UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Estudo da composição química e atividades biológicas do óleo volátil de espécies de *Nectandra* Rol. ex Rottb. e *Schinus lentiscifolius* Marchand

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Estudo da composição química e atividades biológicas do óleo volátil de espécies de *Nectandra* Rol. ex Rottb. e *Schinus lentiscifolius* Marchand

Tese apresentada por **Letícia Jacobi Danielli** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas.

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“Cada um terá a vista da montanha que subir.”

Ícaro Fonseca

RESUMO

Os óleos voláteis e seus compostos isolados têm sido alvo de investigações relacionadas à capacidade de eliminar microrganismos patogênicos, considerando-se que sua função de proteção do vegetal contra o ataque de patógenos é um indicativo de que tais substâncias podem ser potencialmente terapêuticas. Em virtude da limitada terapia antifúngica disponível, associada ao desenvolvimento de resistência e níveis elevados de toxicidade, o emprego de tal classe de metabólitos secundários é investigado tanto na prevenção quanto no tratamento de processos infecciosos. O estudo das atividades biológicas de um óleo volátil está relacionado ainda ao entendimento das alterações na composição química ocasionadas por mudanças sazonais como tentativa de reconhecer fases do desenvolvimento do vegetal potencialmente ativas. Tendo em vista a importância da identificação de novas substâncias com atividade antifúngica a fim de proporcionar alternativas terapêuticas efetivas, este estudo determinou a composição química e as atividades antifúngica, antioxidante e antiquimiotáxica de óleos voláteis de *Nectandra megapotamica*, *Nectandra lanceolata* e *Schinus lentiscifolius* obtidos por hidrodestilação. Além disso, o mecanismo de ação antifúngico dos mesmos foi investigado e a variação da composição química sazonal do óleo volátil de *N. megapotamica* e sua relação com as atividades biológicas foi determinada. Quimicamente, ambos os óleos apresentaram predominância da fração sesquiterpênica, com os compostos biciclogermacreno (33,4%), β-cariofileno (32,5%) e γ-eudesmol (12,8%) identificados como majoritários respectivamente para as amostras de *N. megapotamica*, *N. lanceolata* e *S. lentiscifolius.* Atividade seletiva frente a dermatófitos foi evidenciada para os óleos de todas as espécies, sendo a membrana fúngica o provável alvo de ação. Especificamente para *S. lentiscifolius* há ainda o envolvimento da parede celular, indicado pela presença de dano em estruturas hifais, observadas por microscopia eletrônica, bem como resultado positivo para o ensaio de ergosterol. Através da técnica de *checkerboard* avaliou-se a combinação dos óleos voláteis e antifúngicos comerciais – terbinafina e ciclopirox – frente a isolados dos gêneros *Microsporum* e *Trichophyton*. Resumidamente, a combinação de *N. lanceolata* e ciclopirox apresentou interações

sinérgicas e aditivas para grande parte dos isolados (75%), enquanto que para *S. lentiscifolius* sinergismo foi observado principalmente em associação à terbinafina. Este efeito é devido, provavelmente, à ação das substâncias em alvos distintos da célula fúngica. Em relação à atividade antiquimiotáxica, os óleos de ambas as espécies inibiram significativamente a migração leucocitária, com percentual de atividade de até 95%. O efeito na quimiotaxia dos leucócitos está diretamente relacionado à ação da substância na fase inicial do processo inflamatório. Além das atividades mencionadas, o óleo de *S. lentiscifolius* apresentou efeito inibitório na formação de biofilme por *M. canis*, principal agente causador de tinea capitis. A capacidade de formação de biofilme por este microrganismo foi relatada pela primeira vez e caracterizada pela presença de regiões compactas de matriz extracelular polissacarídica, compatível com biofilme maduro. Em relação ao óleo de *N. megapotamica*, o estudo de variabilidade da composição química indicou forte influência da fração monoterpênica, principalmente limoneno, α- e β-pineno na bioatividade do óleo. Em suma, os resultados indicam que os óleos voláteis testados atuam de forma a sensibilizar a célula fúngica à ação do agente antifúngico resultando em melhora no efeito e redução da concentração ativa do fármaco, minimizando, consequentemente, os efeitos adversos relacionados. Além disso, apresentam a vantagem de um efeito anti-inflamatório associado, reduzindo sintomas e acelerando o processo de cura.

Palavras-chave: atividade antifúngica, mecanismo de ação, *Nectandra lanceolata*, *Nectandra megapotamica*, óleos voláteis, *Schinus lentiscifolius*, variação sazonal.

ABSTRACT

Study of the chemical composition and biological activities of the volatile oil from *Nectandra* Rol. ex Rottb. species and *Schinus lentiscifolius* Marchand

Volatile oils and their isolated compounds have been subject of investigations related to the ability to eliminate pathogenic microorganisms, considering that their function of protecting plant against pathogens attack is an indicative that these substances may be potentially therapeutic. Due to the limited antifungal therapy available, associated with the development of resistance and high levels of toxicity, the use of this class of secondary metabolites is investigated in the prevention and treatment of infectious processes. The study of biological activities of volatile oils is also related to the understanding of changes in the chemical composition caused by seasonal variations as an attempt to recognize potentially active phases of plant development. In view of importance of identifying new substances with antifungal activity in order to provide effective therapeutic alternatives, this study determined the chemical composition and the antifungal, antioxidant and antichemotactic activities of *Nectandra megapotamica*, *N. lanceolata* and *Schinus lentiscifolius* volatile oils obtained by hydrodistillation. In addition, the antifungal mechanism of action of the oils was investigated out and the variation of chemical composition of *N. megapotamica* volatile oil and its relation with the biological activities was determined. Chemically, both oils showed predominance of sesquiterpene fraction, with the compounds bicyclogermacrene (33.4%), β-caryophyllene (32.5%) and γ-eudesmol (12.8%) identified as majorities respectively for *N. megapotamica*, *N. lanceolata* and *S. lentiscifolius*. Selective activity against dermatophytes was evidenced for all species and the fungal membrane is considered the probable target. Specifically for *S. lentiscifolius* there is also the involvement of the cell wall, indicated by the presence of damage in hyphal structures observed by scanning electron microscopy, as well as positive results for the ergosterol assay. The checkerboard technique was employed to evaluate the combination of the volatile oils and commercial antifungal agents - terbinafine and ciclopirox - against isolates of the genera *Microsporum* and *Trichophyton*. Briefly, the combination of *N. lanceolata* and

ciclopirox presented synergic and additive interactions for most isolates (75%), whereas to *S. lentiscifolius*, synergism was observed mainly in association with terbinafine. This effect is probably due to the action of the substances on different targets of the fungal cell. In relation to antichemotactic activity, the oils of all species significantly inhibited leukocyte migration, with activity of up to 95%. The effect on leukocyte chemotaxis is directly related to the action of the substance in the initial phase of the inflammatory process. In addition to the mentioned activities, *S. lentiscifolius* oil showed an inhibitory effect on the biofilm formation by *Microsporum canis*, the main agent of tinea capitis. The capacity of biofilm formation by this microorganism was reported by first time. The formed sctructure was characterized by the presence of compact regions of polysaccharide extracellular matrix, compatible with mature biofilm. In relation to the study of variability in the chemical composition from *N. megapotamica* oil, it was observed a strong influence of the monoterpene fraction, mainly limonene, α- and β-pinene, in the bioactivity of the oil. In summary, the results indicate that the volatile oils tested act as chemosensitizer of the fungal cell to the action of the antifungal agent resulting in an improvement in the effect and reduction of active drug concentration minimizing the related adverse effects. In addition, these substances have the advantage of a combined anti-inflammatory effect, reducing symptoms and accelerating the healing process.

Keywords: antifungal activity, essential oils, mechanism of action, *Nectandra lanceolata*, *Nectandra megapotamica*, *Schinus lentiscifolius*, seasonal variation.

SUMÁRIO

Plantas são consideradas a base do sistema de medicina tradicional e importante fonte de moléculas na investigação de novos fármacos (SOARES et al., 2013; LIU et al., 2016). Caracterizados por ampla diversidade estrutural, responsável por diferentes atividades biológicas, os produtos naturais fornecem, em muitos casos, a base para o desenvolvimento de agentes sintéticos de importância terapêutica (SOARES et al., 2013).

Óleos voláteis e seus compostos isolados estão entre os produtos naturais intensamente investigados, em virtude da ampla gama de bioatividades relacionadas (AMORATI et al., 2013; PERRICONE et al., 2015; SÁ et al., 2015). Esta classe de metabólitos secundários detém a função, entre outras, de proteção do vegetal contra o ataque de herbívoros e microrganismos, o que é considerado como um indicativo de que derivados antimicrobianos podem ser potencialmente terapêuticos (RAUT; KARUPPAYIL, 2014).

Substâncias biologicamente ativas obtidos de produtos naturais, como os óleos voláteis, têm sido alvo de investigações a fim de determinar sua capacidade em eliminar microrganismos patogênicos e superar problemas de toxicidade e resistência aos antimicrobianos, incluindo atividade antibiofilme (NEWMAN et al., 2000; SOARES et al., 2013; NEGRI et al., 2014; PEIXOTO et al., 2017). Considerando a limitada terapia antifúngica disponível no contexto atual, associada ao mecanismo de ação fungistático, desenvolvimento de resistência pelo microrganismo e elevados níveis de toxicidade, intensificou-se a busca por substâncias que possam ser empregadas tanto na prevenção quanto no tratamento de processos infecciosos, inclusive de forma complementar à terapia convencional (DIAS et al., 2013; SOARES et al., 2013; ASDADI et al., 2015; SCORZONI et al., 2016).

Diversos fungos de interesse clínico demonstram-se sensíveis à ação dos óleos voláteis, inclusive de forma associada a fármacos antifúngicos (HOUËL et al., 2013; CASTRO et al., 2015; CARDOSO et al., 2016). A terapia combinada é considerada uma estratégia viável com a abordagem de múltiplos alvos, a fim de alcançar o efeito sinérgico entre substâncias, reduzindo a dose empregada e, consequentemente, no caso dos antifúngicos, os níveis de toxicidade e o retardo ou minimização no surgimento de resistência (BADDLEY; PAPPAS, 2005; CARRILLO-MUÑOZ et al., 2014; ZHANG

et al., 2014). A efetividade de tal estratégia foi descrita para óleos voláteis e compostos isolados combinados a agentes antifúngicos convencionais frente a diversos microrganismos, incluindo dermatófitos e isolados de *Candida* resistentes ao fluconazol (AHMAD et al., 2013; CARDOSO et al., 2016).

Associado ao importante efeito antifúngico, os óleos voláteis desempenham atividade antioxidante e anti-inflamatória, melhorando a efetividade da substância no combate à infecção (CABRAL et al., 2015). Um processo inflamatório é fundamental na defesa do hospedeiro durante um processo infeccioso, contudo, respostas exacerbadas podem causar danos aos tecidos e mesmo aumentar a suscetibilidade aos patógenos oportunistas (ROMANI, 2011). Além disso, a massiva produção de espécies reativas pelas células do sistema de defesa pode gerar um desbalanço no equilíbrio redox e causar danos em tecidos circundantes, contribuindo para o desenvolvimento de doenças inflamatórias crônicas (WRIGHT et al., 2010; ORHAN et al., 2016). Dessa forma, a associação de efeito anti-inflamatório e antioxidante à atividade antifúngica, amplamente descrita para óleos voláteis, torna esta classe de metabólitos como importante alvo na investigação de substâncias com potencial antifúngico, com a finalidade de acelerar o alívio dos sintomas, favorecer a cura e prevenir a propagação da infecção, além de evitar o desenvolvimento de cronicidade (HAVLICKOVA; FRIEDRICH, 2008; HUBE et al., 2015).

É sabido que a composição química de um óleo volátil sofre a influência de diversos fatores genéticos e ambientais, fato que está intimamente relacionado às suas bioatividades (FURTADO et al., 2014; LEMOS et al., 2015; DHOUIOUI et al., 2016). Desta forma, a variabilidade química de óleos tem sido investigada na tentativa de determinar a correlação entre os compostos e as atividades biológicas empregando ferramentas de estatística multivariada. A partir dos resultados obtidos por meios analíticos associados a técnicas quimiométricas é possível verificar a existência de similaridade entre amostras estabelecendo tendências e determinando marcadores e quimiotipos (PINHEIRO et al., 2016; SORO et al., 2016; YANG et al., 2016).

De uma maneira geral, as espécies abordadas neste estudo – *Nectandra megapotamica* (Spreng.) Mez, *Nectandra lanceolata* Ness. e *Schinus lentiscifolius* Marchand - apresentam grande emprego econômico na indústria moveleira e da

construção civil (ZANON et al., 2009; MACÍAS-VILLAMIZAR et al., 2015; LABORATÓRIO DE MANEJO FLORESTAL DA UNICENTRO, 2016). Contudo, óleos voláteis obtidos de espécies da família Lauraceae e Anacardiaceae, estão relacionados a diversas atividades biológicas (TONDOLO et al., 2013; TORRES et al., 2014; PRATTI et al., 2015; DANIELLI et al., 2017b). Desta forma, por tratarem-se de espécies nativas do Sul do Brasil e considerando o envolvimento de representantes dos gêneros *Nectandra* e *Schinus* em importantes investigações de atividade biológica, foram selecionadas para o desenvolvimento deste estudo.

Tendo em vista a importância da identificação de novas substâncias com atividade antifúngica a fim de proporcionar alternativas terapêuticas efetivas, este estudo propõe a determinação da composição química e avaliação das atividades antifúngica, antioxidante e antiquimiotáxica de óleos voláteis de espécies do gênero *Nectandra* Rol. ex Rottb. e de *Schinus lentiscifolius* Marchand, bem como a investigação do mecanismo de ação antifúngico dos mesmos através de ensaios específicos e determinação da variação sazonal na composição química do óleo volátil de *N. megapotamica* e sua relação com as atividades biológicas.

Objetivos específicos incluem:

1. Revisar a literatura sobre estudos de combinação entre óleos voláteis e seus compostos isolados e antifúngicos comerciais (Capítulo II);
2. Determinar a composição química e atividades antifúngica, antioxidante e antiquimiotáxica do óleo volátil de espécies do gênero *Nectandra* Rol. ex Rottb., sua interação com antifúngicos comerciais e mecanismo de ação antifúngico (Capítulo III);
3. Determinar a composição química e atividades antifúngica, antioxidante e antiquimiotáxica do óleo volátil de *Schinus lentiscifolius* Marchand, sua interação com antifúngicos comerciais e mecanismo de ação antifúngico (Capítulo IV);
4. Investigar a capacidade de formação de biofilme por fungos do gênero *Microsporum* (Capítulo V);
5. Avaliar o efeito do óleo volátil de *Schinus lentiscifolius* na formação de biofilme por *Microsporum canis* (Capítulo VI);
6. Investigar a influência da variação da composição química do óleo volátil de *Nectandra megapotamica* nas atividades antiquimiotáxica, antioxidante e antifúngica empregando métodos quimiométricos (Capítulo VII).

