Lamiaceae is recognized by the richness of species containing EOs (ISMAN; MACHIAL, 2006) with antifungal activity (BOULOGNE et al., 2012). Belonging to the Lamiaceae family, the genus *Hesperozygis* Epling has been reported to have fungicidal activity in a study on *Hesperozygis marifolia* Epling. EO (GONZÁLES-CHÁVEZ et al., 2011). *Hesperozygis ringens* (Benth.) Epling is a woody herb known as *espanta-pulga* that occurs in rocky fields of Rio Grande do Sul, Brazil (VON POSER et al., 1996). This species is characterized by a high amount of essential oil (EO) production in its leaves throughout the year and the presence of pulegone, which is an oxygenated monoterpenoid, as a major compound (PINHEIRO et al., 2016). Histochemical tests performed in *H. ringens* leaves have detected EO in glandular trichomes and vascular bundle (PINHEIRO; DE OLIVEIRA; HEINZMANN, 2018). The extractive presents allelophatic, acaricidal, larvicidal, and anesthetic activities (PINHEIRO et al., 2017; RIBEIRO et al. 2010; SILVA et al. 2014; TONI et al. 2014; VON POSER et al., 1996). The inflorescence of *H. ringens* also produces high amounts of EO (PINHEIRO; DE OLIVEIRA; HEINZMANN, 2018).

Antifungal products can interact with different systems in the fungal cell; consequently, they may negatively affect their development and survival. One of the most important mechanisms of antifungal agents is their action on ergosterol content of the microorganism membrane. Ergosterol is the major sterol in the membrane of filamentous

fungi and is necessary for its normal function (DE LIRA MOTA et al., 2012), since it contributes to cellular functions such as membrane fluidity and integrity (LUPETTI et al., 2002). The concentration of this sterol in mycelia can be used to suggest fungal growth (GAO; CHEN; BREUIL, 1993)

Considering the need to find alternative methods to control white and brown rot fungi, this study aimed to analyze the antifungal effect of *H. ringens* EO and pulegone *in vitro* against *Ganoderma applanatum* and *Trametes versicolor* (white-rot fungi) and *Gloeophyllum trabeum* and *Lentinus lepideus* (brown-rot fungi). In addition, this study aims to explain a possible mode of action for the EO and its major component. Therefore, the effects of both the EO and pulegone on ergosterol content in the mycelium were assessed.

MATERIAL AND METHODS

Legal requirements

A legal authorization was required to collect plant material because *H. ringens* is included in the list of endangered species (GOVERNO DO ESTADO DO RIO GRANDE DO SUL, 2014). The authorization to perform scientific activities was granted by *Sistema de Autorização e Informação em Biodiversidade* (SISBIO, number 44197-2). Our group was also granted access to the Brazilian genetic heritage by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, No. 010191/2014-3).

Essential oil extraction

Leaves from *H. ringens* were harvested from four individuals in good phytosanitary conditions, of a population located in São Francisco de Assis (S 29° 35' 43, 1’’; W 055° 07' 33, 4’’), Rio Grande do Sul, Brazil in the spring of 2012. A voucher specimen was identified by Dr. Solon Jonas Longhi and deposited at the Herbarium of the Biology Department, UFSM, Brazil (SMDB 13.427). The EO was extracted from fresh material in triplicate by hydrodistillation with a Clevenger type apparatus for 2h (PINHEIRO et al., 2016), and the pool furnished by the four sampled individuals was stocked at -4°C until biological tests.

Essential oil analysis

The qualitative identification of the EO components occurred by means of chromatographic analysis using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass selective detector (GC-MS) while the quantitative analysis of the extractive was carried out by means of gas chromatography with flame ionization detector (GC-FID) on an Agilent 7890A Gas Chromatograph. The parameters used were as described by Pinheiro et al. (2016). For the identification of the chemical constituents, their retention indices, which were obtained through a calibration curve of *n*-alkanes injected in the same conditions of the samples, and mass fragmentation patterns were compared to data in literature (ADAMS, 2009; NIST, 2010).

Pulegone isolation and identification

Pulegone was isolated by column chromatography (CC) on silica gel 60 0.063- 0.2 mm/ 70- 230 MESH ASTM (Macherey-Nagel). Initially, 10 g of EO was applied over 700 g of adsorbent (58 x 6.3 cm) and eluted with hexane:acetone (95:5) in Flash flow. Fractions of 50 mL were collected and fractions 42-52 were grouped, obtaining 2.605 g of enriched pulegone mixture. Afterwards, a fraction containing 0.9 g of the enriched substance was applied onto a 90 g CC (60 cm x 2.5 cm) eluted hexane:ethyl acetate in gradient (99:1; 98:2; 97:3; 96:4) in a flow of 1 mL. min^{-1}. Fractions of 5 mL were collected. Fractions 160-202 were grouped and 0.707 g of the isolated substance was obtained. In order to obtain a greater amount of pulegone, a third procedure was performed with a fraction containing 0.9 g of the enriched substance which was applied onto a 90 g CC (60 cm x 2.5 cm) eluted with hexane:ethyl acetate (96:4) in a flow of 1 $mL.min^{-1}$. Fractions of 5 mL were collected and from these, fractions 79-95 which correspond to pulegone (0.721 g). Pulegone isolation was verified by GC-MS/ GC-FID in both cases and its structure was confirmed by means of proton and carbon 13 nuclear magnetic resonance spectra.

Antifungal activity bioassay

The antifungal activity of the EO and pulegone was tested against four species of wood-decay fungi, classified as white and brown rot fungi. From the first group, the species chosen were *G. applanatum* and *T. versicolor* while the two representative species for the second group were *G. trabeum* and *L. lepideus*. All species were provided by the *Laboratório*

de Produtos Florestais located at *Universidade Federal de Brasília*. The four fungi were initially cultivated in Petri plates (9 cm of diameter) containing potato-dextrose-agar (PDA) culture medium, sealed with parafilm for a period of 15 days in a Biochemical Oxygen Demand (BOD) chamber at 25°C (±1) in light/dark (12h/12h).

For the antifungal bioassay, a growth control (T0) and a negative control containing ethanol concentration used as a diluent (1 µL mL^{-1}) (T1) were included. The EO was tested at the following concentrations: 0.5, 0.75, 1.00, 1.25, and 1.50 µL mL^{-1} (T2, T3, T4, T5, and T6, respectively). A positive control (T7) containing propiconazole at 1.50 µL mL^{-1} was also evaluated. The concentrations chosen for the major EO component were proportional to their concentration in the EO. Thus, pulegone was tested at 0.35; 0.71, and 1.05 µL mL^{-1}. Both EO and pulegone, previously diluted in ethanol (1 µL mL^{-1}), were added to the medium while in liquid state. Afterwards, the solutions were transferred to the Petri dishes. For each fungal species, 11 mm mycelial discs were removed from 15-day-old cultures and transferred to the Petri dishes containing PDA with the aforementioned treatments. The dishes were sealed, transferred to the BOD chamber and stored in the previously mentioned conditions of temperature and photoperiod.

The fungal growth evaluation started 24 h after the beginning of the experiment by means of two diametrically opposed colony measurements. This procedure was repeated every two days. For the treatments containing pulegone, the measurements were carried out at the end of the experiments. The tests were concluded when the fungal development in the growth controls completely covered the medium in the Petri dishes (BADAWY; ABDELGALEIL, 2014). In order to differentiate fungistatic from fungicidal activities, the mycelial discs were re-inoculated in fresh PDA medium whenever an EO or pulegone treatment completely inhibited the fungal growth. The eventual revival growth was then observed for seven days (KEDIA et al., 2014). The mycelial growth inhibition (MGI) was calculated by the formula used by Badawy and Abdelgaleil (2014): $MGI\ (\%) = [(DC - DT)*100]/\ DC$, where DC and DT are considered average diameters of colony in control and treatment, respectively.