A utilização de plantas como fonte de cura para várias doenças decorre desde a antiguidade. Empregadas por milhares de pessoas, tanto na forma de decocções, como cataplasma ou infusões, são consideradas a base do sistema de medicina tradicional (SOARES et al., 2013; LIU et al., 2016). Devido a fácil obtenção, as plantas são consideradas alternativas econômicas utilizadas na medicina popular para o tratamento de diversas patologias (ROJAS et al., 2006). Suas distintas atividades biológicas atuando através de diferentes mecanismos de ação associadas à ampla diversidade estrutural torna os produtos naturais uma importante fonte de desenvolvimento de novos fármacos (BARROS et al., 2013; HARVEY et al., 2015).

Substâncias de origem natural têm grande contribuição no contexto de novos compostos bioativos, fornecendo base para pesquisa química e descoberta de novos fármacos, além de inspiração para o desenvolvimento de agentes sintéticos de importância terapêutica (SOARES et al., 2013). O antimalárico, artemisinina, isolado de *Artemisia annua*, e taxol, importante fármaco no tratamento do câncer, obtido de *Taxus brevifolia*, são exemplos bem-sucedidos desta abordagem (RAUT; KARUPPAYIL, 2014). Entre 1981 e 2014, 51,2% de novos fármacos consistiam, mimetizavam ou derivavam de produtos naturais (NEWMAN; CRAGG, 2016). Apesar do crescimento de compostos sintéticos ou semissintéticos, as substâncias de origem natural e derivados ainda representam mais de um terço de todas as novas moléculas aprovadas pelo Food and Drug Administration (FDA) (PATRIDGE et al., 2016).

1. Óleos voláteis

Terpenos, fenilpropanoides e alcaloides compõem os principais grupos de uma importante diversidade de metabólitos secundários produzidos pelas plantas (BASSOLÉ; JULIANI, 2012). Devido a propriedades repelentes, tais metabólitos desempenham importante função de proteção do vegetal contra ataque de herbívoros e microrganismos, além de interação planta-planta e atração de disseminadores de sementes e polinizadores, possibilitando a reprodução do vegetal (BASSOLÉ; JULIANI, 2012; HEMMERLIN et al., 2012; REHMAN et al., 2016).

Óleo essencial, também conhecido como óleo volátil, é definido como um produto obtido por hidrodestilação, destilação a vapor ou seca ou por um processo mecânico adequado sem aquecimento (para os frutos de *Citrus*) de uma planta ou partes dela (FARMACOPEIA EUROPEIA, 2008; ISO 9235, 2013). Consistem em misturas complexas de constituintes de baixo peso molecular, alta pressão de vapor ou alta volatilidade em temperatura e pressão normais, e densidade geralmente menor em relação à água (REHMAN et al., 2016). Quimicamente, caracterizam-se pela presença de compostos terpênicos, onde monoterpenos e sesquiterpenos, bem como seus derivados oxigenados, constituem a fração predominante, muito embora fenilpropanoides, ácidos graxos e seus ésteres também ocorram (BAKKALI et al., 2008; BASSOLÉ; JULIANI, 2012). Além disso, são consideradas substâncias extremamente complexas devido à presença de compostos altamente funcionalizados e de diferentes classes químicas (REHMAN et al., 2016).

Em geral, apresentam-se líquidos em temperatura ambiente, com ocorrência nas formas resinosa ou sólida em menor frequência, de coloração característica amarelo pálido, podendo variar a verde, azul ou vermelho. Tais metabólitos podem ser biossintetizados em todos os órgãos do vegetal, de acordo com as características da planta: folhas, flores, caules, sementes, raízes, rizomas e frutos (ZUZARTE et al., 2011). Tricomas glandulares representam as mais comuns estruturas secretoras e de armazenamento de óleos voláteis, no entanto, tais processos também são verificados em células secretoras e epidérmicas, dutos e cavidades (BAKKALI et al., 2008).

Os óleos voláteis podem ser extraídos por diversos métodos, como fluido supercrítico, *headspace*, extração por solvente ou prensagem, no entanto, hidrodestilação e destilação por arraste de vapor caracterizam-se como as técnicas comumente empregadas (BASSOLÉ; JULIANI, 2012). Devido às propriedades de volatilidade e polaridade dos constituintes presentes nos óleos, a cromatografia em fase gasosa, através de determinação do índice de Kovats, índice de retenção linear e tempo de retenção relativo, associada a dados espectrais, é considerada padrão-ouro na identificação dos constituintes químicos presentes nestas matrizes (RUBIOLO et al., 2010).

A constituição química de um óleo pode ser afetada por diversos fatores, sendo eles extrínsecos ou intrínsecos ao vegetal. Os principais componentes podem constituir até 70% do óleo total, enquanto que a presença de compostos minoritários é representativa de quantidades vestigiais (BAKKALI et al., 2008). Proporções distintas de metabólitos secundários em plantas, bem como variações temporais e espaciais no conteúdo total podem ocorrer (GOBBO-NETO; LOPES, 2007). Fatores edafoclimáticos, tais como características e quantidade de nutrientes, estresse hídrico, sazonalidade, radiação ultravioleta, altitude, poluição atmosférica, temperatura e clima, influenciam de forma significativa no rendimento e atividades biológicas desempenhadas por estas substâncias (GOBBO-NETO; LOPES, 2007; LIU et al., 2011; DJERRAD et al., 2015; CHRYSARGYRIS et al., 2016).

Diversos gêneros, distribuídos em cerca de 60 famílias são caracterizados pela produção de óleos voláteis. Alliaceae, Apiaceae, Asteraceae, Lamiaceae, Myrtaceae, Poaceae e Rutaceae são amplamente conhecidas pela produção de óleos com alto valor industrial e medicinal (CARSON; HAMMER, 2011). Aproximadamente 3000 óleos voláteis já foram identificados e cerca de 300 apresentam valor agregado na indústria (BAKKALI et al., 2008; CARVALHO et al., 2016). O emprego de grande parte dos terpenos, principalmente mono e sesquiterpenos, está vinculado ao mercado cosmético e alimentício, especialmente como agentes aromatizantes em alimentos, bebidas, perfumes e cosméticos, além da utilização como conservantes naturais (BAKKALI et al., 2008; LLANA-RUIZ-CABELLO et al., 2015; CARVALHO et al., 2016; LOW et al., 2016). Em 2015, o mercado global de óleos voláteis ultrapassou US$ 6 bilhões, com destaque para os óleos de espécies dos gêneros *Citrus* L., *Mentha* L. e *Eucalyptus* L'Hér. (GRAND VIEW RESEARCH, 2016). O Brasil, acompanhado da Alemanha, da China, do Japão, da França, dos Estados Unidos, do Reino Unido, da Espanha e da Itália, está entre os principais mercados mundiais de plantas medicinais e aromáticas, aplicadas principalmente na indústria farmacêutica, agronômica, cosmética, alimentícia e sanitária (BAKKALI et al., 2008).

1.1. Biossíntese dos óleos voláteis

Terpenoides ou terpenos constituem uma ampla família de metabólitos secundários contemplando aproximadamente 30.000 membros, classificados em hemiterpenos (C_5), monoterpenos (C_{10}), sesquiterpenos (C_{15}), diterpenos (C_{20}), triterpenos (C_{30}) e politerpenos $(C_5)n$, onde "n" pode estar entre 9 e 30.000 (MCGARVEY; CROTEAU 1995; DEWICK, 2002).

A determinação de isoprenos como sendo uma repetitiva unidade em produtos naturais ocorreu em 1887 por Wallach, numa tentativa de isolamento desta molécula em produtos da pirólise do óleo de turpetina (CROTEAU, 1998). Mais tarde, em 1953, Ruzicka propôs um papel biogenético ao isopreno que foi consistente com as estruturas conhecidas de isoterpenoides. Já a elucidação da biossíntese do ergosterol e colesterol desencadeou a descoberta da rota do mevalonato, determinada na época, como única via de biossíntese de terpenos. A presença de uma rota alternativa foi considerada a partir de discrepâncias observadas na via do mevalonato, onde então, considerou-se a condensação de D-gliceraldeído-3-fosfato e piruvato ativado por acetaldeído, em ligação cabeça-cabeça, a qual resulta na síntese de 1-deoxi-D-xilulose-5-fosfato (DOXP), como o primeiro precursor desta nova via chamada de deoxixilulose fosfato (ZUZARTE; SALGUEIRO, 2015).

Terpenos derivam da via do mevalonato, no citosol e nos cloroplastos, da via deoxixilulose fosfato (Figura 1) (ZUZARTE; SALGUEIRO, 2015). Ambas biossintetizam unidades de 5 carbonos, conhecidas como pirofosfato de isopentanila (PIP) e seu isômero, difosfato de dimetilalina (DDMA), estruturas básicas e precursoras de todos os terpenos. Prenil transferases, em ambas as vias, utilizam PIP e DDMA em reações de condensação para a produção de prenil difosfatos, como o precursor de monoterpenos ($C_{10}H_{16}$), difosfato de geranila, de sesquiterpenos ($C_{15}H_{24}$), difosfato de farnesila, de diterpenos ($C_{20}H_{32}$) e difosfato de geranilgeranila, para os carotenoides (AHARONI et al., 2005). Duas unidades condensadas de difosfato de farnesila originam esqualeno, precursor dos triterpenos e esterois. A via do mevalonato, na qual PIP é formado a partir de acetil CoA via ácido mevalônico, é responsável pela síntese de fitoesterois e ubiquinona. Hidroximetilglutaril-CoA

redutase é a enzima chave regulatória desta via na formação de ácido mevalônico a partir de hidroximetilglutaril-CoA (ENFISSI et al., 2005). Ácido mevalônico é então convertido em PIP pela ação sequencial de ácido mevalônico quinase, fosfomevalonato quinase e fosfomevalonato descarboxilase (MCGARVEY; CROTEAU, 1995). Difosfato de farnesila forma-se a partir de PIP através da ação de difosfato de farnesila sintase, enquanto que esqualeno sintase catalisa a reação de condensação cabeça-cabeça de duas moléculas de difosfato de farnesila para formar esqualeno, no primeiro passo para a formação do esterol (ENFISSI et al., 2005).

Já na via dos cloroplastos, também conhecida como via metileritritol-4-fosfato ocorre a produção de precursores de monoterpenos, diterpenos e tetraterpenos (AHARONI et al., 2005). Esta via utiliza piruvato e gliceraldeído-3-fosfato para a formação de 1-deoxixilulose-5-fosfato (DXP), catalisada por DXP sintase (ZUZARTE; SALGUEIRO, 2015). Apesar da compartimentalização destas vias, fortes evidências indicam cooperação mútua na formação de determinados isoprenoides, provavelmente devido ao metabolismo cruzado entre elas (BICK; LANGE, 2003). Os precursores estão sujeitos a modificações estruturais através de reações de redução, oxidação, conjugação, isomerização, entre outras transformações que originam a ampla variedade de terpenos conhecidos hoje (MCGARVEY; CROTEAU, 1995). Entre os compostos biossintetizados, monoterpenos e sesquiterpenos ocorrem com maior frequência nos óleos voláteis (Figura 2) (BAKKALI et al., 2008).

Fenilpropanoides derivados do aminoácido L-fenilalanina constituem uma importante classe de compostos voláteis envolvidos na reprodução e defesa das plantas (SÁ et al., 2014). Estes compostos podem conter uma ou mais unidades de C_6-C_3, sendo C_6 um anel benzênico (Figura 2) (ZUZARTE; SALGUEIRO, 2015). Basicamente, o aminoácido L-fenilalanina é convertido em ácido *trans*-cinâmico, na via do ácido chiquímico, numa reação catalisada por L-fenilalanina amonialiase (Figura 1). As etapas conseguintes originam uma variedade de ácidos hidroxicinâmicos, aldeídos e álcoois a partir do ácido *trans*-cinâmico correspondente (HUMPHREYS; CHAPPLE, 2002). Alguns destes intermediários podem ser convertidos em compostos voláteis, como o eugenol e isoeugenol, formados a partir de acetato de coniferil catalisados por eugenol sintase e isoeugenol sintase (KOEDUKA

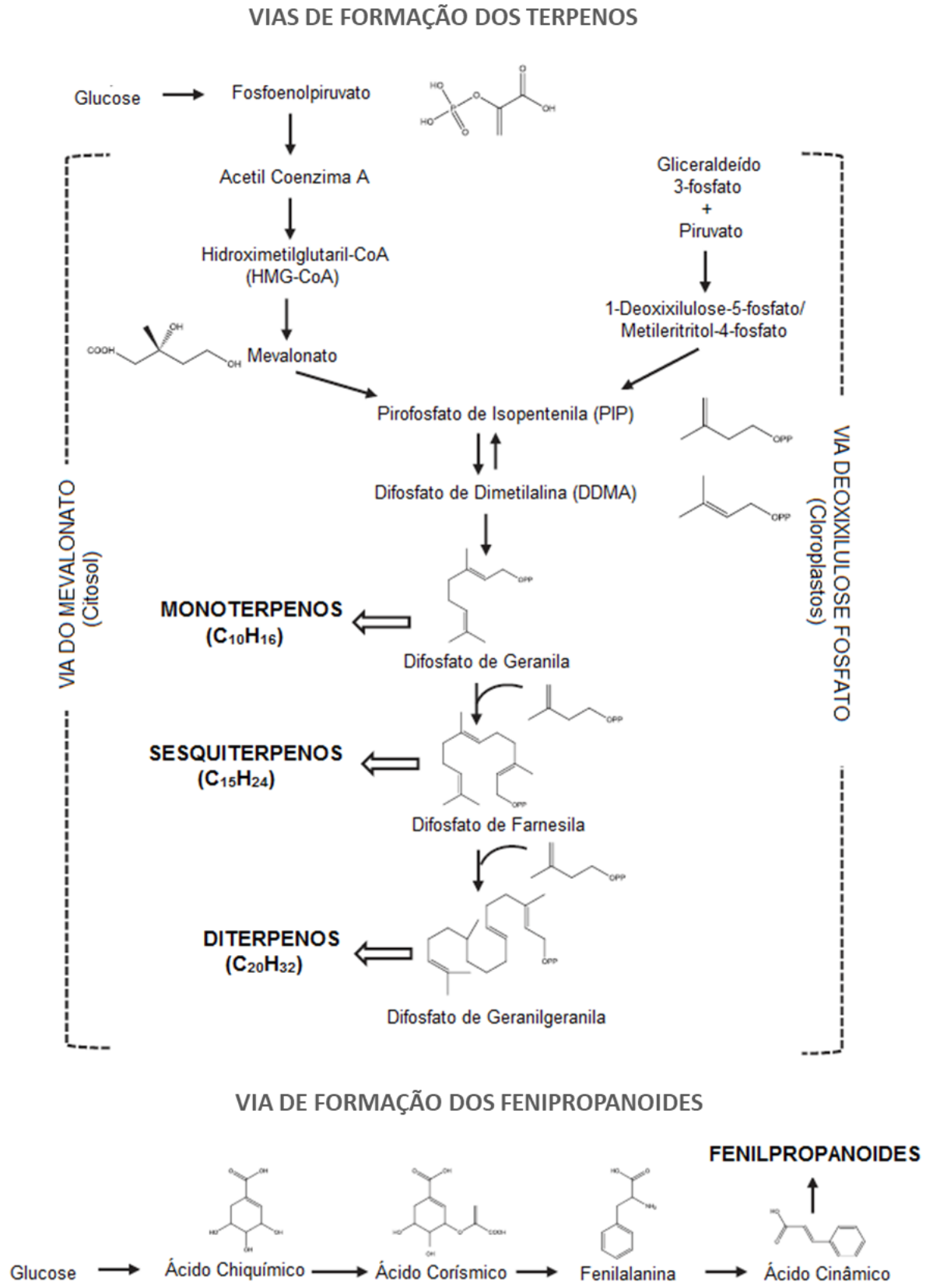

Figura 1. Representação esquemática da biossíntese dos terpenos e fenilpropanoides em vegetais. Adaptado de ZUZARTE e SALGUEIRO (2015).

et al., 2006). Por sua vez, tais compostos podem sofrer metilação e gerar metileugenol e isometileugenol, e assim sucessivamente (DUDAREVA et al., 2006).

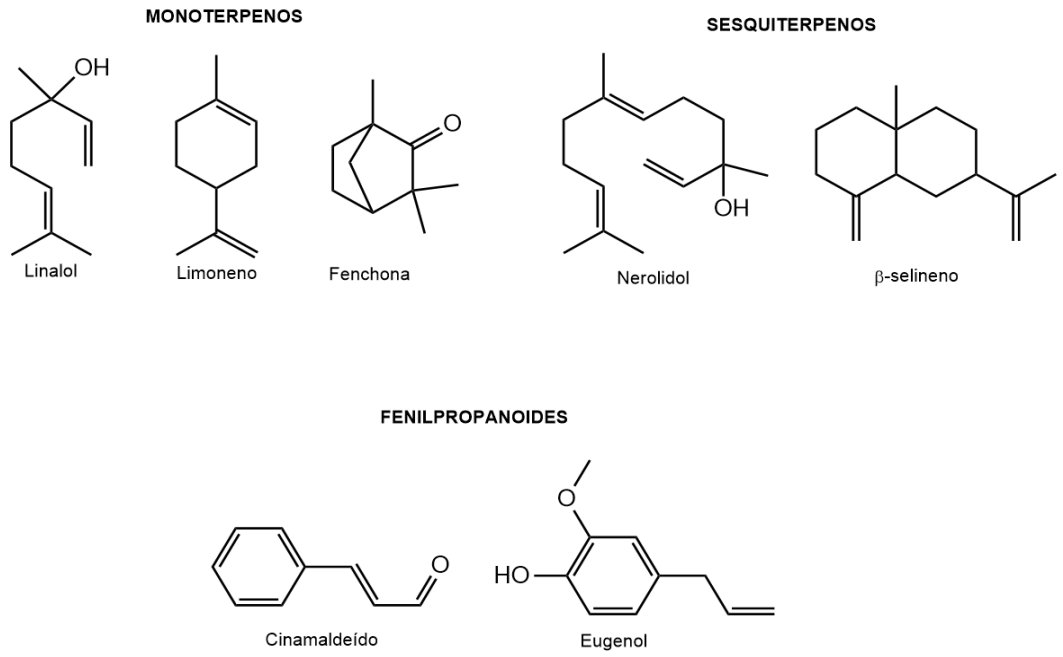

Figura 2. Estruturas representativas das diferentes classes químicas presentes nos óleos voláteis: terpenos e fenilpropanoides.

2. Dermatofitoses

Milhares de pessoas são vítimas de infecções fúngicas superficiais em todo o mundo (TABASSUM; VIDYASAGAR, 2013). O risco estimado de aquisição de uma infecção por dermatófitos durante a vida ocorre entre 10% e 20% (BAJPAI et al., 2009). Em pacientes imunocompetentes o processo infeccioso é considerado superficial, contudo, acarreta importantes implicações físico-sociais ao paciente (CASSELLA et al., 2002; TABASSUM; VIDYASAGAR, 2013). Historicamente, dermatofitoses têm menor prioridade na clínica e pesquisa médica, entretanto, com vistas ao aumento exponencial de pacientes neutropênicos, transplantados e com Síndrome da Imunodeficiência Adquirida (SIDA), quadros infecciosos causados por estes microrganismos têm se tornado preocupantes (CASSELLA et al., 2002). Embora rara, a infecção invasiva por dermatófitos pode ocorrer, principalmente em pacientes imunocomprometidos (VENTURI et al., 2012).

Dermatófitos são fungos altamente especializados em invadir tecidos queratinizados, como pelo, pele e unhas de humanos e animais (AALA et al., 2010). Espécies dos gêneros *Trichophyton* e *Microsporum* causam processos infecciosos superficiais, conhecidos como tinea capitis, tinea pedis, tinea corporis e onicomicoses (BAJPAI et al., 2009). As espécies *T. rubrum* e *T. mentagrophytes* var. *interdigitale* são consideradas como as mais comuns causas de infecções em tecidos queratinizados (CASSELLA et al., 2002; SANTOS; HAMDAN, 2005). Segundo Peres e colaboradores (2010), espécies zoofílicas, como *M. canis* e *T. mentagrophytes*, são responsáveis por cerca de 30% das dermatofitoses humanas, ocasionando, geralmente, um quadro de inflamação aguda associada, enquanto que as espécies antropofílicas, como *T. rubrum* e *T. tonsurans*, representam cerca de 70% das infecções nestes hospedeiros, provocando um processo crônico e de progressão lenta.

Desordens patogênicas causadas por estes microrganismos denotam tratamento longo e oneroso, além de efeitos adversos exacerbados, considerando-se a semelhança de sua estrutura celular às células do hospedeiro (BAJPAI et al., 2009; RAUT; KARUPPAYIL, 2014). A associação destes fatores ocasiona reduzidas taxas de eliminação da infecção (CASSELLA et al., 2002).

Fármacos das classes de azóis e alilamilas são comumente utilizados para o tratamento de dermatofitoses, incluindo formulações de administração oral e tópica. Além destes, derivados de morfolina e ciclopirox também têm sido amplamente empregados (ZUZARTE et al., 2011).

3. Terapia antifúngica convencional

A terapia antifúngica disponível está baseada, principalmente, em fármacos que atuam na membrana citoplasmática (poliênicos, azóis e alilaminas), parede celular (equinocandinas) ou síntese de DNA e proteínas (flucitosina e griseofulvina) (Tabela 1) (BADDLEY; PAPPAS, 2005). Em alguns casos, combinações de antifúngicos de uso tópico e anti-inflamatórios de administração oral têm sido empregadas com a finalidade de aumentar as taxas de cura (PERES et al., 2010; HUBE et al., 2015).

Entre os poliênicos, anfotericina B é, sem dúvida, o representante mais utilizado. Apresenta estrutura lipofílica e liga-se avidamente ao ergosterol, principal esterol da membrana citoplasmática, causando alterações na integridade da membrana, o que resulta em extravasamento do conteúdo intracelular e morte celular (GHANNOUM; RICE, 1999; KAMINSKI, 2014). Entretanto, este fármaco possui afinidade também pelos esteróis de células humanas, o que resulta em efeitos adversos e toxicidade. Já os azóis, como fluconazol, itraconazol, voriconazol e cetoconazol, são empregados como alternativa à anfotericina B, por exibir menor toxicidade. Atuam inibindo a enzima 14-α-demetilase, interrompendo a conversão de lanosterol em ergosterol, resultando também em alteração da permeabilidade da membrana e extravasamento do conteúdo celular (GHANNOUM; RICE, 1999; VALDÉS, 2005). Embora efetivos, também apresentam diversos efeitos adversos e relatos de toxicidade (AALA et al., 2010). Entre as alilaminas, terbinafina é comumente utilizada como primeira escolha no tratamento de infecções causadas por dermatófitos, atuando através da inibição da enzima esqualeno epoxidase, envolvida na síntese do ergosterol da membrana (BADDLEY; PAPPAS, 2005).

Equinocandinas são lipopeptídeos semissintéticos que atuam na parede celular inibindo a 1,3-β-glucana, obtidas como metabólitos da fermentação fúngica (VALDÉS, 2005). Glucanas não estão presentes nas células dos mamíferos, mas são consideradas componentes essenciais da parede celular de fungos patogênicos. Sua depleção leva à instabilidade osmótica e lise na parede celular (ERNST, 2001; VALDÉS, 2005). A flucitosina tem propriedades antimetabólicas. Após sua captação pelo fungo, é convertida em fluoracila por uma reação de deaminação, e posteriormente, em 5-fluorouridina trifosfato, que é então incorporada ao RNA fúngico e inibe a síntese de proteínas. Outro mecanismo refere-se à conversão de fluoracila em fluordeoxirudina monofosfato, que interfere na síntese de DNA inibindo a enzima timidilato sintetase (VERMES et al., 2000). A ocorrência de resistência à flucitosina se dá rapidamente quando utilizada como monoterapia, por esta razão é comumente combinada com anfotericina B (DORSTHORST et al., 2002). Griseofulvina é utilizada para o tratamento de infecções por dermatófitos, atuando por

inibição da mitose fúngica através da ligação à tubulina e a uma proteína associada aos microtúbulos, rompendo, assim, a organização do fuso mitótico (DIAS et al., 2013).

Amorolfina e ciclopirox têm demonstrado importante efetividade frente às onicomicoses em formulações tópicas do tipo esmalte (WELSH et al., 2010). O primeiro pertence à classe das morfolinas e atua inibindo duas enzimas na via biossintética do ergosterol (DIAS et al., 2013; GUPTA et al., 2013). Já o ciclopirox apresenta atividade antifúngica por diferentes mecanismos de ação: por dispor de alta afinidade a cátions trivalentes, atua na inibição da maioria das enzimas dependentes de metal que são responsáveis pela degradação de peróxidos no interior da célula fúngica, além de afetar a produção de energia do transporte mitocondrial de elétrons, a captação de nutrientes e a síntese de proteínas e ácidos nucleicos (GUPTA et al., 2013; ELSAYED, 2015). Ciclopirox e seu sal olamina estão disponíveis em diversas formulações tópicas, para administração na pele, unhas e mucosas (SUBISSI et al., 2010).

Importantes efeitos adversos desencadeados por fármacos antifúngicos, como toxicidade renal para anfotericina B, por exemplo, têm dificultado a terapia. Formas farmacêuticas de administração oral podem ser responsáveis pelos principais efeitos adversos desta classe de fármacos, incluindo hepatotoxicidade, neurotoxicidade, nefrotoxicidade, reações hematológicas e problemas raros de pele, como síndrome de Stevens-Johnson (ZUZARTE et al., 2011).

Além do reduzido arsenal terapêutico e da ocorrência de importantes efeitos adversos, o desenvolvimento de resistência pelos microrganismos tem dificultado o tratamento de infecções fúngicas. Formação de biofilmes, resistência inata a antifúngicos, mutações espontâneas até superexpressão de bombas de efluxo estão entre os mecanismos empregados pelos patógenos a fim de driblar a ação dos fármacos (SANGLARD, 2003; MONK et al., 2008; NIGAM, 2015). Em um estudo comparativo entre antifúngicos empregados no tratamento de onicomicoses, terbinafina demonstrou-se o fármaco mais efetivo, provendo a cura de 70-80% dos casos. O estudo constatou ainda que este antifúngico tem um excelente perfil de tolerabilidade (BENGER et al., 2004). Mutação no gene que codifica a enzima esqualeno epoxidase,

Tabela 1. Principais fármacos utilizados na terapia antifúngica, dispostos em relação à classe, ao mecanismo de ação, à via de administração e aos efeitos adversos.

Classe	Mecanismo de ação	Representantes	Via de Adminstração	Efeitos Adversos
Derivados da Morfolina	Inibição enzimática na via de biossíntese do ergosterol	Amorolfina	Via tópica	Ardor, prurido, vermelhidão e irritação
Hidroxipiridona	Ligação a cátions trivalentes, como Fe^{3+}, inibindo enzimas dependentes de metal	Ciclopirox	Via tópica	Irritação, ardor, edema eritematoso e pigmentação nas unhas
Alilaminas	Inibição da enzima esqueleno epoxidase na via de biossíntese do ergosterol	Terbinafina	Via oral e tópica	Insuficiência hepática, distúrbios de gosto e cheiro, sintomas depressivos, neutropenia grave, síndrome de Stevens-Johnson e necrólise dérmica tóxica
		Butenafina	Via tópica	Prurido, dermatite de contato e eritema
Triazóis e Imidazóis	Inibição da enzima lanosterol 14-α-demetilase na via de biossíntese do ergosterol	Itraconazol	Via oral e intravenosa	Gastrite, diarreia, uticária, síndrome de Stevens-Johnson, tontura, fotosenssibildiade, raramente insuficiência hepatica, entre outros
		Fluconazol	Via oral, tópica e intravenosa	Dor de cabeça, náuseas, dor abdominal, diarreia, erupção acneiforme, neutropenia, hemorragia ocular, entre outros
		Voriconazol	Via oral e intravenosa	Anafilaxia, eritema multiforme, fotossensibilidade, fototoxicidade, síndrome de Stevens-Johnson, alopecia, distúrbios gastrointestinais, entre outros
		Posaconazol	Via oral	Náuseas, vômitos, diarreia, elevação discreta de enzimas hepáticas, e raramente disfunção ou insuficiência hepática
		Miconazol e clotrimazol	Via tópica	Eritema, ardor, dermatite de contato alérgica e prurido
		Cetoconazol	Via oral e tópica	Prurido, vômitos, náuseas, erupção cutânea, anemia hemolítica, hepatotoxicidade, entre outros
Equinocandinas	Inibição da 1,3-β-glucana na parede celular	Caspofungina	Via intravenosa	Efeitos gastrointestinais mínimos e rubor
		Micafungina	Via intravenosa	Reações de anafilaxia, hiperidrose, prurido, hipotensão, dispneia, artralgia, sintomas gastrointestinais, entre outros
		Anidulafungina	Via intravenosa	*Rash*, urticária, rubor, prurido, dispneia, hipotensão, eventual elevação das enzimas hepáticas, entre outros
Poliênicos	Ligação ao ergosterol da membrana fúngica	Nistatina	Via oral e tópica	Irritação local, nauseas e vômitos
		Anfotericina B	Via tópica Via intravenosa	Urticária, alopecia, prurido, hipertensão, delírios, febre, hepato e nefrotoxicidade, entre outros
		Anfotericina B lipossomal, em dispersão coloidal e complexo lipídico	Via intravenosa	Aumento nos níveis de creatinina e distúrbios eletrolíticos. Menor nefrotoxicidade em relação à anfotericina convencional
Antimetabólitos	Interfere na síntese de DNA e proteínas	Flucitosina	Via oral	Leucopenia e trombocitopenia, náuseas, vômitos, diarreria e disfunção hepática
-	Interfere na síntese de DNA	Griseofulvina	Via oral	Dor abdominal e de cabeça, urticária, toxicidade hepática, erupção liquenoide, porfiria aguda intermitente, entre outros

ESPINEL-INGROFF, 2009; DIAS et al., 2013; GUPTA et al., 2013; SINGHAL, 2013.

alvo da terbinafina, provocou alta resistência de *Trichophyton rubrum* a este fármaco (MUKHERJEE et al., 2003). A ocorrência espontânea de resistência da espécie *T. rubrum* à terbinafina, ao itraconazol, ao ciclopirox e à amorolfina foi avaliada em exposição a concentrações subinibitórias destes fármacos. Os resultados indicaram alta incidência de mutantes resistentes ao itraconazol (GHELARDI et al., 2014).