From the checked averages of fungal growth, mycelial growth rate (MGR) calculation was obtained by the formula proposed by Oliveira and described by Barbosa, Vieira and Teixeira (2015): $MGR = \Sigma(D - Dp)/\ N$, where D and Dp are the average diameter of current fungal colony and average diameter of fungal colony from the previous day, respectively. N is the number of days after the beginning of the experiment. A completely randomized experimental design was used, where all treatments were performed in triplicate (BADAWY; ABDELGALEIL, 2014), each repetition composed by two experimental units.

Effect of essential oil and pulegone on ergosterol content in the plasma membrane of the wood-decay fungi

Ganoderma applanatum, *T. versicolor*, *G. trabeum*, and *L. lepideus* were initially cultivated at the previously conditions described. Aiming to verify the effect of both samples on ergosterol content in the fungal plasma membrane, each fungal species was cultivated at 0.5 µL mL^{-1} EO and 0.35 µL mL^{-1} pulegone, both samples were diluted in ethanol (1 µL mL^{-1}) and then added to the medium, as previously described. A negative control was also considered and all treatments were performed in triplicate. The inoculation of mycelia discs was carried out according to a method adapted from Bento et al. (2014). The Petri plates were transferred to a BOD chamber and remained there for 5 days. After incubation, mycelia of each species were harvested and their wet weight was determined.

Ergosterol extraction from mycelia occurred according to a method adapted from Tian et al. (2012). The collected mycelia were transferred to test tubes and 25% alcoholic potassium hydroxide (5 mL), sterile distilled water (2 mL) and *n*-heptane (5 mL) were added. The content layers of each tube were separated, the *n*-heptane one was then collected and analyzed by spectrophotometry (Biospetro sp-220) between 230 and 300 nm. The reading carried out at 282 nm is characterized by the presence of ergosterol in *n*-heptane layer while the reading at 230 and 282 nm indicate the presence of the late sterol intermediate 24(28) dehydroergosterol (TIAN et al., 2012).

Ergosterol concentration was calculated by formulas adapted from Tian et al. (2012): *% 24 (28) dehydroergosterol = (A230/ 518)/ mycelia wet wt*; *% ergosterol + % 24 (28) dehydroergosterol = (A282)/ 290)/ mycelia wet wt*.

For these formulas, 290 and 518 are considered E (%/ cm) values for crystalline ergosterol and 24 (28) dehydroergosterol. The percentage reduction in the amount of ergosterol was calculated with a formula described by Kedia et al. (2014): *(C – T)/ C x 100*, where C and T are the percentages of ergosterol in control and treatment, respectively.

Statistical analysis

In case of antifungal activity bioassay, the data normality and homogeneity of variances were checked for each parameter. The ANOVA one-way test was performed and the means were compared by Tukey test ($P<0.01$) or Kruskall Wallis non-parametric test ($P<0.01$), when necessary. For each fungal species, the linear regression of each parameter

was also analyzed considering the results obtained for the concentration curves of EO (0.5, 0.75, 1.00, 1.25, and 1.50 μL mL^{-1}) and pulegone (35; 0.71, and 1.05 μL mL^{-1}). The half-maximal inhibitory concentration (IC50) values for EO and pulegone were calculated by probit analysis. After that, the t-test ($P<0.05$) was carried out. All statistical analyses were performed by means of Microsoft Excel and SigmaPlot 11.0.

For mycelia wet wt, the ANOVA one-way test was performed and the means were compared by Tukey test. Absorbance values obtained at 282 nm for each fungus were submitted to the ANOVA one-way test followed by Tukey test. All statistical analyses were performed by means of Microsoft Excel and SigmaPlot 11.0.

RESULTS

Besides the major constituent, which is pulegone (70.42%), *H. ringens* EO contained limonene (1.58%), β-*Z*-ocimene (1.44%), linalool (1.39%), and caryophyllene oxide (1.64%). Twenty-four compounds were identified, totalizing 82.74% of its chemical composition.

Considering mycelial growth inhibition (MGI), *G. applanatum* had its development significantly impaired ($P<0.01$) by T5 when compared to negative control (T1) (Table 1). Both white-rot species were significantly affected ($P<0.01$) by the highest EO concentration (T6), not differing from the positive control (T7). *Trametes versicolor* was completely inhitibed by T6. In this case, mycelia discs were re-inoculated in plates containing fresh PDA and the species revival could be observed after seven days, with a mean growth of 32.23%. Thus, the EO activity can be characterized as fungistatic. Both brown-rot fungi had their mycelial growth significantly affected ($P<0.01$) by T3 when compared to negative control (T1) (Table 1). The highest EO concentration (T6) did not differ from the positive control for *G. trabeum* (T7).

Considering mycelial growth rate (MGR), *G. applanatum* was previously affected ($P<0.01$) by the lowest EO concentration (T2), differing from control groups (T0 and T1) (Table 1). For both white-rot fungi, the highest EO concentration (T6) did not differ from positive control (T7). Both brown-rot species were significantly affected ($P<0.01$) by the lowest EO concentration (T2) (Table 1). The treatment characterized by the highest EO concentration (T6) did not differ from positive control (T7).

Regarding the antifungal effect of pulegone (Table 2), *G. applanatum* was completely inhibited by the highest concentration (P4) while *T. versicolor* was inhibited by the two highest compound concentrations (P3 and P4). The mycelia discs of both inhibited fungi were

re-inoculated in plates containing fresh PDA and *G. applanatum* revival could be observed after seven days, presenting a growth mean of 50.5%. Thus, pulegone activity can be characterized as fungistatic. However, the revival of *T. versicolor* did not occur, indicating the fungicidal effect of the oxygenated monoterpenoid. The two highest pulegone concentrations (P3 and P4) significantly affected (P<0.01) the mycelia growth of *G. trabeum* when compared to negative control (P1). *Lentinus lepideus* was significantly impaired (P<0.01) by the highest substance concentration (P4) (Table 2). The IC50 demonstrated that, for both white-rot fungi, pulegone presented values significantly lower (P<0.05) than *H. ringens* EO (Table 3). For both brown-rot species, no differences were detected between the EO and its major compound.

Table 1 - Mycelial growth inhibition (%) and mycelial growth rate of *Ganoderma applanatum*, *Trametes versicolor, Gloeophyllum trabeum*, and *Lentinus lepideus* under different treatments of *Hesperozygis ringens* essential oil (EO).