4. Atividades biológicas dos óleos voláteis

Propriedades biológicas de plantas aromáticas têm sido exploradas na medicina tradicional desde a antiguidade. Efeito fungicida, bactericida, inseticida, virucida, antiparasitário, antioxidante, anticâncer, anti-inflamatório e em doenças cardiovasculares e diabetes, foram reportadas (EDRIS, 2007; MIGUEL, 2010b; BAKKALI et al., 2008; SÁ et al., 2014; CABRAL et al., 2015). Apesar da diversidade de atividades biológicas relacionadas, propriedades antimicrobianas, antioxidantes e anti-inflamatórias destacam-se pelo interesse da indústria farmacêutica, cosmética e de alimentos (MIGUEL 2010b; AMORATI et al., 2013; LLANA-RUIZ-CABELLO et al., 2015; PERRICONE et al., 2015; SÁ et al., 2015; CARVALHO et al., 2016). Ação quimiosensibilizante também tem sido observada para óleos e seus derivados. Neste caso, o emprego destes compostos tem a finalidade de potencializar a atividade de uma segunda substância associada (RAUT; KARUPPAYIL, 2014). Associado a estas propriedades, a reduzida toxicidade e genotoxicidade e capacidade de atuação em múltiplos alvos são algumas vantagens vinculadas à utilização de óleos voláteis como agentes terapêuticos (RAUT; KARUPPAYIL, 2014).

De uma forma geral, apesar de tratar-se de uma matriz complexa, a responsabilidade pela bioatividade de um óleo é atribuída a presença de compostos majoritários. Contudo, estudos evidenciam a ocorrência de sinergismo entre componentes individuais, onde majoritários e minoritários, atuando de forma combinada, potencializam o efeito do óleo total em relação aos compostos isolados (HARRIS, 2002; CAVALEIRO et al., 2006).

4.1. Atividade antifúngica

No contexto da terapia antifúngica, o arsenal terapêutico disponível apresenta limitações (KATHIRAVAN et al., 2012). Quase todos os fármacos representantes apresentam limitações terapêuticas, como interações medicamentosas, biodisponibilidade insuficiente, mecanismo de ação fungistático e, principalmente, alta toxicidade e desenvolvimento de resistência pelos microrganismos (PAUW; PICAZO, 2008; DIAS et al., 2013; SOARES et al., 2013; SCORZONI et al., 2016). Por esta razão, intensificou-se a busca por substâncias que possam ser empregadas tanto na prevenção quanto no tratamento dos processos infecciosos, inclusive de forma complementar à terapia convencional (AHMAD et al., 2014; ASDADI et al., 2015). Para Negri e colaboradores (2014), o desenvolvimento de novas opções para fármacos antifúngicos é mandatório em vista do crescente número de infecções e sua alta taxa de mortalidade.

Compostos biologicamente ativos, obtidos de produtos naturais, têm sido alvo de investigações com a finalidade de avaliar sua capacidade em eliminar microrganismos patogênicos e superar problemas de toxicidade e resistência aos antimicrobianos (NEWMAN et al., 2000; SOARES et al., 2013; NEGRI et al., 2014). A ausência ou redução de casos de infecções nos vegetais pode ser considerada como um indicativo de que estes organismos desenvolvem efetivos sistemas de defesa, sugerindo que antimicrobianos derivados de plantas podem ser potencialmente terapêuticos (RAUT; KARUPPAYIL, 2014). Segundo Hemaiswarya e colaboradores (2008), plantas produzem diversas moléculas antibióticas, conhecidas como fitoalexinas (terpenoides, polifenois, flavonoides e glicosteroides), que mesmo em menor potência, controlam e combatem de forma efetiva infecções nos próprios vegetais, muito provavelmente, como resultado de um efeito de sinergia entre elas.

O efeito antifúngico dos óleos voláteis foi descrito pela primeira vez em 1927 por Myers (CASSELLA et al., 2002). Desde então, diversos fungos patogênicos ao homem, incluindo leveduras e fungos filamentosos, demonstraram-se sensíveis à ação destas substâncias (BENGER et al., 2004; PINTO et al., 2006; CHEE; LEE, 2007; AHMADI et al., 2010; BASSOLÉ; JULIANI, 2012; AHMAD et al., 2013; ALVES-SILVA et al., 2013; MELO et al., 2013; ASDADI et al., 2015). Diferentes graus de

inibição estão associados aos distintos mecanismos de ação e composição química do óleo, bem como a influência de fatores intrínsecos relacionados aos microrganismos (RAUT; KARUPPAYIL, 2014).

O mecanismo comumente aceito para o efeito antifúngico de óleos voláteis refere-se ao seu caráter lipofílico, que facilitaria o ingresso na célula, através de permeação ou rompimento da membrana, ocasionando redução de potencial, perda de íons, colapso na bomba de prótons e esgotamento de ATP (BAKKALI et al., 2008). Distorções da membrana celular tornam o microrganismo mais suscetível ao extravasamento de constituintes vitais da célula, afetando a respiração celular e outros sistemas enzimáticos (BURT; REINDERS, 2003; ASDADI et al., 2015). Além disso, inibição enzimática, comprometimento do material genético do patógeno, formação de hidroperoxidase de ácidos graxos pela oxigenação de ácidos graxos insaturados, coagulação do citoplasma, dano a lipídios e proteínas, fluxo de elétrons e/ou transporte ativo, também são considerados como meio de ação antimicrobiana empregado pelos óleos (VIUDA-MARTOS et al., 2011). Em leveduras, atuam ainda inibindo a formação de tubo germinativo (ZUZARTE et al., 2012).

Grande parte desta atividade é atribuída à presença de terpenos oxigenados (como álcoois e compostos fenólicos), contudo há relatos de hidrocarbonetos exibindo efeito antimicrobiano (BURT, 2004; LANG; BUCHBAUER, 2012). Óleos caracterizados pela presença de aldeídos ou fenóis, como timol, carvacrol e cinamaldeído, geralmente apresentam maior eficácia, seguidos daqueles que contêm álcoois terpênicos (BASSOLÉ; JULIANI, 2012).

4.1.1. Atividade antidermatofítica

Atividade antidermatofítica foi relatada para óleos voláteis de diversas espécies vegetais (NARDONI et al., 2015). *Metasequoia glyptostroboides* inibiu o crescimento e germinação dos esporos de isolados de *T. rubrum*, *T. mentagrophytes* e *M. canis* (BAJPAI et al., 2009). Além de atividade antidermatofítica, *Otanthus maritimus* demonstrou efeito anti-inflamatório associado (CABRAL et al., 2013). Autores atribuem a atividade inibitória ao crescimento de dermatófitos demonstrada por *Thyus*

villosus subsp. *lusitanicus* a um efeito sinérgico entre os monoterpenos presentes no óleo (PINTO et al., 2013). Estudo do mecanismo de ação de *Cymbopogon winterianus* frente a dermatófitos indicou envolvimento de membrana, e não evidenciou efeito à nível de parede celular fúngica (PEREIRA et al., 2011), sugerindo, mais uma vez, que o caráter lipofílico do óleo pode particionar a membrana fúngica, alterando sua integridade e causando morte micelial (VIUDA-MARTOS et al., 2011).

4.1.2. Estudos de combinação entre fármacos antifúngicos e óleos voláteis

Com o objetivo de suprir a falta de novos agentes antimicrobianos e combater a resistência ao tratamento convencional, a terapia combinada se torna uma estratégia viável com a abordagem de múltiplos alvos (MUKHERJEE et al., 2003; CARRILLO-MUÑOZ et al., 2014). Uma terapia combinada busca alcançar efeito sinérgico entre substâncias, reduzindo a dose empregada, e consequentemente níveis de toxicidade, além de prevenir/minimizar ou retardar o surgimento de resistência ao fármaco, bem como ampliar o espectro de atividade (BADDLEY; PAPPAS, 2005; CHEN et al., 2010; ZHANG et al., 2014). Além disso, trata-se de uma abordagem com importantes implicações financeiras, considerando que o desenvolvimento de um novo fármaco pode ser muito mais oneroso em relação à reformulação daqueles existentes ou utilização de combinações (VAN VUUREN; VILJOEN, 2011).

Os primeiros relatos de desenvolvimento de resistência aos antifúngicos, como *Cryptococcus* à monoterapia por flucitosina (HOSPENTHAL; BENNETT, 1998), motivaram a avaliação de terapia combinada. Fatores como crescimento de infecções invasivas por fungos oportunistas com consequente acréscimo nas taxas de morbidade e mortalidade também contribuíram para pesquisa de diferentes opções terapêuticas (BADDLEY; PAPPAS, 2005).

Dois fármacos atuando concomitantemente em diferentes alvos de uma célula fúngica podem resultar em aumento de eficácia. Exemplo disso refere-se à combinação de anfotericina B e flucitosina, considerando que o primeiro é um agente de membrana citoplasmática enquanto que o segundo atua na síntese de proteínas ou DNA (BADDLEY; PAPPAS, 2005).

Além de sinérgicas, as interações podem ser aditivas, antagonistas ou de caráter indiferente. Efeito sinérgico é caracterizado pela potencialização da atividade em relação à soma dos agentes individuais. Quando o contrário ocorre, ou seja, a atividade combinada é considerada menor em relação àquela obtida com os agentes de forma individual, é caracterizado um quadro de antagonismo. Um efeito aditivo refere-se a igual eficácia da combinação em relação à soma do efeito dos fármacos individualmente. Por fim, indiferença caracteriza-se quando o efeito da combinação não supera àquele de um agente empregado de forma isolada (LEWIS et al., 2002; KONTOYIANNIS; LEWIS, 2003).

Em geral, a combinação entre substâncias antifúngicas é determinada pelo método de *checkerboard*, associado à determinação da curva de morte ou superfície de resposta (LEWIS et al., 2002). Pelo ensaio de *checkerboard* obtém-se o Índice de Concentração Inibitória Fracional (ICIF). Este índice determina a natureza da interação, classificada como sinérgica (ICIF $\leq$ 0,5), aditiva (0,5 < ICIF < 1), indiferente (1 $\leq$ ICIF< 4) ou antagonista (ICIF $\geq$ 4) (WHITE, et al., 1996; LEWIS et al., 2002).

O ensaio de curva de morte ou *time-kill assay* indica a dinâmica do efeito inibitório da substância, apresentando maiores detalhes ao longo do tempo do efeito antifúngico em relação ao *checkerboard* (LEWIS et al., 2002). Trata-se de uma técnica muito dispendiosa, em virtude da necessidade de intensa contagem de unidades de formação de colônia, além de diversas combinações e concentrações a serem testadas concomitantemente (CUENCA-ESTRELLA, 2004).

Uma das vantagens do emprego combinado de compostos naturais e fármacos sintéticos encontra-se na redução dos efeitos adversos destes antifúngicos (AALA et al., 2010). Alvos incluem modificação no sítio receptor, inibição enzimática, aumento da permeabilidade da membrana e inibição de bombas de efluxo (VAN VUUREN; VILJOEN, 2011).

Estudos têm demonstrado efeito sinérgico entre óleos voláteis e seus derivados com antifúngicos utilizados na terapia convencional. Sinergismo foi relatado para a combinação entre o óleo volátil de *Ocimum sanctum* e os antifúngicos fluconazol e cetoconazol frente a isolados do gênero *Candida* (AMBER et al., 2010). O mesmo foi

observado para *Myrtus communis* e anfotericina B frente a *C. albicans* e *Aspergillus*, o que representa grande relevância considerando o importante perfil de toxicidade deste antifúngico (MAHBOUBI; BIDGOLI, 2010). Além destes, outros exemplos de efeito sinérgico entre óleos voláteis e antifúngicos foram observados: espécies de *Allium* e cetoconazol frente a *Trichophyton* (PYUN; SHIN, 2006); *Melaleuca alternifolia*, *Origanum vulgare* e *Pelargonium graveolens* combinado a anfotericina B (ROSATO et al., 2008); *Origanum vulgare* e *Pelargonium graveolens* associados à nistatina frente a *Candida* (ROSATO et al., 2009); geraniol, citronelol e óleo de *P. graveolens* combinado à anfotericina B e cetoconazol frente a espécies de *Aspergillus* e *Trichophyton* spp. (SHIN, 2003; SHIN; LIM, 2004); *Coriandrum sativum* e anfotericina B e *Piper bredemeyeri* Jacq e itraconazol frente a isolados do gênero *Candida* (SILVA, 2011; TANGARIFE-CASTAÑO et al., 2011); *Otacanthus azureus* combinado com azóis frente a dermatófitos (HOUËL et al., 2013); timol e nistatina frente a espécies de *Candida* (CASTRO et al., 2015); *Ocimum basilicum* var. Maria Bonita e seus principais compostos, linalol e geraniol, com fluconazol frente a isolados sensíveis e resistentes de *Candida albicans* e *Cryptococcus neoformans* (CARDOSO et al., 2016); e *Nectandra lanceolata* associada a ciclopirox e terbinafina frente a espécies dos gêneros *Trichophyton* e *Microsporum* (DANIELLI et al., 2017b).

Óleo de *Mentha piperita* L. e de espécies do gênero *Cymbopogom* combinados a íons de prata também apresentaram efeito sinérgico antimicrobiano (AHMAD et al., 2014; AHMAD; VILJOEN, 2015). Segundo os autores, a interação dos óleos com a membrana celular tornou-a porosa, facilitando o acesso dos íons de prata ao interior da célula (AHMAD; VILJOEN, 2015). O mesmo mecanismo de particionamento da bicamada lipídica da membrana celular fúngica, devido à hidrofibicidade dos óleos, foi sugerido para justificar o efeito sinérgico observado entre *Thymus maroccanus* e *T. broussonetti* com anfotericina B e fluconazol frente a isolados de *Candida* (SAAD et al., 2010).