(to be continued)

	Mycelial Growth Inhibition (%)					
Treatments	*Ganoderma applanatum*	*Trametes versicolor*			*Gloeophyllum trabeum*	*Lentinus lepideus*
	Mean (SEM)	Median	25%	75%	Mean (SEM)	Mean (SEM)
T1	2.387±2.387**c	0*b	0	0	0±0**d	0±0**f
T2	4.776±2.424 c	0 ab	0	3.401	24.250±12.181 cd	11.234±0.159 ef
T3	9.863±2.587 c	11.265 ab	4.438	19.528	45.268±13.446 bc	18.220±1.747 e
T4	16.139±5.642 c	69.819 ab	67.536	73.002	48.174±4.472 bc	32.327±2.59 d
T5	42.031±2.511 b	83.731 ab	79.333	93.730	55.125±1.560 bc	48.610±5.638 c
T6	77.719±11.517 a	100 a	100	100	75.440±4.949 ab	71.323±2.770 b
T7	100±0 a	100 a	100	100	100±0 a	100±0 a
CV (%)	24.91	10.57			25.52	11.41

Equation	Y = 46.36292948* X - 13.14948764	Y = 77.34810127*X - 19.48101266			Y = 47.61338156* X + 1.698764316	Y = 46.57950573*X -8.530213984
R^2	0.729692396	0.841205522			0.967793922	0.917160565
	Mycelial Growth Rate					
T0	71.772±0.329** a	55.191*a b	54.361	56.055	62.732±2.095** a	66.382±1.623** ab
T1	70.684±1.718 a	56.286 a	55.759	56.895	63.374±3.386 a	69.951±1.14 a
T2	57.310±1.408 b	43.992 ab	41.585	44.454	41.404±4.186 b	56.001±0.674 b
T3	44.501±3.781c	28.059 ab	27.841	37.245	23.814±2.316 c	43.551±3.489 c
T4	34.437±3.110 c	7.798 ab	6.084	7.833	22.382±2.476 c	27.995±1.391 d
T5	17.334±2.513 d	2.777 ab	0.912	3.712	12.458±0.559 cd	18.547±5.39 de
T6	4.511±2.390 e	0 b	0	0	4.223±0.775 de	8.409±1.759 ef
T7	0±0 e	0 b	0	0	0±0 e	0±0 f
CV (%)	10.44	11.42			14.39	11.99
Equation	Y = -45.08307217* X + 75.69914909	Y = -42.39173951 + 58.81144381			Y = -38.99035853* X + 60.43489983	Y = -42.91804074*X + 73.1746412
R^2	0.97353869	0.934271736			0.9694275	0.984761553

Source: The authors.

Data are presented as mean (SEM = standard error of the mean) and median. ** Means followed by different lowercase letters in columns indicate statistical differences (P<0.01) by Tukey test; * Means followed by different lowercase letters in columns indicate statistical differences (P<0.01) by Kruskall Wallis test; T0= control; T1= Negative control; T2= EO at 0.5 µL mL^{-1}; T3= EO at 0.75 µL mL^{-1}; T4= EO at 1.0 µL mL^{-1}; T5= EO at 1.25 µL mL^{-1}; T6= EO at 1.5 µL mL^{-1}; T7= Positive control (Propiconazole at 1.5 µL mL^{-1}); CV=coefficient of variation.

Table 2 - Mycelial growth inhibition (%) of *Ganoderma applanatum*, *Trametes versicolor* *Gloephyllum trabeum*, and *Lentinus lepideus* under different treatments of pulegone.

Treatments	*Ganoderma applanatum*			*Trametes versicolor*			*Gloephyllum trabeum*	*Lentinus lepideus*
	Median	25%	75%	Median	25%	75%	Mean (SEM)	Mean (SEM)
P1	0*b	0	0	0*b	0	0	0±0**c	0±0**b
P2	10.506 ab	8.178	23.890	6.854 ab	5.017	8.340	0±0 c	3.693±0.548 b
P3	27.195 ab	27.152	27.369	100 a	100	100	23.495±6.618 b	13.181±3.431 b
P4	100 a	100	100	100 a	100	100	61.683±7.130 a	36.468±5.184 a
CV (%)	15.85			2.15			39.56	40.53
Equation	Y = 88.44159464*X - 10.98472931			Y = 112.5042399*X -7.671408472			Y = 59.27465302* X - 9.972548242	Y = 33.76985912* X - 4.477828004
R^2	0.817923191			0.832757092			0.851176847	0.871722923

Source: The authors.
Data are presented as mean (SEM = standard error of the mean) and median. ** Means followed by different lowercase letters in columns indicate statistical differences (P<0.01) by Tukey test; * Means followed by different lowercase letters in columns indicate statistical differences (P<0.01) by Kruskall Wallis test; P1= Negative control; P2= Pulegone at 0.35 µL mL^{-1}; P3= Pulegone at 0.71 µL mL^{-1}; P4= Pulegone at 1.05 µL mL^{-1}; CV=coefficient of variation.

Table 3 - Half-maximal inhibitory concentration (IC50) (µL/mL) of *Hesperozygis ringens* essential oil and pulegone on *Ganoderma applanatum*, *Trametes versicolor, Gloephyllum trabeum*, and *Lentinus lepideus*.

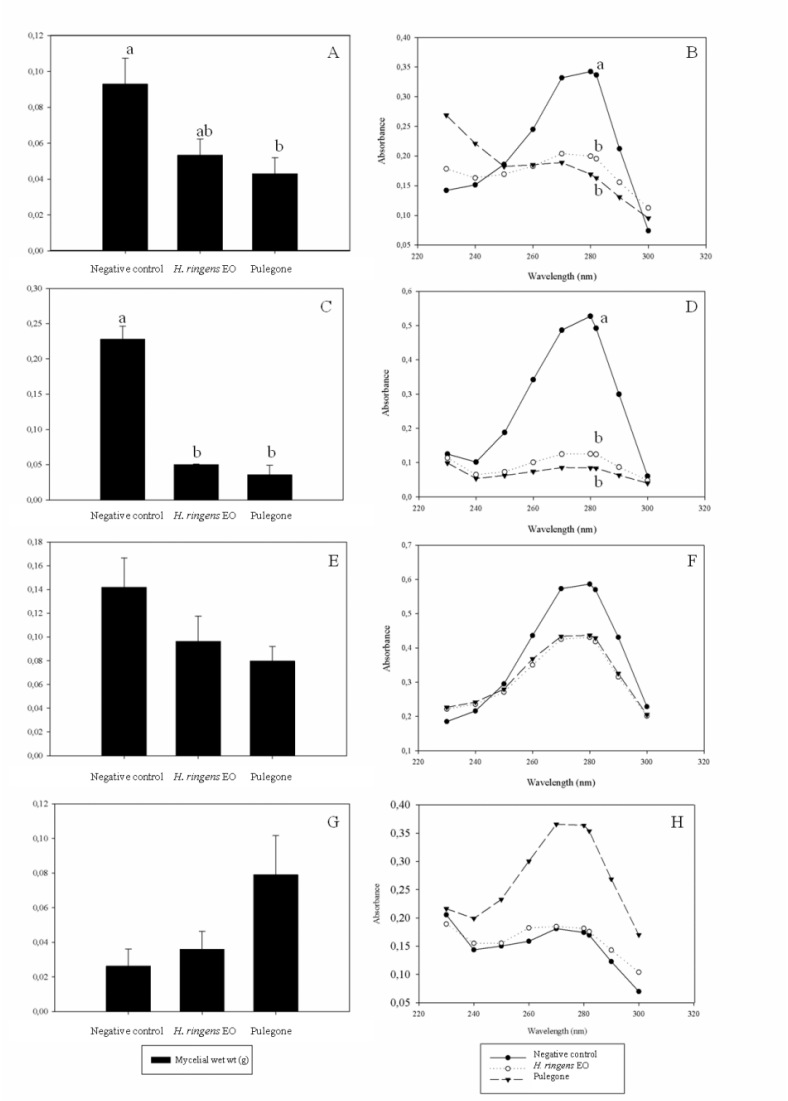
	Ganoderma applanatum	*Trametes versicolor*	*Gloephyllum trabeum*	*Lentinus lepideus*
Hesperozygis ringens EO	1.291±0.140*	0.904±0.137*	0.879±0.017	1.219±0.002
Pulegone	0.757±0.001	0.452±0.014	0.962±0.141	1.640±0.257

Source: The authors.
Data are presented as mean ± standard error of the mean. * Statistical differences (P<0.05) by t- test.