Timol, eugenol e metileugenol em combinação com fluconazol apresentaram importante efeito sinérgico frente a cepas de *Candida* spp., inclusive para isolados resistentes a este azol (AHMADI et al., 2010; AHMAD et al., 2013). Enquanto que o fluconazol atua na biossíntese de ergosterol, timol apresenta habilidade em alterar a

permeabilidade da membrana fúngica, além de exercer efeito quimiossensibilizante das células do microrganismo ao fármaco e impedir a ação das bombas de efluxo Cdr1 e Mdr1, importante mecanismo de resistência aos azóis (AHMAD et al., 2013).

A combinação de fármacos com óleos voláteis e seus compostos isolados, principalmente monoterpenos, também tem sido empregada no desenvolvimento de formas farmacêuticas transdérmicas por favorecer a penetração da substância ativa na pele e, consequentemente, melhorar sua eficácia em virtude do aumento na permeação (HARRIS, 2002).

A seleção de um composto natural para atuar de forma sinérgica em combinação com determinado fármaco requer o conhecimento prévio sobre o mecanismo de ação do mesmo na ausência e presença deste composto. O ponto crítico recai na falta de informações sobre biodisponibilidade, seletividade, interações adversas, estabilidade e até mecanismo de ação destas substâncias (HEMAISWARYA et al., 2008), reforçando a necessidade de estudos aprofundados a fim de determinar tais propriedades em produtos naturais.

4.2. Atividade antibiofilme

Biofilme pode ser definido como uma complexa comunidade de microrganismos envoltos por uma matriz extracelular de polissacarídeos, aderidos um ao outro numa superfície ou interface (DONLAN; COSTERTON, 2002; PERCIVAL et al., 2012). Estima-se que 95% dos microrganismos encontrados na natureza estão ligados a algum tipo de biofilme (SARDI et al., 2014). Técnicas moleculares e de microscopia têm indicado a formação de biofilme como forma natural e preferida de crescimento fúngico (SARDI et al., 2014). O reconhecimento destas estruturas contribuiu para a caracterização de diversas doenças infecciosas de caráter persistente, já que a matriz polimérica mantém o biofilme coeso, dificultando o tratamento (COSTERTON et al., 1999; RAMAGE et al., 2014).

Inicialmente a formação de biofilme estava relacionada às infecções bacterianas, entretanto, a habilidade de colonizar superfícies foi confirmada como fator de virulência também para espécies fúngicas (SARDI et al., 2014). Grande parte dos

estudos associa a formação de biofilme a *Candida albicans*, no entanto o envolvimento de outras espécies de *Candida*, bem como diferentes gêneros leveduriformes como *Cryptococcus* spp. e *Trichosporum* spp. tem sido reportado (DESAI et al., 2014; SARDI et al., 2014; PIERCE et al., 2015).

A formação de biofilmes por fungos filamentosos foi descrita recentemente e as investigações concentram-se em espécies do gênero *Aspergillus* e *Fusarium* (PEIQIAN et al., 2014; GONZÁLEZ-RAMÍREZ et al., 2016; KVASNIČKOVÁ et al., 2016). Contudo, Costa-Orlandi e colaboradores (2014) demonstraram que os dermatófitos *Trichophyton rubrum* e *Trichophyton mentagrophytes* apresentam habilidade em produzir biofilme, e recentemente nosso grupo de pesquisa relatou estruturas compatíveis a biofilme maduro para a espécie *Microsporum canis* (DANIELLI et al., 2017a).

Grande parte das infecções humanas, de uma forma geral, envolve a formação de biofilmes, principalmente relacionadas ao emprego de materiais na prática médica, como cateteres, por exemplo, e aumento no número de pacientes imunocomprometidos (RAMAGE et al., 2005; COS et al., 2010). Biofilmes estão envolvidos com resistência e tolerância, incluindo lenta penetração do agente antimicrobiano através da rede fúngica, resposta adaptativa ao estresse, alterações na química do microambiente e presença de células tolerantes, conhecidas como *persisters* (STEWART; COSTERTON, 2001). Neste contexto, infecções com a presença de biofilme apresentam um grande desafio na medicina, considerando sua importante resistência aos agentes antimicrobianos e ao sistema imune do hospedeiro (JABRA-RIZK et al., 2004; ACKER et al., 2014; SARDI et al., 2014).

A capacidade de inibição de formação de biofilme por óleos voláteis e compostos isolados foi relatada para o gênero *Candida*, incluindo espécies com sensibilidade reduzida aos azóis (CANNAS et al., 2014; CURVELO et al., 2014; PEIXOTO et al., 2017). A atividade antibiofilme descrita para os óleos de *Cinnamon cassia* e *Citronella witerianus* indicou remoção tanto de células aderentes do biofilme de *C. albicans*, quanto de matriz polimérica extracelular em tempo inicial de tratamento (ALMEIDA et al., 2016a). Já o óleo de *Piper claussenianum* demonstrou efeito sobre a estabilidade de biofilme fúngico maduro, indicando promissora

capacidade de causar desarranjo nas estruturas pré-formadas (CURVELO et al., 2014). A possibilidade dos óleos voláteis de atuar em células planctônicas e sésseis estabelece uma alternativa promissora no tratamento de infecções fúngicas persistentes, inclusive de forma a complementar a terapia convencional.

4.3. Atividade anti-inflamatória

Inflamação caracteriza-se como um processo de resposta biológica do organismo aos estímulos nocivos desencadeados por fatores injuriantes promotores de danos às células e aos tecidos (SERHAN, 2007). Esta resposta pode ser rápida (aguda) ou longa (crônica). A resposta aguda inicia-se, basicamente, através da liberação de fatores quimiotáxicos e consequente migração leucocitária ao local da injúria ou infecção (MEDZHITOV, 2008). Além de ser mais abundante na corrente sanguínea entre os leucócitos, neutrófilos caracterizam-se como primeiras células de defesa recrutadas ao local da injúria, a partir de fatores quimioatrativos liberados por microrganismos ou células necróticas (MCDONALD et al., 2010). A etapa seguinte ao ingresso dos neutrófilos no tecido lesado consiste na emissão de gradientes quimioatrativos secundários os quais os guiarão até locais próximos à inflamação, onde gradientes quimioatrativos primários os recrutam ao núcleo do processo inflamatório (MEDZHITOV, 2008). Neste contexto, substâncias capazes de inibir a migração leucocitária ou ainda, reduzir a produção de citocinas pró-inflamatórias envolvidas no processo de recrutamento celular, podem regular ou modular a resposta inflamatória e imunológica, reduzindo, desta forma, o dano tecidual (MELO et al., 2011).

A ativação do sistema imune, desencadeando um processo inflamatório, é uma importante etapa na defesa do hospedeiro contra infecções microbianas. Entretanto, de forma contraditória, respostas inflamatórias exacerbadas podem causar danos aos tecidos e mesmo aumentar a suscetibilidade aos patógenos oportunistas. Em alguns casos, uma infecção fúngica pode tornar-se crônica em virtude de uma resposta inflamatória acentuada, por comprometer a capacidade do hospedeiro em enfrentar o patógeno (ROMANI, 2011).

Outro fator importante a ser ponderado refere-se ao fato de que a massiva produção de espécies reativas de oxigênio (incluindo ânion superóxido - O_2 - e peróxido de hidrogênio - H_2O_2) pelos neutrófilos é considerada um dos principais mecanismos pelos quais tais células eliminam os microrganismos (NAUSEEF, 2007). O ponto crucial é que, ao mesmo tempo em que a geração de espécies reativas é crítica para a efetiva defesa do hospedeiro, sua produção inapropriada pode gerar um desbalanço no equilíbrio redox e causar danos em tecidos circundantes, contribuindo para o desenvolvimento de doenças inflamatórias crônicas (WRIGHT et al., 2010).

Deste modo, processos inflamatórios incontrolados podem comprometer tanto o tratamento da infecção quanto o manejo de doenças relacionadas. Estudos recentes têm investigado moléculas capazes de inibir as funções dos fagócitos, incluindo, neste caso, a produção de espécies reativas de oxigênio (BOUDIAF et al., 2016; ORHAN et al., 2016). Mais uma vez, a redução no recrutamento dos leucócitos com o uso de fármacos ou outras substâncias, representa uma interessante estratégia terapêutica para o tratamento de determinadas doenças (ESTEVÃO-SILVA et al., 2014).

O emprego de plantas medicinais e seus compostos isolados na medicina popular com finalidade de tratamento de desordens inflamatórias é recorrente (SÁ et al., 2013; LIU et al., 2016). Além da capacidade de eliminação de radicais livres, há evidências de que alguns óleos voláteis dispõem de propriedades anti-inflamatórias associadas a efeitos antimicrobianos (SÁ et al., 2014). Diversos monoterpenos (1,8-cineol, mentol, mentona, neomentol, timoquinona, borneol, acetato de bornila, terpineol, timol, carvacrol, linalol, acetato de linalila, geraniol, citronelol, mirceno, limoneno, α-pineno, entre outros), sesquiterpenos (farnesol, cariofileno e óxido de cariofileno, α-humuleno, bisabolol, entre outros) e fenilpropanoides (cinamaldeido, safrol, eugenol, metileugenol, elemicina, mirsticina, asarone, anetol, entre outros) isolados de óleos voláteis apresentaram efeito anti-inflamatório por diferentes mecanismos (SÁ et al., 2013; ARIGESAVAN; SUDHANDIRAN, 2015; SÁ et al., 2015).

Nestes casos, o efeito anti-inflamatório é atribuído, em geral, à interação com cascatas de sinalização, envolvendo citocinas e fatores de regulação, bem como na expressão de genes pró-inflamatórios (MIGUEL, 2010b). Inibição da migração

leucocitária *in vitro* estimulada por lipopolissacarídeo de *Escherichia coli* (LPS) foi reportada para óleos voláteis do gênero *Stenachaenium* Benth. (DANIELLI et al., 2016). Os óleos voláteis de *Citrus latifolia*, *Artemisia kotuchovii*, e compostos isolados, como limoneno e eugenol exibiram efeito inibitório similar a partir da utilização de n-formil-metionil-leucil-fenilalanina (fMLP) e leucotrieno B4 como quimioatrativos (KUMMER et al., 2013; ESTEVÃO-SILVA et al., 2014; SCHEPETKIN et al., 2015). Da mesma forma, o óleo de *Zingiber officinale* exibiu efeito antiquimiotáxico *in vitro* e *in vivo* a partir da estimulação por caseína (MELO et al., 2011). Além da migração leucocitária induzida por carregenina, γ-terpineno, reduziu, *in vivo*, a produção de interleucina-1-β e fator de necrose tumoral-α (RAMALHO et al., 2015).

4.4. Atividade antioxidante

Evidências indicam que a geração de espécies reativas de oxigênio/nitrogênio causa danos oxidativos que estão relacionados a diversas patologias, como doenças neurodegenerativas, cardíacas e aterosclerose, em virtude de um desequilíbrio entre a produção de espécies reativas e o sistema antioxidante do organismo (FINKEL; HOLBROOK, 2000; SINGH et al., 2009; JIA et al., 2010).

Uma substância com propriedade antioxidante pode atuar por diversos mecanismos, incluindo sequestro de radicais livres, quelação a metais de transição ou oxigênio singleto, doação de hidrogênio, inibição de peroxidação lipídica e decomposição de peróxidos (NIKOLIĆ et al., 2014; LUÍS et al., 2016; UD-DAULA et al., 2016). Por esta razão, diferentes métodos *in vitro* têm sido empregados na determinação de propriedades antioxidantes a partir de produtos naturais (AMORATI et al., 2013; EMBUSCADO, 2015). É importante considerar que devido à alta especificidade dos métodos relacionados à investigação de determinado mecanismo de ação, distintas técnicas devem ser utilizadas em conjunto e com aplicação de diferentes substratos (BOUNATIROU et al., 2007; MIGUEL, 2010a).

É sabido que substâncias de ocorrência natural exibem propriedades antioxidantes. Para os óleos voláteis e seus compostos isolados, esta particularidade

tem sido alvo de investigação devido ao seu potencial na indústria farmacêutica, cosmética e alimentícia (BAJPAI et al., 2009; BOURGOU et al., 2010; MIGUEL, 2010b; ALVES-SILVA et al., 2013; ASDADI et al., 2015).

Durante a fagocitose do microrganismo, em um processo infeccioso, ocorre importante aumento no consumo de oxigênio e por consequência, formação de espécies reativas. Da mesma forma, a formação de radicais hidroxila e peroxila, e de óxido nítrico também pode ocorrer. A geração destes radicais pelos fagócitos é importante para neutralizar os organismos injuriantes, entretanto, se em excesso, pode ser responsável por danos em locais inflamatórios (MIGUEL, 2010b). Neste sentido, considerando que a explosão oxidativa de ocorrência em células relacionadas ao sistema imune, como monócitos e neutrófilos, atua como um dos gatilhos para a resposta inflamatória, uma substância com propriedade anti-inflamatória associada à ação antioxidante, como é o caso de muitos óleos voláteis, atuará de forma muito mais efetiva no combate à infecção (CABRAL et al., 2015).

5. Análise multivariada aplicada às analises de óleos voláteis

A composição química de plantas medicinais e aromáticas, bem como as atividades biológicas decorrentes são influenciadas por fatores genéticos e ambientais (FURTADO et al., 2014; LEMOS et al., 2015; DHOUIOUI et al., 2016). Estas variações podem ser correlacionadas com distintas regiões de origem (VERMA et al., 2014; ZHANG et al., 2015; KARIMI et al., 2016), estádio de desenvolvimento do vegetal (AMARAL et al., 2015a; KARIMI et al., 2016), condições ambientais e agronômicas (OLMEDO et al., 2015; ALMEIDA et al., 2016b) e método de extração (DANH et al., 2013). O entendimento das alterações de um óleo volátil ocasionadas por mudanças sazonais é importante para estudos farmacológicos e, recentemente, também aplicado em áreas de engenharia metabólica como tentativa de melhorar o rendimento e facilitar a acessibilidade de um determinado composto (LANGE; AHKAMI, 2013; LANGE; TURNER, 2013; ALMEIDA et al., 2016b; KARIMI et al., 2016; TIWARI, 2016).

Análises multivariadas são frequentemente empregadas no tratamento de dados analíticos para estudo de variabilidade química dos óleos voláteis. A partir de ferramentas computacionais são explorados resultados provenientes de análises químicas, a fim de verificar existência de similaridade entre amostras, o que corresponde às semelhanças na composição do óleo (CORREIA; FERREIRA, 2007).

Neste contexto são desenvolvidos algoritmos, como análise por agrupamento hierárquico (HCA) e análise de componentes principais (PCA), com a finalidade de elaborar gráficos representativos da maior quantidade possível das informações contidas em um grupo de dados analíticos (MOITA NETO; MOITA, 1997).