Considering *G. applanatum* mycelial wet wt, negative control presented a value significantly higher ($P<0.05$) than the one found for pulegone, but not differing from the one observed for *H. ringens* EO (Fig. 1A). The absorbance obtained at 282 nm showed a mean which was significantly lower ($P<0.05$) in EO and pulegone when compared to negative control (Fig. 1B). For *T. versicolor*, the negative control presented a mycelial wet wt which was significantly higher ($P<0.05$) than the ones found for EO and pulegone (Fig. 1C). The absorbance mean obtained at 282 nm for this species was significantly lower ($P<0.05$) in EO and pulegone when compared to negative control (Fig. 1D). For both brown rot fungi, the extractive and oxygenated monoterpenoid did not differ from negative control.

For *G. applanatum* cultured in the presence of *H. ringens* EO, a decrease of 35.33% in ergosterol content could be observed while pulegone reduced this sterol concentration in 64.13%. *Trametes versicolor* had 31.92% of its ergosterol concentration decreased by the EO whereas pulegone provided a decrease of 47.34%. For *G. trabeum*, the EO decreased the ergosterol concentration in 4.70% whereas pulegone did not impair this sterol content. The ergosterol concentration in *L. lepideus* was not influenced by EO and the oxygenated monoterpenoid.

Figure 1 - *Ganoderma applanatum*: mycelial wet wt **(A)** and UV spectrophotometric ergosterol profile **(B)**; *Trametes versicolor*: mycelial wet wt **(C)** and UV spectrophotometric ergosterol profile **(D)**; *Gloeophyllum trabeum*: mycelial wet wt **(E)** and UV spectrophotometric ergosterol profile **(F)**; *Lentinus lepideus*: mycelial wet wt **(G)** and UV spectrophotometric ergosterol profile **(H)**. Different lowercase letters indicate statistical differences (P<0.05) by Tukey test.



Source: The authors.

DISCUSSION

Although the American Society for Testing and Materials (2005) indicates the use of Malt Agar Substrate for wood decay fungi cultivation, our group chose the PDA medium since previous tests had demonstrated no difference between the fungi growth in both medium

(data not shown). Additionally, the literature supports the use of the chosen medium for the four fungal species assayed in this study (KIRN et al., 2000; OSEMWEGIE; OGHENEKARO; OWOLO, 2010; RINI; OHTANI; ICHIURA, 2012; TASCIOGLU et al., 2013). There are many ways to evaluate the antifungal activity of a natural product. Mycelial growth rate and the percentage of inhibition are two common criteria found in literature (BARBOSA; VIEIRA; TEIXEIRA, 2015; PRAKASH et al., 2012). Thus, we chose both methods in order to better understand the antifungal effect.

Numerous defensive phytochemicals, which have already been identified in plants, such as terpenoids, have antimicrobial activities. Therefore, these species are not totally helpless when confronted by pathogenic agents (VARMA; DUBEY, 1999). The *H. ringens* EO analyzed in our study is mainly composed by monoterpenoids. Many compounds included in this class are known for their antifungal activities (BOULOGNE et al., 2012; JING et al., 2014; ZHANG et al., 2016).

The antifungal activity of the EO rich in pulegone obtained from *H. marifolia* against *Aspergillus flavus* Link has been described by Gonzáles-Chávez et al. (2011), which suggests a fungitoxic potential of the EO obtained from species classified in this genus. As observed in our study, three of the fungal species evaluated were not completely eliminated by the EO at the tested concentrations, but they had their development affected by this extractive. In addition, a fungistatic effect was detected when the EO was tested against *T. versicolor*.

Results of the *in vitro* assays indicate a high antifungal activity for *H. ringens* EO and the effect was detected mostly at the same concentration than the conventional antifungal used as positive control (Table 1). When considering MGR, the highest EO concentration differed from positive control only for *L. lepideus*. However, when MGR was observed, no differences were detected among the extractive and propiconazole, both assayed at 1.5 µL/mL.

No reports about pulegone activity against wood-decay fungi were found in literature. In the present study, the isolated substance affected all fungi species, especially the white rot ones, demonstrating fungistatic effect against *G. applanatum* and fungicidal activity against T. *versicolor*. This oxygenated monoterpenoid is well known by its biological activities, among them are the antifungal effect against *A. flavus* and *Cladosporium cucumerinum* (GATA-GONÇALVES et al., 2003; GONZÁLES-CHÁVEZ et al., 2011). Moreover, other monoterpenoids found in *H. ringens* EO, as limonene and linalool as well as caryophyllene oxide, which is a sesquiterpenoid, have had their antifungal effects previously described (CHENG et al., 2004; QUINTANA-RODRIGUEZ et al., 2015), indicating that the antifungal effect presented by the EO may occur due to the combined action of its constituents.

Pulegone was more effective than *H. ringens* EO against white-rot fungi, which may be related to the antagonistic effect between pulegone and other extractive constituents. Antagonism occurs when two or more antimicrobial agents interact, resulting in a lower potency than would be expected from the individual effects (BELL, 2005). The antagonism occurred by combinations of EO chemical constituents has already been mentioned in the literature (VAN ZYL; SEATLHOLO; VILJOEN, 2010). Interestingly, the antagonistic effect of the EO components observed for white-rot fungi did not occur in brown-rot representatives. These diverse behaviors found in the results may also be related to the characteristics of each fungal group as white and brown rot representatives have different features (MARTÍNEZ et al., 2005).

Plant secondary metabolites act as antimicrobial agents through a series of action mechanisms (LV et al., 2011) and many of these chemical constituents have their effect against fungi species related to their ability to dissolve or disrupt the integrity of membranes or cell walls (ISMAN; MACHIAL, 2006). Besides, EO may present antioxidant compounds such as terpenoids and phenolic components, whose antiseptic action is often described in the literature (BAKKALI et al., 2008). No reports about antifungal mechanisms of *Hesperozygis* EO and pulegone could be found in the literature. Therefore, we opted for the analysis of the possible effects of the extractive and its major compound on the ergosterol concentration in the membrane of the four wood-decay fungal species.

The amounts of ergosterol detected in negative controls were not the same in all fungal species analyzed, even though they were all evaluated at the same growth period and culture medium. Besides the growth stage and culture condition, ergosterol contents may vary depending on factors such as species and strains (BARAJAS-ACEVES et al., 2002; GAO; CHEN; BREUIL, 1993). For both white-rot fungi, the mycelial wet weight was impaired by pulegone and the *T. versicolor* ergosterol content was also affected by the EO. One of the possible explanations for the decrease of the ergosterol content is that this membrane lipid is decomposed in dead hyphae (SCHEU; PARKINSON, 1994).

Over the years, the possible effects of EO rich in monoterpenoids and this class of constituents in the fungal plasma membrane has been considered an important mechanism of antimicrobial action (AVANÇO et al., 2017; PARVEEN et al., 2004; TIAN et al., 2012; URIBE; RAMIREZ; PEÑA, 1985). According to Cowan (1999), their mechanism of action may involve membrane disruption by lipophilic components, although this is not completely understood. Since terpenoids present low molecular weight, they may diffuse across cell membranes and stimulate biological reactions (CHAO et al., 2005).

In this study, *H. ringens* EO and pulegone decreased the ergosterol contents of the white-rot fungi and the extractive also impaired the sterol content of *G. trabeum*. Monoterpenoids may cause ergosterol biosynthesis inhibition as already reported for α-pinene in *Saccharomyces cerevisiae* (PARVEEN et al., 2004). According to these authors, this monoterpenoid derivative affected sterol uptake, transcriptional induction of genes related to lipid metabolism, cell wall structure, detoxification, and cellular transport in yeast.

Essential oil and pulegone increased ergosterol content of at least one brown-rot fungus. The increase in the fungal sterol amount by an extractive has already been reported by Avanço et al. (2017), which described a gain in ergosterol production of *Fusarium verticillioides* by *Curcuma longa* L. EO. Monoterpenoids are lipophilic, so their presences in PDA medium should induce stress in the environment of cellular lipids (PARVEEN et al., 2004). According to the authors, this effect must stimulate an adaptive response capable of reprogramming the genomic expression in order to protect the architecture of the cell wall. Another possibility is the occurrence of the hormesis effect. This is a phenomenon characterized by a beneficial effect of toxic constituents when applied at low concentrations (FORBES, 2000). Some chemical constituents provided by EO can induce a hormesis effect in fungi (STEFFEN et al., 2013), as described for the monoterpenoids α-pinene and β-pinene (LUDLEY et al., 2009).

According to Kumar and Gupka (2006), a new biological wood preservative should be easily stored, manipulated and applied as well as provide competitive stability when compared to traditional products. Despite the demonstrated potential for the use of *H. ringens* EO and pulegone as preservatives against wood-decay fungi, further research is needed in order to study the extractive behavior when in contact with wood and exposed to environmental factors. Some monoterpenoids are considered chemically stable at ambient temperatures (ISMAN; MACHIAL, 2006); however, many of these compounds present high volatility. This feature may result in a short-term effect and can hinder the development of a botanical preservative against wood-decay fungi. Therefore, investments for developing a product with appropriate characteristics are also needed. In addition, given that *H. ringens* is an endangered species and produces high amount of EO (PINHEIRO et al., 2016; PINHEIRO et al., 2018), the discovery of possible uses for its extractive may promote future studies aiming to preserve and reproduce the plant.

To the best of our knowledge, this study has reported antifungal activities of pulegone and *H. ringens* EO against wood-decay fungi for the first time. The extractive and its major constituent affected white and brown rot fungi, indicating that the EO activity is related to the

oxygenated monoterpenoid. In general, the white-rot representatives were more susceptible to pulegone and EO activities. The effect of the extractive and its major compound on this fungal group may be, at least in part, due to the impairment of ergosterol content in the plasma membrane. However, other studies should be carried out to show possible interference with the ergosterol biosynthesis. The results obtained in this study indicate the potential uses of *H. ringens* EO and pulegone as active components in wood preservatives to control wood-decay fungi.

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6 DISCUSSÃO

Os campos do Rio Grande do Sul apresentam uma grande diversidade de espécies vegetais endêmicas, como *H. ringens* (FREITAS et al., 2010). Para a realização da presente tese, material vegetal de *H. ringens* foi coletado de duas populações, localizadas em Santa Maria e São Francisco de Assis. Ambas as populações se encontram em regiões de clima Subtropical, segundo classificação de Köppen (MORENO, 1961). Sabe-se que *H. ringens* pertence à lista de espécies ameaçadas de extinção (GOVERNO DO ESTADO DO RIO GRANDE DO SUL, 2014). Tanto em Santa Maria quanto em São Francisco de Assis, os locais de ocorrência de indivíduos da espécie são caracterizados por apresentarem terrenos arenosos com elevações rochosas. Segundo Fracaro (2006), essa peculiaridade de ocorrência, ou seja, em áreas rochosas, associada à renovação de pastagens para a alimentação do gado, a coleta da espécie para uso popular, e sua baixa eficiência de dispersão por sementes, são fatores prejudiciais que elevam o risco de extinção de *H. ringens*.

A literatura apresenta poucas informações a respeito de caracterização estrutural dos órgãos vegetativos das representantes de *Hesperozygis*. Informações gerais sobre o gênero foram encontradas em Harley et al. (2004), e as espécies *H. spathulata*, *H. nitida* e *H. rhododon* são diferenciadas por Pereira e Pereira (1973). Entretanto, nenhum estudo foi encontrado a respeito da caracterização estrutural dos órgãos vegetativos de *H. ringens*. O Artigo 1 descreve características marcantes da espécie, como a presença de tricomas tectores e glandulares em limbo foliar, pecíolo e caule, além da detecção de dois tipos de cristais na mesma espécie, algo não encontrado nas informações sobre Lamiaceae: inulina em folha e caule; hesperidina em raiz. Espera-se que as informações relatadas no referido manuscrito sobre caracterização de limbo foliar, pecíolo, caule e raiz possam agregar conhecimento a respeito dos órgãos vegetativos do gênero *Hesperozygis*, fornecendo informações sobre as particularidades estruturais de *H. ringens*, além de auxiliar em sua identificação.

As classes químicas de metabólitos secundários produzidos por uma espécie vegetal podem apresentar diferentes funções que auxiliam a sobrevivência do organismo em seu local de ocorrência. Muitos testes histoquímicos são realizados em espécies pertencentes à família Lamiaceae, visando à detecção das classes de metabólitos produzidas por essas plantas. Dentre essas classes, podem ser citados os lipídios totais, ácidos e neutros; OEs; resinas; oleorresinas; proteínas; amido; compostos fenólicos; flavonoides; alcaloides; entre outras (BOTTEGA; CORSI, 2000; MOTA et al., 2013; TOZIN; RODRIGUES, 2017). Essas

substâncias podem ser armazenadas em diferentes órgãos vegetais (GIULIANI; BINI, 2008; MOTA et al., 2013), como foi possível observar nos resultados do Artigo 2.

O presente artigo ainda demonstra a importância da realização de testes histoquímicos em material vegetal fresco. Além da análise histoquímica em material nesse estado, folhas e pétalas de *H. ringens* também passaram por procedimento de inclusão, conforme método descrito. Na sequência, testes buscando detectar a presença de lipídios totais, compostos fenólicos e flavonoides foram realizados em materiais incluídos (resultados não apresentados). A análise dos cortes histológicos demonstrou que as classes de constituintes não foram preservadas após os procedimentos de inclusão. Por esse motivo, apenas resultados detectados em materiais frescos foram considerados para o Artigo 2. Testes histoquímicos em espécies de Lamiaceae são comumente realizados em material vegetal fresco, como pode ser constatado em estudos envolvendo *Hyptis villosa* Pohl ex Benth., *Micromeria pulegium* (Rochel) Benth., *M. thymifolia* (Scop.) Fritsch e *Ocimum obovatum* E. Mey. ex Benth. e *Plectranthus grandidentatus* Gürke (MARIN; JASNIĆ; ASCENSÃO, 2013; MOTA et al., 2013; NAIDOO et al., 2013; STOJIČIĆ et al., 2016; TOZIN; RODRIGUES, 2017).

Em espécies dessa família é comum a produção de substâncias químicas em tricomas glandulares, muitas vezes capazes de promover defesas contra agentes prejudiciais, como herbívoros e patógenos, visando à sobrevivência da planta, bem como proporcionar a atração de agentes polinizadores, com o intuito de facilitar a propagação da espécie vegetal (WERKER, 1993; WERKER, 2000). Nos Artigos 1 e 2 é descrita a localização de tricomas glandulares classificados como peltados em limbo foliar, pecíolo, caule e pétala de *H. ringens*, um tipo de estrutura muito comum de ser encontrado em espécies pertencentes a essa família botânica (JING et al., 2014; MARIN; JASNIĆ; ASCENSÃO, 2013; MOTA et al., 2013; NAIDOO et al., 2013; STOJIČIĆ et al., 2016; TOZIN; RODRIGUES, 2017; WERKER, 2000). Além de peltados, outros tipos de tricomas glandulares como capitados e digitiformes também foram descritos em espécies pertencentes à Lamiaceae, como *Hyptis villosa* Pohlex Benth., *Plectranthus grandidentatus* Gurke, *Micromeria thymifolia* (Scop.) Fritsch, *M. pulegium* (Rochel) Benth. e *Satureja horvatii* Silic (MARIN; ASCENSAO; LAKUŠIĆ, 2012; MARIN; JASNIĆ; ASCENSÃO, 2013; MOTA et al., 2013; STOJIČIĆ et al., 2016; TOZIN; RODRIGUES, 2017).