A HCA agrupa amostras em classes a partir da similaridade dos participantes e diferenças entre membros de classes diferentes, resultando em uma representação gráfica do tipo dendograma (MOITA NETO; MOITA, 1997). Já a PCA objetiva reduzir a dimensionalidade do grupo original de dados, preservando a maior quantidade de informação possível, através do estabelecimento de novas variáveis ortogonais entre si, denominadas componentes principais (PCs) (CHRISTIE, 1995; MOITA NETO; MOITA, 1997).

A complexidade química dos óleos voláteis dificulta o estabelecimento de uma relação entre um grande número de amostras por observação direta. Contudo, a análise multivariada permite a medida quantitativa de diferenças e similaridades entre amostras a partir de critérios matemáticos (WIKLUND et al., 2008; MASHIGO et al., 2015). Em geral, modelos quimiométricos são construídos a partir dos conjuntos de dados obtidos de coletas em diferentes fases do desenvolvimento do vegetal, condições climáticas ou posicionamento geográfico, a fim de estabelecer tendências e padrões de agrupamento dentro do conjunto de dados, determinar marcadores e quimiotipos (MASHIGO et al., 2015; DAOUDI-MERBAH et al., 2016; PINHEIRO et al., 2016; SORO et al., 2016; YANG et al., 2016).

6. Considerações sobre o gênero *Nectandra* Rol. Ex Rottb.

A família Lauraceae apresenta, no Brasil, 22 gêneros e 390 espécies distribuídas entre restinga, cerrado e florestas pluviais (ALVES; SARTORI, 2009).

Entre os gêneros, *Nectandra* Rol. ex Rottb encontra-se representado por 43 espécies, com ampla diversidade na floresta Amazônica e Atlântica (MACÍAS-VILLAMIZAR et al., 2015). Espécies pertencentes a este gênero são consideradas importantes do ponto de vista econômico, através do emprego da madeira na indústria moveleira e construção civil (ZANON et al., 2009; MACÍAS-VILLAMIZAR et al., 2015).

A espécie *N. megapotamica* (Spreng.) Mez, conhecida popularmente como "canela-preta" ou "canela-imbuia" é utilizada na medicina tradicional como analgésica, antirreumática, anti-inflamatória e para tratamento de tosse, resfriados e abcessos (SILVA-FILHO, 2004b; MELLO; YOSHIDA, 2005; ALVES et al., 2008). Estudos indicam atividade hemolítica, coagulante e proteolítica contra o veneno de *Bothrops diporus*, anestésica, antimicrobiana, anti-inflamatória e antitumoral para esta espécie (APEL et al., 2006; BRITO, 2009; TONDOLO et al., 2013; TORRES et al., 2014). Entre os metabólitos secundários bioativos, foram observadas, no extrato etanólico das folhas, lignanas hexahidrobenzofurânicas e tetrahidrofurânicas, para as quais foi detectada atividade frente a *Trypanosoma cruzi* (SILVA-FILHO et al., 2004a), além de atividade leishmanicida e antimalárica (BÖHLKE et al., 1996; SILVA-FILHO et al., 2008), e citotoxicidade contra células leucêmicas humanas (PONCI et al., 2015). Os fenilpropanoides (GARCEZ et al., 2009) elemicina, isoelemicina, (±)eritro-1-(3,4,5-trimetoxifenil)-1,2-propanodiol e (±)treo-1-(3,4,5-trimetoxifenil)-1,2-propanodiol isolados das cascas, exibiram atividade antifúngica (KRUGEL et al., 2006). A presença de alcaloides com atividade tripanocida, inibindo o crescimento de *Crithidia fasciculata*, também foi reportada para esta espécie (SANTOS-FILHO; GILBERT, 1975).

Grande parte dos estudos relacionados ao óleo volátil de *N. megapotamica* aborda a variação química sazonal entre os compostos (BARRA, 2009; ROMOFF et al., 2010; AMARAL et al., 2015a, AMARAL et al., 2015b). Contudo, atividade anestésica avaliada em robalos-pava (*Centropomus parallelus*), anti-inflamatória, antitumoral, antitrombótica, antileucêmica, antimicrobiana e inibidora de hemólise induzida pelo veneno de jararaca, foi reportada (APEL et al., 2006; BRITO, 2009; TONDOLO et al., 2013; TORRES et al., 2014; MACÍAS-VILLAMIZAR et al., 2015).

A espécie *N. lanceolata* Ness., conhecida popularmente como “canela-branca”, ocorre na floresta ombrófila mista e densa, de Minas Gerais ao Rio Grande do Sul. Da mesma forma que outras espécies do gênero, exibe importante valor econômico com emprego na construção civil, caibros, forro, ripas, obras internas e móveis (LABORATÓRIO DE MANEJO FLORESTAL DA UNICENTRO, 2016). Recentemente nosso grupo de pesquisa relatou atividade antifúngica e anti-inflamatória para o óleo volátil desta espécie (DANIELLI et al., 2017b).

7. Considerações sobre o gênero *Schinus* L.

O gênero *Schinus* L. pertence à família Anacardiaceae e contém aproximadamente 600 espécies distribuídas em regiões subtropicais e tropicais (GEHRKE et al., 2013). *S. terebinthifolius* Raddi, *S. molle* Hort. ex Engl., *S. polygama* (Cav.) Cabr., e *S. lentiscifolius* Marchand são nativos de biomas do Rio Grande do Sul e também encontrados na Argentina, Paraguai e Uruguai. Entre as espécies deste gênero, há relatos do emprego popular de *S. terebinthifolius* como antimicrobiano, anti-inflamatório, adstringente e hemostático (AMORIN; SANTOS, 2003; SANTOS et al., 2010)

Diversas bioatividades foram relatadas para óleos voláteis de espécies do gênero *Schinus*. *S. molle* apresentou atividade antimicrobiana, anticâncer, antioxidante e repelente (HAYOUNI et al., 2008; BENZI et al., 2009; GUALA et al., 2009; BENDAOUD et al., 2010; SANTOS et al., 2010; HOSNI et al., 2011; LÓPEZ-MENESES et al., 2015). Há também relatos de atividade antioxidante e anticâncer para o óleo da espécie *S. terebinthifolius*, bem como antimicrobiana, fungicida frente a fitopatógenos, inseticida contra vetores da malária (*Anopheles gambiae* s.s., *An. arabiensis* e *Culex quinquefasciatus*) e larvicida frente ao vetor da dengue (*Stegomyia aegypti*) (GUNDIDZA et al., 2009; BENDAOUD et al., 2010; SANTOS et al., 2010; KWEKA et al., 2011; COLE et al., 2014; PRATTI et al., 2015). O óleo da espécie *S. areira* estimula a liberação de mediadores pró e anti-inflamatórios nos pulmões e modifica parâmetros cardiovasculares e nas vias aéreas (BIGLIANI et al., 2012), além

de apresentar atividade antimicrobiana frente a *Staphylococcus aureus* (CELAYA et al., 2014).

S. lentiscifolius é uma árvore heliófita (necessita de total exposição solar) de ocorrência em regiões de altitude do Rio de Janeiro ao Rio Grande do Sul. Popularmente conhecida como “aroeira-cinzenta” e “aroeira-do-campo”, não apresenta importante valor econômico, considerando-se que se trata de uma espécie recomendada apenas para arborização ou utilização da lenha (LABORATÓRIO DE MANEJO FLORESTAL DA UNICENTRO, 2016).

Escassos são os relatos de estudos envolvendo a espécie *S. lentiscifolius*. Gehrke e colaboradores (2013) observaram amplo espectro antimicrobiano do extrato aquoso das folhas desta espécie. Da fração acetato de etila foram isolados nonadeanol, metil éster do ácido gálico, ácido gálico, quercetina, quercitrina e ácido morônico, sendo que o último apresentou concentrações inibitórias mínimas menores em relação aos outros compostos isolados, quando avaliados quanto ao seu efeito antimicrobiano, indicando que seria um dos metabólitos responsáveis por essa ação.

Diante do exposto o desafio ocorre na busca por substâncias capazes de substituir a terapia convencional ou ainda complementá-la, atuando de forma sinérgica aos fármacos existentes, a fim de reverter quadros de resistência, ou ainda potencializar seu efeito. Uma das principais fontes de alternativa ao tratamento está relacionada à investigação de atividade farmacológica em substâncias obtidas de plantas, incluindo óleos voláteis e seus componentes isolados, cujos relatos demonstram importantes propriedades biológicas, dentre elas, ação antibacteriana e antifúngica, o que os torna potenciais no tratamento destas infecções.

CAPÍTULO II – Combinação entre óleos voláteis e antifúngicos: Uma revisão

Essential oils in combination with antifungals: a review

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Abstract

In recent years, the incidence and severity of fungal diseases have increased significantly, especially in patients with weakened immune system. Furthermore, the current antifungal agents have several disadvantages in terms of activity spectrum, toxicity, safety, pharmacokinetic properties and resistance. In view of this, the development of new options for antifungal therapy is mandatory. Essential oils are secondary metabolites of plants with high volatility and they have been long recognized for their biological properties such as antioxidant, anti-inflammatory and antimicrobial activities. The antifungal effect of essential oils has been described against several fungi pathogenic to man. However, the mechanism of action has not yet been fully elucidated. In addition, the antifungal activity of essential oils combined with commercial drugs has been extensively investigated as a viable strategy with multiple targets in order to address the lack of new antimicrobial agents. This review aims to provide an overview of studies *in vitro* investigating the use of essential oils and their compounds alone in order to potentiate the effect of antifungal drugs. The mechanisms involved in the antifungal action of these substances alone and in combination are also reported.

Keywords: Essential oil, antifungals, resistance, combination, synergism.

1. Introduction

In recent years, the incidence and severity of fungal diseases have increased significantly, especially in patients with weakened immune systems.[1] Invasive fungal infections (IFIs) represent a serious threat to human health and they are the cause of at least 1.5 million deaths each year.[2] Mortality caused by these microorganisms exceeds malaria and is approximately equal to drug-resistant *Mycobacterium tuberculosis*.[3] IFIs are frequently caused by yeast pathogens belonging to genera *Candida* and *Cryptococcus*; or filamentous fungi such as *Aspergillus*.[4,5] However, other opportunistic fungi are emerging as etiological agents and are changing this epidemiology, such as species of Zygomycetes, *Fusarium* or *Scedosporium*.[2]

Despite serious consequences for human health, surveillance levels IFIs remain low. IFIs only represent part of the magnitude of fungal diseases. Fungi also produce mucosal infections and superficial, cutaneous and subcutaneous mycoses.[6] These infections are mostly caused by *Candida* spp., *Malassezia* spp, dermatophytes and *Sporothrix schenckii*, respectively.[2,7] Although the contribution of these diseases to morbidity is unchartered, such mycoses by far exceed the frequency of IFIs and diminishing the quality of life of affected individuals.[2,3]

The antifungal drug development is more challenging than antibacterial drugs once fungi are eukaryotes and several potential targets for therapy are also found in human cells with significant host toxicity risk. Five classes of antifungal agents (azoles, echinocandins, polyenes, allylamines and pyrimidine analogs) are used orally, topically or intravenously for the treatment of fungal infections. However, the current antifungal agents have several disadvantages in the activity spectrum, toxicity, safety and pharmacokinetic properties.[2] Furthermore, resistance to antifungals is an increasingly frequent problem and exists to all of the currently available classes of antifungal agents.[8-11] *Candida*, *Aspergillus* and *Cryptococcus* strains have a high prevalence of azole resistance.[1] A few years ago, echinocandins were considered effective therapy for most *Candida* clinical isolates. However, with increased use of these drugs, echinocandin resistance has also become frequent.[8,10] The increase in infections caused by intrinsically drug-resistant fungi, such as *Scedosporium* species, has also concerned health professionals.[12]

Therefore, the emergence of strains resistant to these drugs has lead researchers to develop new antifungal, with different mechanisms of action that target the biosynthesis of fungal proteins, lipids and cell walls.[13] The development of new options for antifungal drugs is mandatory given the increasing number of infections and their high mortality rate.[14] Thus, combination therapy becomes a viable strategy with a multiple target approach in order to address the lack of new antimicrobial agents and combat resistance to conventional treatment.[15-17] A combination therapy seeks to achieve a synergic effect between two or more substances, reducing the dose used and consequently the toxicity levels, as well as preventing, minimizing or delaying the induction of drug resistance and enlarging the activity spectrum.[18-20]

Furthermore, it is an approach with important financial implications, considering that the development of a new drug becomes much more costly in relation the use of combinations.[21]

Early reports of development of resistance to antifungals, such as *Cryptococcus* to flucytosine,[22] motivated the evaluation of combined therapy. Two substances acting concomitantly on various targets of a fungal cell may result in increased efficacy.[18] In this context, the antifungal activity of commercial drugs combined with natural products has also been extensively investigated.[23-25] This therapeutic strategy has the purpose to eliminate pathogenic microorganisms and overcome problems of toxicity and resistance to antimicrobial.[14,26,27] Absence or reduction of infections in plants is considered as an indicative that these organisms develop effective defense systems, suggesting that its antimicrobials derivatives may be potentially therapeutic.[28] In this way, an overview of studies *in vitro* investigating the use of essential oils and their compounds isolate in order to potentiate the effect of antifungal drugs will be explored in this review. The mechanisms of antifungal action of these substances in isolation and in combination are also addressed.