Em um dos locais de coleta de material vegetal, em São Francisco de Assis, foi possível observar sinais no solo que sugeriam a presença de gado, entretanto, nenhum dos indivíduos da espécie apresentou marcas indicativas de que havia sido predado por esses animais. Schiestl (2010) sugere que os monoterpenoides, principais componentes do OE de *H.*

ringens, evoluíram primariamente com a função de defesa contra herbívoros em angiospermas e gimnospermas, sendo a atração de agentes polinizadores uma função posterior da classe de constituintes. Portanto, uma das funções da classe dos terpenoides na espécie, poderia ser a repelência de herbívoros, como forma de defesa.

Através de observações realizadas nos dois locais de coleta de *H. ringens*, foi possível constatar que a mesma é dominante na área em São Francisco de Assis, e uma das dominantes na área em Santa Maria. Von Poser et al. (1996) já haviam observado a dominância da espécie nos seus locais de ocorrência, sugerindo estar relacionada a um efeito alelopático. Esse efeito foi relatado pelos últimos autores, quando o OE de *H. ringens* foi testado em sementes de *L. sativa*. Posteriormente, Pinheiro et al. (2017) avaliaram o potencial do extrativo da espécie sobre sementes de dicotiledôneas (*G. max*, *L. sativa* e *B. pilosa*) e monocotiledôneas (*O. sativa*, *A. strigosa* e *L. multiflorum*). Os autores constataram que ambos os grupos foram afetados pelo OE, e as plantas daninhas analisadas, *B. pilosa* e *L. multiflorum*, apresentaram maior susceptibilidade em relação às culturas *G. max* e *O. sativa*. O potencial alelopático do extrativo de *H. ringens* encontrado nesses estudos, somado às observações a campo realizadas, sugere que a mistura de metabólitos secundários que compõe seu OE também poderia apresentar função de defesa contra plantas competidoras nos locais de ocorrência.

O rendimento obtido para o OE extraído de folhas e a distribuição abundante desse extrativo no respectivo órgão da espécie, detectado através dos testes histoquímicos descritos no Artigo 2, estão de acordo com os elevados teores de OE relatados para as folhas de *H. ringens*, em estudo realizado durante as quatro estações do ano (PINHEIRO et al., 2016). No presente estudo, o OE de folhas apresentou o monoterpenoide oxigenado pulegona como constituinte majoritário. Esse resultado condiz com relatos obtidos de outros estudos relacionados ao extrativo da espécie, onde a pulegona também foi mencionada como o constituinte mais representativo na composição do extrativo (PINHEIRO et al., 2016; PINHEIRO et al., 2017; RIBEIRO, et al., 2010; SILVA et al., 2014; TONI et al., 2014; VON POSER et al., 1996). Além da produção de OE em folhas de *H. ringens*, o Artigo 2 ainda descreve rendimento e composição química do extrativo produzido por inflorescências, informações não encontradas na literatura até então. Assim como ocorreu para o OE de folhas, o extrativo obtido de inflorescências também apresentou um elevado rendimento e a pulegona como constituinte majoritário. Uma vez que os OEs obtidos de ambos os órgãos demonstraram características semelhantes, essas informações poderão auxiliar na decisão de futuras coletas de partes aéreas de *H. ringens*, visando à extração de OE. Entretanto, para que

esse extrativo seja explorado, é fundamental que, primeiramente, a planta seja reproduzida com o intuito de retirá-la da lista de espécies ameaçadas de extinção.

Em se tratando da produção de OEs, a família Lamiaceae demonstra diversas espécies com altos rendimentos (AGOSTINI et al., 2009; DRIS; TINE-DJEBBAR; SOLTANI, 2017; MECHERGUI et al., 2010; RAMZI et al., 2017). Pertencente a essa família, o gênero *Hesperozygis* se destaca pelos elevados potenciais para produção de OE de suas representantes. *Hesperozygis rhododon* apresentou rendimento de 1% para o OE de suas folhas (VON POSER et al., 1996). Para partes aéreas de *H. marifolia* foi relatado o dobro dessa produção (GONZÁLEZ-CHÁVES et al., 2011). As folhas de *H. myrtoides* apresentaram rendimentos variando de 1,01 a 1,72% em OE (MARTINI, 2011), enquanto que as partes aéreas da mesma espécie demonstraram produções entre 0,74 e 3,0% (CASTILHO et al., 2016). *Hesperozygis ringens* apresenta elevada produção de OE durante todo o ano, com rendimentos variando de: 3,01 a 3,46% no outono; 1,15 a 1,91% no inverno; 2,32 a 4,38% na primavera; e 2,64 a 3,33% no verão (PINHEIRO et al., 2016). Em espécies de Lamiaceae, é comum a produção de OE em tricomas glandulares (GIULIANI e BINI, 2008; WERKER, 1993; MOTA et al., 2013). Entretanto, essa tese relata que, em *H. ringens*, a ocorrência abundante do extrativo não ocorre apenas em tricomas, mas também no mesofilo. Isso está correlacionado com os elevados rendimentos apresentados por folhas (PINHEIRO et al., 2016; RIBEIRO et al., 2010; SILVA et al., 2014; VON POSER et al., 1996). Por outro lado, a constatação da produção de OEs em tricomas glandulares poderá auxiliar o procedimento pós-colheita de amostras de material vegetal. Uma vez que a cutícula dessas estruturas pode ser rompiada com facilidade, cuidados devem ser tomados durante sua manipulação, com o objetivo de evitar a volatilização do extrativo produzido.

A caracterização histoquímica também detectou a presença de compostos fenólicos em folhas e pétalas de *H. ringens*. Segundo Boulogne et al. (2012), a produção dessa classe de compostos poderia estar relacionada a uma forma de defesa da planta contra insetos e fungos, uma vez que essas funções biológicas já foram relatadas para derivados dessa classe em outras espécies pertencentes à mesma família. Por outro lado, os compostos fenólicos também podem exercer outras funções. Entre elas destaca-se a atuação dos flavonoides como filtro contra radiação UV, para proteção do mesofilo (CALDWELL; ROBBERECHT; FLINT, 1983). Outra função bem estabelecida para os compostos fenólicos é a sua participação nas defesas do vegetal contra estresse oxidativo, através da atividade antioxidante (AGATI et al., 2012).

Assim como descrito no Artigo 2, a composição química do OE de folhas de *H. ringens* apresentada no Artigo 4 evidenciou a pulegona como constituinte majoritário. Enquanto o extrativo caracterizado no primeiro trabalho é representado por 79.94% da substância, o OE utilizado no segundo estudo apresenta 70,42% desse monoterpenoide oxigenado. Em ambos os casos, os percentuais de pulegona foram superiores ao encontrado no OE de inflorescências (63,86%), verificado no Artigo 2. Em trabalho realizado por Pinheiro et al. (2016), essa substância foi observada como majoritária no OE de folhas de *H. ringens* nas quatro estações do ano, apresentando teores de: 81,17% no outono, 53,93% no inverno, 76,91% na primavera, e 79,02% no verão. No mesmo estudo, merecem destaque outros monoterpenoides, como limoneno, β-*Z*-ocimeno e linalol, substâncias também encontradas nos OEs relatados nos Artigos 2 e 4 da presente tese.

Tratamentos em madeira podem ser realizados visando combater o apodrecimento, muitas vezes causado por organismos xilófagos, como os fungos apodrecedores. Para que esses organismos se desenvolvam apropriadamente na madeira, são necessários níveis adequados de umidade, substrato, temperatura e oxigênio (JAYANETTI, 1986). De acordo com De Belie et al. (2000), os fungos apodrecedores são divididos em: fungos de podridão-branca e parda, classificados como basidiomicetos; e os fungos de podridão-mole, classificados como ascomicetos. Para os experimentos descritos nos Artigos 3 e 4 foram escolhidos apenas os basidiomicetos, uma vez que nessa classe estão classificados fungos apodrecedores da madeira considerados importantes (HUMAR; KOSMERL; POHLEVE, 2011; LESAR; KRALJ; HUMAR, 2009). A relevância dessas espécies devido aos danos causados aos produtos madeireiros justifica a grande quantidade de estudos visando descobrir alternativas para controlar fungos de podridão-branca e parda (BENTO et al., 2014; RODRIGUES et al., 2012; ZHANG et al., 2016).

Os OEs de espécies pertencentes à família Lamiaceae apresentam diversas funções biológicas descritas, dentre elas, antifúngica/fungicida (BOULOGNE et al., 2012; LIMA; CARSOSO, 2007). Apesar disso, nenhum relato foi encontrado sobre os extrativos produzidos pela família contra os quatro fungos apodrecedores utilizados nos ensaios apresentados na presente tese. Além disso, nenhum relato foi encontrado sobre os possíveis efeitos dos OEs de *Hesperozygis* contra fungos apodrecedores da madeira. Através dos resultados obtidos no Artigo 4, foi possível observar que o OE de *H. ringens* possui atividade antifúngica frente aos representantes de podridão-branca *G. applanatum* e *T. versicolor*, e podridão-parda *G. trabeum* e *L. lepideus*. Óleos essenciais de outras espécies pertencentes à mesma família já foram testados e apresentaram efeito sobre fungos apodrecedores. O extrativo de *Thymus*

zygis L. apresentou efeito inibitório sobre o fungo de podridão-branca *Coriolus versicolor* (Linnaeus) Quélet e as espécies de podridão-parda *Coniophora puteana* (Schumacher ex Fries) Karsten, *Poria placenta* (Fries) Cooke sensu J. Eriksson e *G. trabeum* (AMARTI et al., 2011). Os OEs de *Origanum vulgare* L. e *Rosmarinus officinalis* L. apresentaram atividade antifúngica frente aos fungos de podridão-branca *Hexagonia apiaria* (Pers.) Fr.e *Ganoderma lucidum* (Curtis) P. Karst. (MOHAREB; BADAWY; ABDELGALEIL, 2013).

Uma vez que a pulegona já possui efeito antifúngico descrito na literatura (GATA-GONÇALVES et al., 2003), inclusive quando encontrada como principal constituinte na composição química do OE de outra espécie do gênero *Hesperozygis* (GONZÁLEZ-CHÁVES et al., 2011), uma hipótese considerada inicialmente foi a possibilidade desse monoterpenoide oxigenado ser um dos responsáveis pela atividade antifúngica observada para o OE de *H. ringens*. No Artigo 4 foi possível constatar a atividade fungicida da pulegona frente a *T. versicolor*, fungistática frente a *G. applanatum* e o efeito nocivo da substância sobre as duas espécies de podridão-parda analisadas. Esses resultados confirmam a hipótese inicial considerada e sugerem a futura utilização do monoterpenoide oxigenado como componente ativo de um antifúngico botânico.

Em relação aos sesquiterpenoides contidos no extrativo obtido na primavera, cuja análise química é relatada no Artigo 4, foi possível detectar o óxido de cariofileno, representando mais de 1,5% da composição, constituinte cuja atividade antifúngica contra o fungo de podridão-parda *Laetiporus sulphureus* já é conhecida (CHENG et al., 2004). Portanto, com relação às substâncias ativas, além dos monoterpenoides, o efeito antifúngico também pode estar sendo causado pelos sesquiterpenoides presentes no OE. Pinheiro et al. (2016) já haviam observado a presença de sesquiterpenoides, que é mais acentuada no inverno. Pinheiro (2014) sugere que o aumento da produção dessa classe na estação mais fria do ano poderia estar relacionado à defesa que os sesquiterpenoides promovem contra o ataque de fungos patogênicos, que podem ser favorecidos pelas condições de alta umidade e temperaturas amenas.

O uso do fungicida comercial propiconazole como controle positivo contra fungos apodrecedores da madeira foi previamente relatado (HSU; CHANG; CHANG, 2007; YEN; CHANG, 2008). Uma vez que uma das propostas do trabalho é demonstrar a possibilidade da utilização do OE de *H. ringens* como antifúngico botânico, optou-se por apresentar uma comparação entre sua atividade e o efeito de um produto comercial de uso convencional. Em se tratando da velocidade de crescimento dos fungos apodrecedores estudados, os resultados encontrados indicam que o efeito do OE não diferiu do propiconazole. Considerando o

crescimento micelial das quatro espécies fúngicas, apenas *L. lepideus* demonstrou maior resistência ao extrativo, quando comparado ao produto comercial. Essas constatações sugerem a possibilidade de uma futura utilização do OE de *H. ringens* como componente ativo de um antifúngico com potencial para controlar fungos apodrecedores da madeira, principalmente os representantes de podridão-branca, grupo que, em geral, se mostrou mais suscetível ao efeito do extrativo.

A avaliação do mecanismo de ação antifúngica de uma substância permite compreender a forma com que a mesma age sobre as espécies fúngicas. A verificação dos mecanismos inibitórios de constituintes naturais é fundamental para o futuro desenvolvimento de produtos antifúngicos (BENTO et al., 2014). Para o Artigo 4, optou-se pela escolha da averiguação dos possíveis efeitos do OE de *H. ringens* e da pulegona sobre a concentração do ergosterol presente em membranas plasmáticas dos fungos apodrecedores da madeira. Contudo, o método inicialmente escolhido para extração desse esterol (TIAN et al., 2012) não foi eficaz para nenhuma das espécies avaliadas. Até o momento da realização dos ensaios, nenhuma informação havia sido encontrada na literatura sobre a utilização desse método em fungos apodrecedores da madeira. Dessa forma, o método de Tian et al. (2012) precisou ser otimizado e o Artigo 3 propõe uma técnica para extração de ergosterol de fungos de podridão-branca e parda. Os resultados apresentados para as massas do micélio dos fungos e as curvas de absorbâncias verificadas comprovam a validação do método proposto. Uma vez padronizada, a técnica foi utilizada no Artigo 4 para avaliar os possíveis efeitos do OE e da pulegona sobre *G. applanatum*, *T. versicolor*, *G. trabeum* e *L. lepideus*. Os resultados encontrados confirmam que tanto o OE de *H. ringens* quanto a pulegona interferiram negativamente na quantidade de ergosterol nas membranas plasmáticas das duas espécies de fungos de podridão-branca analisadas. Todavia, efeito similar não foi detectado quando extrativo e substância foram testados contra as espécies de podridão-parda.

Estudo realizado com extratos de espécies de *Casearia* Jacq. demonstrou que um dos mecanismos de ação de extrativos sobre fungos apodrecedores da madeira é o fato destes causarem estresse oxidativo nos microrganismos (BENTO et al., 2014). Entretanto, nenhuma informação foi encontrada a respeito dos mecanismos de ação antifúngica de OEs sobre fungos de podridão-branca e parda. Os resultados obtidos no Artigo 4 demonstram que esses extrativos podem influenciar a quantidade de ergosterol presente na membrana plasmática de um fungo apodrecedor da madeira, sendo esse, um mecanismo de ação antifúngica a ser considerado em futuros trabalhos envolvendo espécies de podridão-branca e parda.