2. Essential oils

Essential oils are secondary metabolites of plants defined as products obtained by hydrodistillation, steam or dry distillation or by a suitable mechanical process without heating (for *Citrus* fruits) of a plant or parts thereof.[29,30] These substances are complex mixtures of low molecular weight constituents with high volatility.[31] Due to its repellent properties, they play an important function in the protection of the plants against the attack of herbivores and microorganisms, besides plant-plant interaction and the attraction of disseminators and pollinators.[31-33]

The chemical composition presents predominantly terpenic compounds, such as monoterpenes and sesquiterpenes, besides to phenylpropanoids and fatty acids, to a lesser extent.[32,33] The major components may constitute up to 70% of the total oil, while minor constituents may represent trace amounts.[32] This variation is related to extrinsic and intrinsic factors to the vegetable. climatic conditions, such as nutrient characteristics and quantity, water stress, seasonality, ultraviolet radiation, altitude, air

pollution, temperature and climate, have a significant influence on the yield and biological activities performed by these substances.[34-36]

The use of these substances is closely related to their biological properties. A high number of terpenes is investigated due to properties employed in the cosmetic and food industry, mainly as flavoring agents in foods, beverages, perfumes and cosmetics, besides being considered natural preservatives.[32,37,38] Antioxidant, anti-inflammatory and antimicrobial activities are among the most commonly reported, however, researchers also showed anti-ulcer effect, anxiolytic, insecticidal, antinociceptive, antitumor, among others.[39-44] Many of these effects are related to the isolated constituents and despite the complex chemical composition, some oils are characterized by the presence of a major compound. In this case, the direct relationship with biological activity is facilitated. However, minority compounds also exhibit biological effects, characterizing synergism. The occurrence of this event among essential oil components contributes significantly to the biological effect. In some cases, the total oil is considered to be more potent when compared to its isolated constituents.[45]

3. Antifungal mechanism of the essential oils

The antifungal effect of essential oils was described for the first time in 1927 by Myers.[46] Since then, several fungi pathogenic to man, including yeasts and filamentous fungi, have been shown to be sensitive to the action of these substances.[47-50] In spite of numerous studies confirming this activity, the mechanism of action is still widely investigated, suggesting that it occurs in large part because of its hydrophobic characteristic that improves the penetration in the cellular membrane of the microorganism, aiding in the antimicrobial action of its components.[51-53]

Damage to the fungal cell membrane resulting in severe injury and cell death is one of the major mechanisms described for antifungal activity of essential oils.[54,55] Similarly to the action of azole, the effect on cell membrane integrity may be related to alteration in the ergosterol biosynthesis,[52,56] the main sterol of fungal species, indispensable for normal cell growth and function.[51] Compounds such as methyl chavicol and linalool have been described inhibiting the ergosterol biosynthesis and

causing pores in the cytoplasmic membrane of *Candida* spp.[51] Transmission electron microscopy images of *Candida albicans* cells treated with essential oil of *Satureja montana* and *Thymus capitatus* demonstrated disorganization of cytoplasmic organelles, a nucleus with non-condensed chromatin and membrane disarrangement, associated with the presence of autophagic vacuoles.[53] Wall thickening and cell membrane irregularities suggest an effect on budding in *C. albicans* from treatment with geraniol.[24]

The enzymatic system of the microorganisms is among the cellular targets. *Lippia alba* oil rich in linalool induced inhibition of peptidases and keratinases in dermatophytes,[57] whereas *Thymus vulgaris* and *Carum copticum* reduced more than 80% the elastase activity in *Aspergillus fumigatus*.[49] Whereas enzymes produced by fungi contribute to pathogenicity by host tissue degradation,[58] its inhibition can reduce fungal virulence.[49,57] *Foeniculum vulgare* induced to decrease in the mitochondrial activity of succinate dehydrogenase, malate dehydrogenase and ATPase in *Trichophyton rubrum*.[59] Inhibition of the activity of mitochondrial dehydrogenases through the perturbation of the citric acid cycle and inhibition of ATP synthesis in mitochondria in *C. albicans* was observed after exposure to the essential oil of *Anethum graveolens*.[54] Moreover, a mitochondrial dysfunction, caused by mitochondrial hyperpolarization, promoted the accumulation of reactive oxygen species (ROS) in the fungal cell, causing cell death through oxidative damage to biomacromolecules or mediation of apoptosis.[54] A similar effect was observed for thymoquinone.[50] The antifungal action of citronellol is also associated with increased ROS production, as well as interference with membrane homeostasis, by increasing the hypersensitivity of the fungi to membrane-perturbing agents, reducing ergosterol levels and decreasing glucose-induced H^+ extrusion.[60]

Other factors of fungal virulence are described as the target of essential oils and their compounds isolated. The oil of *Ocimum basilicum*, linalool and geraniol reduced the capsule size of *Cryptococcus neoformans*, considered as the major virulence factor of this fungus and, therefore, an important target of antifungal substances.[24] *T. vulgaris* and thymol presented inhibitory activity against arthroconidia and macroconidia of *Trichophyton rubrum*, hyphal elements capable of exacerbating

the damage causing by infection and to penetrate the deeper layers of the keratinized tissues of the host.[49]

4. Assay methods used for detecting interactions

There are several experimental models for measuring the *in vitro* efficacy of the antifungal combination. The most used method is the checkerboard, due to its relative ease of execution.[61] The experiment simultaneously evaluates a broad combination of antifungal concentrations from two substances distributed horizontally and vertically in a microwell plate.[62] The checkerboard results are interpreted by calculation of the fractional inhibitory concentration index (FICI) to demonstrate synergism, additivity, indifference and antagonism among antifungal agents.[62,63] Generally authors consider the nature of the interaction synergic when FICI values are less or equal than 0.5 or antagonic when greater than 4,[23-25,64] although some studies indicates that antagonism requires a FICI greater than 2.[65-67] The main disagreement occurs in the FICI range between 0.5 and 4, considered by some authors as additive and indifference effects,[25,64] while others do not consider additivity and interpret this interval as no interaction.[48,68]

Additional methods for synergy testing include time-kill studies and E-test.[62,69, 70] Time-kill assay indicates the dynamics of the inhibitory effect of the substance, presenting greater details over time of the antifungal activity about the checkerboard.[71] However, this is a very expensive technique due to the need for intense counting of colony formation units, in addition to several combinations and concentrations to be tested concomitantly.[72] The E-test consists of standardized strips at different concentrations of antimicrobial agents which are interpreted by the formation of a zone of elliptic inhibition. Due of the ease of performing the technique, the authors believe that the standardization of this method for synergism evaluation would be able to incorporate this technique as routine in clinical laboratory.[61]

5. Combination between essential oils and antifungals

The concept of antimicrobial synergism is based on the fact that the combination of a drug with a second substance should increase therapeutic efficacy, reduce toxicity and minimize adverse effects, associated with the advantage of

delaying or limiting the occurrence of microbial resistance.[19,20,72] *In vitro* studies indicate that the combination of natural products with antifungal agents is able to reduce the minimum inhibitory concentration (MIC) in relation to the substances tested alone,[17,24] so thus being considered an important source in the discovery of antimicrobial substances. An overview of the published studies on interactions between essential oils and antifungals and between essential oils compounds and antifungals is presented in Tables 1 and 2, respectively.

Azoles are considered the main class of antifungal drugs used in the clinic and therefore more prone to the development of resistance. Because of this concentrates the largest number of combination studies. The essential oil from *Ocimum sanctum* presented a synergic effect in combination with fluconazole and ketoconazole against *C. albicans, C. tropicalis* and *C. glabrata*.[23] For fluconazole-resistant *C. albicans* isolates, the combination of *O. basilicum* essential oil with this azole showed synergism, with a reduction in MIC of fluconazole from 500 to 1.01 μg/mL. However, for the sensitive isolates the combination was not effective.[24] A similar result was observed for *C. neoformans* isolates.[24] Transmission electron microscopy images of *C. albicans* strains resistant to fluconazole and treated with the synergic combination of essential oil and fluconazole indicated an increase in the thickness of the fungal cell wall, in addition to membrane invaginations.[24] Fluconazole and amphotericin B combined with *Syzygium aromaticum*, *Cinnamomum verum*, *Cymbopogon citratus* and *Cymbopogon martini* oils against multi-resistant *C. albicans* showed variable levels of interaction, with synergic association of fluconazole and *C. martini* for all the isolates tested.[68] The variation in the biological effect of an essential oil is directly related to its nature, chemical constitution and concentration. The constituents present in this matrix may exhibit different modes of action by interacting alone or in combination with other compounds.[68]

Preliminary exposure of fluconazole-resistant *C. albicans* isolates to *Melaleuca alternifolia* in subinhibitory concentration (¼ of MIC) and fluconazole for 24 hours increased the susceptibility of the isolates to azole with a reduction in MIC from 244 to 38.46 μg/mL. After pretreatment, 62.25% of the microorganisms were considered sensitive, 25% showed intermediate sensitivity and only 12.5% still had resistance.[17]

Boswellia serrata oil presented potent antifungal synergism with ketoconazole, fluconazole, posaconazole and voriconazole against *C. albicans* isolates resistant to this class of drugs and additive effect against sensitive isolates. However, the combination with butenafine and terbinafine against resistant isolates did not show synergism. For sensitive strains, the association between terbinafine and the oil exhibited antagonistic effect.[73] Synergism was also observed against *Candida* isolates sensitive and resistant to fluconazole by the combination of this azole and *Mentha piperita* oil.[56] The synergic effect of combining antifungals with essential oils against resistant isolates, by reducing MIC of the drug, minimizes adverse effects of administration of high concentrations, in addition to turn the isolates more susceptible to the combination.[24]

Treatment of *C. albicans* with *Mentha suaveolens* oil combined with fluconazole resulted in a reduction of the individual MIC of the drug. The hypothesis for this action is related to the involvement of the monoterpenes present in the oil in the damage to the membrane lipid bilayer, increasing the permeability to the drug and, thus, facilitating its action in ergosterol biosynthesis and consequent cellular damage.[67] Significant ultra-structural modifications were observed by transmission electron microscopy (TEM) in *Candida* cells exposed to the combined treatment of *M. suaveolens* oil and fluconazole. Alterations were evident both in the cytoplasm and in the cell wall, especially the complete destruction of the cytoplasmic material.[67]

Minimum inhibitory concentration (MIC 80%) of amphotericin B against *C. albicans* was reduced when combined to a number of different concentrations of *Thymus vulgaris* oil (thymol chemotype), however, with presence of antagonism for the lowest concentrations of oil.[74] Similar effect was observed for *Cinnamomum cassia* oil.[75] Although the association between oil and amphotericin B resulted in synergism with *C. albicans*, the same was inactive for *C. tropicalis*.[76] In this case, it is assumed that the mechanism of action of the oil is similar to that of the drug and is related to the alteration of the cell membrane permeation, causing extravasation of the intracellular components, besides acting in the inhibition of the formation of germ tube, known effect of the amphotericin B and which justifies the lack of effect in *C. tropicalis*, considering that this species does not present this propriety.[76] Besides *Coriandrum*

sativum, the essential oils of *Myrtus communis* and *Pelargonium graveolens* exhibited a synergic effect combined with amphotericin B against *Candida* species, with a reduction in MIC of the drug.[66,76,77] These results are especially important due of the serious toxic effects of amphotericin B therapy. In many cases, this drug cannot be administered in doses sufficiently high for the treatment of infection and therefore requires the co-administration of other antifungals.[76]

In relation to filamentous fungi, the essential oil of *Otacanthus azureus* presented a synergic effect when used in combination with azoles, itraconazole, fluconazole and ketoconazole against *Trichophyton mentagrophytes* isolates (FICI in the range of 0.4 to 0.5), at the same time, indifference was observed against *Microsporum gypseum* (FICI in the range of 0.6 to 0.8).[48] For the species *T. rubrum*, synergism was verified for combination of *C. verum*, *S. aromaticum* and *C. martini* with fluconazole, however against multi-resistant *A. fumigatus* isolates only the association with the oil of *S. aromaticum* presented synergic.[52] Isobolograms constructed from the synergic concentrations resulting from the combination of essential oil of *Allium sativum* and ketoconazole against *Trichophyton* spp., indicated that effective concentrations were significantly reduced by the combination.[65] Itraconazole associated with *Angelica koreana* oil showed important synergism against *Aspergillus versicolor, A. fumigatus* and *A. terreus* and additivity for *Trichophyton tonsurans, T. mentagrophytes* and *T. rubrum*.[78] An interaction of synergic nature resulted from the combination of *Agastache rugosa* and ketoconazole versus *Trichophyton* spp, with FICI values ranging from 0.05 to 0.27.[79] On the other hand, the oil of *P. graveolens* associated with ketoconazole showed a significant synergic inhibition against *Trichophyton soudanense* and *Trichophyton schoenleinii* and an interaction of the additive type against *Trichophyton erinacei*.[80] It is known that a synergic effect derives predominantly from the action of substances on multiple cellular targets.[16] In the case of combined activity to the azoles whose mechanism is involved with the inhibition of 14α-demethylase resulting in interruption of the ergosterol biosynthesis, it is suggested that the oils act by altering the integrity of the cell wall which may result in an increase in intracellular levels of the drug and greater amount available for action.[52]

The combination of the essential oil of *Nectandra lanceolata* and ciclopirox against isolates of the genera *Trichophyton* and *Microsporum* indicated predominantly additive effect, being synergic only for two isolates. The indifference described for *Nectandra megapotamica* associated with terbinafine may be related to similarity in the mechanism of action of the substances.[25] The *M. communis* and *P. graveolens* oils combined with amphotericin B showed a synergic and additive effect, respectively, against *Aspergillus niger* and *Aspergillus flavus*.[66,81] The same was observed for the combination of *P. graveolens* and ketoconazole.[81] Synergic interaction resulted from the association of *Agastache rugosa* and ketoconazole against *Blastoschizomyces capitatus*, rare fungal presenting severe and fatal mycoses in immunocompromised patients.[82] The combination of itraconazole with *M. alternifolia*, *M. piperita* and *Origanum vulgare* exhibited synergism in 95%, 80% and 60% of the isolates of *Pythium insidiosum*, respectively, whereas antagonism was evidenced for some combinations with terbinafine. These variations can be attributed to different bioactive compounds present in the oils.[83] In some combinations certain substances can act as sequestrant of the antifungal drug, preventing its action and resulting in antagonism.[73]

6. Combination between essential oils compounds and antifungals

Thymol (1), eugenol (2) and methyleugenol (3) in combination with fluconazole demonstrated an important synergic effect against *Candida* strains, including for isolates resistant to this azole.[84,85] While fluconazole acts in the ergosterol biosynthesis, thymol has the ability to alter the permeability of the fungal membrane, as well as to exert a chemosensitizing effect of the cells of the microorganism to drug action and to prevent the action of the Cdr1 and Mdr1 efflux pumps, an important mechanism of resistance to azoles.[85] Pre-treatment of fluconazole-resistant *C. albicans* with terpinen-4-ol (4) turn all isolates tested drug-sensitive.[17] The association of fluconazole with linalool (5) and geraniol (6) against resistant *Candida* isolates resulted in synergic interaction and reduction of the drug MIC from 500 to 2.02 and 1.4 μg/mL, respectively.[24] Transmission electron microscopy images showed important alterations and formation of vesicles on the cytoplasmic membrane of the yeasts treated with the synergic combination between

fluconazole and geraniol (6).[24] Synergic interactions have also been reported for combination of eugenol (2) and cinnamaldehyde (7) with amphotericin B, and carvone (8), mentone (9) and menthol (10) associated with fluconazole against multi-resistant *C. albicans*.[56,68]

Eugenol (2), cinnamaldehyde (7) and geraniol (6) exhibited synergic interactions with fluconazole against *T. rubrum*, however, synergism was verified only in the combination of azole with eugenol (2) and cinnamaldehyde (7) against multi-resistant *A. fumigatus*, with emphasis on the association of fluconazole with cinnamaldehyde (7), with FICI of 0.156 and 0.187 for *T. rubrum* and *A. fumigatus*, respectively.[86] Thymol (1) showed an important synergic effect with fluconazole against to *A. fumigatus* and *T. rubrum* azole strains, reducing the MIC of fluconazole by 4-fold.[49] Itraconazole in combination with *m*-cresol (11) exhibited interactions of additivity against *Trichophyton* spp. and *Aspergillus* spp. with FICI values ranging from 0.5 to 1. The combination was synergic only against *A. niger* isolates (FICI 0.26).[78] Association of amphotericin B with geraniol (6) and citronellol (12) caused MIC reduction of the compounds against *Aspergillus* and FICI indicating additive effect. Both compounds associated with ketoconazole produced additive effects, with FICI of 0.52 and 0.625, respectively for *A. flavus* and *A. niger*,[81] and strong synergism against *T. soudanense* and *T. schoenleinii*.[80]

Synergic effect was also observed in the combination of geraniol (6) with fluconazole against *C. neoformans*, with MIC reduction of the substances from 31.25 to 4.14 μg/mL and 76 to 19 μg/mL respectively.[24] The synergic interactions between thymol (1) and itraconazole against *P. insidiosum* strains reached 96%. Similar results were observed for carvacrol (13) and thymol (1) associated with amphotericin B, caspofungin and terbinafine.[87] Isobologram curves indicated synergism between ketoconazole and estragole (14) combined against *B. capitatus* and *T. mentagrophytes*.[79,82] The structures of the compounds mentioned are presented in Figure 1.