Preservantes são considerados substâncias químicas utilizadas na madeira para aumentar o seu tempo de vida útil, tornando o material tóxico ou repelente a organismos prejudiciais, como os fungos (JAYANETTI, 1986). Segundo o autor, além da toxidez, os preservantes devem apresentar outras características: não ser lixiviáveis; conseguir penetrar na madeira e ser inofensivos a ela; não corroer metais; ser baratos; poder ser obtidos em grandes quantidades; não apresentar odor; não apresentar cor; conseguir repelir umidade. A falta de preservação em estruturas madeireiras pode ocasionar perda de materiais e despesas com mão de obra e exigir a substituição de produtos (DE BELIE et al., 2000). Os prejuízos proporcionados pelos fungos apodrecedores da madeira justificam a necessidade da utilização de produtos preservantes com potencial antifúngico.

A indústria utiliza preservantes que, apesar da proteção proporcionada, acabam ocasionando poluição ambiental e oferecendo riscos à saúde dos trabalhadores (KUMAR; GUPKA, 2006). A literatura apresenta outras possíveis formas de controle de fungos apodrecedores da madeira, como OEs, constituintes químicos isolados e extratos obtidos com o uso de solventes orgânicos. Os monoterpenoides carvacrol, eugenol, timol e citral apresentaram efeito tóxico contra as espécies causadoras de podridão-branca *Trametes hirsuta* (Wulfen) Pilát, 1939, *Schizophyllum commune* Fries, 1815 e *Pycnoporus sanguineus* (L.) Murrill. (1904) (ZHANG et al., 2016). Extratos de espécies de *Juniperus* foram testados contra os fungos de podridão-parda *G. trabeum* e *Postia placenta* (Fr.) M. J. Larsen & Lombard, e de podridão-branca *T. versicolor* e *Irpex lacteus* (Fr.: Fr.) Fr., e apresentaram maior efeito contra as os fungos do segundo grupo (TUMEN et al., 2013). Comportamento semelhante foi detectado para os extrativos de *H. ringens*, apresentado no Artigo 4 da presente tese. A partir dos resultados observados, pode-se concluir que, no geral, os fungos de podridão-branca foram mais suscetíveis aos efeitos do OE de *H. ringens* e pulegona, sendo *T. versicolor*, a espécie mais afetada.

Existem muitos aspectos positivos a serem considerados na substituição de pesticidas sintéticos, habitualmente utilizados, por pesticidas botânicos. Uma vez que a composição química de um OE geralmente é caracterizada por apresentar misturas complexas de monoterpenoides e sesquiterpenoides, essas classes de constituintes promoveriam uma rápida volatilização de um pesticida desenvolvido a partir desse extrativo (ISMAN; MACHIAL; 2006). Como foi possível observar nas caracterizações químicas do OE de folhas de *H. ringens* descritas nos Artigos 2 e 4, o extrativo é composto majoritariamente por monoterpenoides, cujo integrante majoritário é a pulegona. Entretanto, sesquiterpenoides também foram identificados. A detecção dessas classes químicas reforça a futura possibilidade

da utilização do OE da planta para o desenvolvimento de um pesticida botânico. De acordo com Isman (2000), um dos fatores atraentes para a utilização de um OE no controle de pragas é o nível de toxicidade favorável, evitando efeitos prejudiciais aos mamíferos. Uma vez que é frequente o efeito dos diferentes componentes por mecanismos de ação distintos, via de regra, as misturas complexas que caracterizam o extrativo ainda postergam a resistência adquirida pelas pestes (ISMAN; MACHIAL, 2006), em comparação aos produtos sintéticos que apresentam uma única substância ativa.

Os resultados obtidos no Artigo 4 sugerem a possibilidade da utilização do OE de espanta-pulga e da pulegona como componentes de antifúngicos botânicos para controlar fungos apodrecedores da madeira. Todavia, para que ambos venham a serem utilizados como preservantes da madeira, estudos posteriores devem ser realizadas envolvendo corpos de prova, e, futuramente, testes a campo, com o objetivo de verificar os possíveis efeitos desse extrativo e seu constituinte majoritário sobre as propriedades da madeira, bem como visando definir como serão realizadas suas manipulações e aplicações.

Em estudo sobre variabilidade genética de populações de *H. ringens*, foi identificado que a espécie apresenta baixa variabilidade intrapopulacional (FRACARO; ECHEVERRIGARAY, 2006). Segundo os autores, os baixos níveis de heterozigose detectados indicam endogamia e apresentam riscos para a espécie. Para que o OE de *H. ringens* venha a ser utilizado no futuro como preservante da madeira devido as suas propriedades antifúngicas, é fundamental que esse extrativo seja obtido em grandes quantidades. Estudos sugerem a criação de microrreservas e áreas de conservação de *H. ringens*, além da transferência de genes entre populações e o monitoramento da variabilidade genética (FRACARO, 2006; FRACARO; ECHEVERRIGARAY, 2006).

A realização de testes histoquímicos em folhas e pétalas de *H. ringens* possibilitou a detecção de quantidades expressivas de OEs, oleorresinas e compostos fenólicos nesses órgãos vegetais. O OE de folhas foi extraído, caracterizado quimicamente e teve sua atividade antifúngica avaliada frente a fungos apodrecedores da madeira. Espera-se que os resultados obtidos na presente tese estimulem novas pesquisas envolvendo extração e identificação química de outras classes de constituintes produzidas por *H. ringens*, além da descoberta de possíveis novas bioatividades para o seu OE. Também se espera que estudos visando à reprodução da espécie sejam impulsionados, com o objetivo de eliminar a ameaça de sua extinção.

7 CONCLUSÃO

- Através da caracterização estrutural de órgãos vegetativos de *H. ringens*, foram encontradas características marcantes, como a presença de dois tipos de cristais e tricomas tectores e glandulares;

- Além de OEs, folhas e pétalas de *H. ringens* produzem diversas classes de constituintes com potenciais a serem explorados;

- Óleos essenciais e oleorresinas foram detectados em abundância tanto em tricomas glandulares quanto no mesofilo de folhas e pétalas de *H. ringens*, explicando o elevado rendimento de OE nos dois órgãos vegetais;

- As composições químicas dos OEs de folhas e inflorescências de *H. ringens* apresentaram o monoterpenoide oxigenado pulegona como constituinte majoritário;

- O método otimizado para extração e quantificação de ergosterol presente nas membranas plasmáticas de fungos apodrecedores da madeira foi bem sucedida em ambos os grupos, causadores de podridão-branca e parda.

- Nos testes para avaliação de atividade antifúngica, tanto o OE de *H. ringens* quanto a pulegona isolada afetaram negativamente os fungos de podridão-branca e parda;

- O OE de *H. ringens* apresentou efeito fungistático sobre a espécie *T. versicolor*;

- Pulegona apresentou efeito fungistático sobre *G. applanatum* e fungicida sobre *T. versicolor*;

- Considerando os resultados de IC50 encontrados, os fungos de podridão-branca foram mais suscetíveis ao efeito da pulegona, em comparação ao OE de *H. ringens*;

- Tanto o OE de *H. ringens* quanto a pulegona afetaram negativamente a quantidade de ergosterol presente nas membranas plasmáticas dos fungos de podridão-branca;

- O OE de *H. ringens* e pulegona apresentam potencial para desenvolvimento de pesticidas botânicos preservantes da madeira.

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