7. Combination between essential oils and isolated compounds

In addition to the interaction with commercial drugs, essential oils and isolated compounds demonstrate a potential antifungal effect against several pathogens when combined (Table 3). The association between *Cinnamomum zeylanicum*, *S. aromaticum*, *T. vulgaris*, *Cymbopogon nardus* and *Mentha spicata* resulted in additive interactions against species of *Candida* spp., *A. terreus*, *Lichtheimia corymbifera* and *R. microsporus*, with emphasis on the combination of *C. zeylanicum* and *S. aromaticum* which demonstrated additivity to all species investigated.[88] Synergic effect was observed between linalool and geraniol against *C. neoformans* and *Candida* isolates sensitive and resistant to fluconazole.[24] The same occurred for the combined treatment of the essential oil of *O. vulgare* and *T. vulgaris* in *Aspergillus* spp.; *T. vulgaris* and *C. zeylanicum* against *A. flavus*; and *O. vulgare* and *C. zeylandicum* for *Penicillium chrysogenum*.[64] Mixing in equal ratios between the oil of *Allium cepa* and *Allium sativum* exhibited a synergic effect against *A. versicolor*, completely inhibiting mycelial growth of the fungus, in addition to reducing the production of mycotoxin.[89] Synergism between salicylaldehyde and linalool, respectively major and minor constituents from *Filipendula vulgaris* essential oil, was detected in mixtures of ratio 60:40 and 80:20 against *A. niger* and *C. albicans*. At the same time, it has been found that pure compounds in corresponding amounts are dramatically less active.[90] Minority compounds also exhibit biological effects or favor the action, characterizing synergism. The occurrence of this event among the components contributes significantly to the bioactivity of the oil.

8. Conclusion

In general, the show present synergic effect between essential oils obtained from the most varied genera and drugs used in the treatment of fungal infections. Most of investigations are related to determination of effect between oils and azoles, known as main drug class employed in the clinic. The association of amphotericin B with essential oils and isolated compounds is considered promising to improve the drug action and reduce the adverse effects. Studies involving multiresistant microorganisms have also showed important results by increasing susceptibility to previously resistant drugs and considerable reductions in the minimum inhibitory concentration. Generally,

the synergic effect observed in the combinations is related to oil effect on the disruption of the fungal membrane, facilitating the access of the drug to cell and consequently benefiting its effect. The same occur for the isolated compounds. Due this, the use of combination between essential oils and antifungal agents may be considered as a means of contributing to more effective therapies from drugs conventionally used.

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Table 1. Combination of essential oils and antifungals and their antimicrobial interactions against several fungal species.

Essential oil	Antifungal	Microorganism	Method	Interaction	Reference
Rosmarinus officinalis *Melaleuca alternifolia* *Thymus vulgaris* *Mentha piperita*	Amphotericin B	*C. albicans*	Isobolograms	SYN or ANT	21
Ocimum sanctum	Ketoconazole Fluconazole	*C. albicans* *C. tropicalis* *C. glabrata* *C. parapsilosis*	Checkerboard	IND or SYN	23
Ocimum basilicum	Fluconazole	*C. neoformans* *C. albicans*	Checkerboard	IND IND or SYN	24
Nectandra lanceolata *Nectandra megapotamica*	Terbinafine Ciclopirox	*T. rubrum* *T. mentagrophytes* *M. canis* *M. gypseum*	Checkerboard	ADD, IND or SYN	25
Otacanthus azureus	Itraconazole Fluconazole Ketoconazole	*T. mentagrophytes* *M. gypseum*	Checkerboard	SYN IND	48
Thymus vulgaris	Fluconazole	*T. rubrum* *A. fumigatus*	Checkerboard	SYN	49
Carum copticum				IND	
Mentha piperita	Fluconazole	*C. albicans* *C. glabrata* *C. tropicalis*	Checkerboard	SYN	56
Allium sativum	Ketoconazole	*T. rubrum* *T. ernacei* *T. soudanense*	Checkerboard	SYN	65
Myrtus communis	Amphotericin B	*C. albicans* *A. niger*	Checkerboard	SYN	66
Mentha suaveolens	Fluconazole Micafungin	*C. albicans*	Checkerboard	SYN, IND or ADD	67
Syzygium aromaticum *Cinnamomum verum* *Cymbopogon citratus* *Cymbopogon martini*	Fluconazole Amphotericin B	*C. albicans*	Checkerboard	IND or SYN	68
Boswellia serrata	Ketoconazole Fluconazole Posaconazole Voriconazole Terbinafine	*C. albicans*	E-test	ADD, IND or SYN	73

SYN, Synergism. ADD, Additivity. IND, Indifference. ANT, Antagonism.

Table 1. *Cont.*

Essential oil	Antifungal	Microorganism	Method	Interaction	Reference
Coriandrum sativum	Amphotericin B	*C. albicans* *C. tropicalis*	Checkerboard	SYN ADD	76
A. koreana	Itraconazole	*Aspergillus spp.* *Trichophyton spp.*	Checkerboard	ADD or SYN ADD	78
Agastache rugosa	Ketoconazole	*Trichophyton spp.*	Checkerboard	SYN	79
Pelargonium graveolens	Ketoconazole	*Trichophyton spp.*	Checkerboard	ADD or SYN	80
Pelargonium graveolens	Ketoconazole Amphotericin B	*A. niger* *A. flavus*	Checkerboard	ADD	81
Agastache rugosa	Ketoconazole	*B. capitatus*	Checkerboard	SYN	82
Melaleuca alternifolia *Mentha piperita* *Origanum vulgare*	Itraconazole Terbinafine	*P. insidiosum*	Checkerboard	IND, SYN or ANT	83
Syzygium aromaticum *Cinnamomum verum* *Cymbopogon martini*	Fluconazole	*A. fumigatus* *T. rubrum*	Checkerboard	IND or SYN	86
Santolina chamaecyparissu	Clotrimazole	*C. albicans*	Mixture	SYN	92

SYN, Synergism. ADD, Additivity. IND, Indifference. ANT, Antagonism.

Table 2. Combination of essential oils coumpouds and antifungals and their antimicrobial interactions against several fungal species.

Compound	Antifungal	Microorganism	Method	Interaction	Reference
Linalool	Fluconazole	*C. neoformans*	Checkerboard	IND	24
		C. albicans		IND or SYN	
Geraniol		*C. neoformans*		SYN	
		C. albicans		IND or SYN	
Carvone Menthol Menthone	Fluconazole	*C. albicans* *C. glabrata* *C. tropicalis*	Checkerboard	SYN	56
Eugenol	Fluconazole	*C. albicans*	Checkerboard	SYN	68
Citral					
Cinnamaldehyde				IND or SYN	
Geraniol					
Eugenol Cinnamaldehyde Citral Geraniol	Amphotericin B	*C. albicans*	Checkerboard	SYN IND or SYN	68
m-Cresol	Itraconazole	*Aspergillus spp.*	Checkerboard	ADD or SYN	78
		Trichophyton spp.		ADD	
Estragole	Ketoconazole	*Trichophyton spp.*	Checkerboard	SYN	79
Citronellol Geraniol	Ketoconazole	*Trichophyton spp.*	Checkerboard	ADD or SYN	80
Geraniol	Ketoconazole	*A. niger*	Checkerboard	ADD	81
Citronellol	Amphotericin B	*A. flavus*			
Estragole	Ketoconazole	*B. capitatus*	Checkerboard	SYN	82
Eugenol	Fluconazole	*A. fumigatus*	Checkerboard	SYN	86
Cinnamaldehyde		*T. rubrum*			
Geraniol				IND or SYN	
Carvacrol Thymol	Amphotericin B Caspofungin Itraconazole Terbinafine	*P. insidiosum*	Checkerboard	SYN	87

SYN, Synergism. ADD, Additivity. IND, Indifference. ANT, Antagonism.

Table 3. Combination of essential oils and isolated coumpouds and their antimicrobial interactions against several fungal species.

Combination	Microorganism	Method	Interaction	Reference
Linalool/geraniol	*C. neoformans* *C. albicans*	Checkerboard	SYN	24
Salicylaldehyde/linalool Salicylaldehyde/methyl salicylate	*A. niger* *C. albicans*	Disk-diffusion	SYN ANT	40
Ocimum basilicum/Cinnamomum zeylandicum/ Eucalyptus globulus/Citrus reticulata/Origanum vulgare/Mentha piperita/Melaleuca alternifolia/ Thymus vulgaris	*A. niger* *A. flavus* *A. parasiticus* *P. chrysogenum*	Checkerboard	ADD, IND or SYN	64
Cinnamomum zeylanicum/ Cymbopogon nardus	*C. albicans* *A. fumigatus*	Checkerboard	ADD or IND	88
Cinnamomum zeylanicum/Syzygium aromaticum	*A. terreus* *L.corymbifera*		ADD	
Cinnamomum zeylanicum/Thymus vulgaris	*A. fumigatus* *L. corymbifera*		ADD or IND	
Cymbopogon nardus/Syzygium aromaticum	*C. albicans* *C. parapsilosis*		ADD	
Cymbopogon nardus/Thymus vulgaris	*C. albicans* *C. parapsilosis*		ADD	
Thymus vulgaris/Mentha spicata	*R. microsporus*		ADD	

SYN, Synergism. ADD, Additivity. IND, Indifference. ANT, Antagonism.

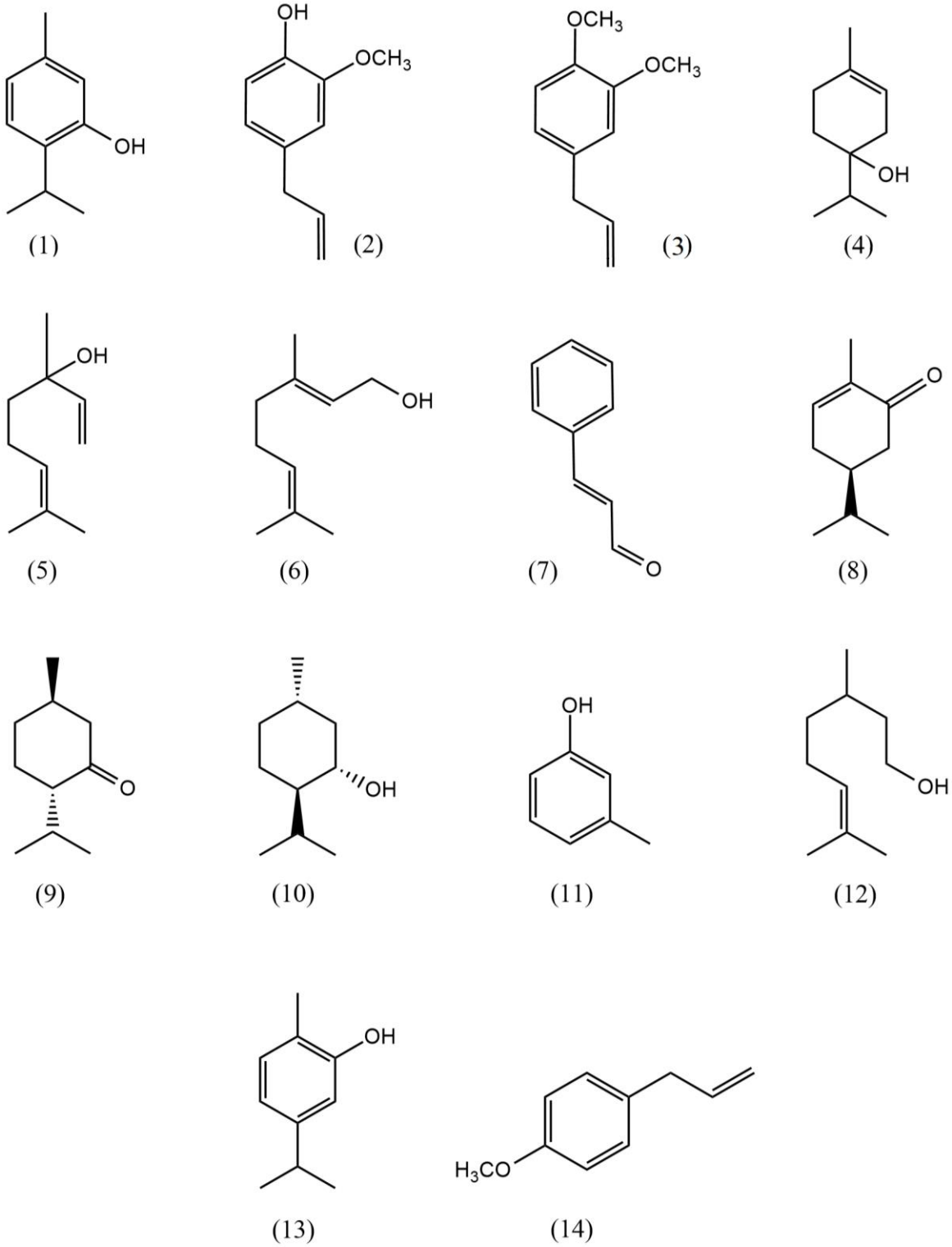

Fig. 1 Chemical structure of essential oils compounds tested in combination with antifungals agents. Thymol (1), eugenol (2), methyleugenol (3), terpinen-4-ol (4), linalool (5), geraniol (6), cinnamaldehyde (7), carvone (8), mentone (9), menthol (10), *m*-cresol (11), citronellol (12), carvacrol (13) and estragole (14).

CAPÍTULO III - Atividades biológicas do óleo volátil de espécies do gênero *Nectandra* Rol. ex Rottb. e sua interação *in vitro* com terbinafina e ciclopirox

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Chemosensitization of filamentous fungi to antifungal agents using *Nectandra* Rol. ex Rottb. species essential oils

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ABSTRACT

Chemosentizing of pathogens using natural compounds has been shown to be a promising alternative, resulting in increased efficacy of classical antifungal therapy. This study determines the chemical composition and antifungal, antichemotactic and antioxidant activities of *Nectandra* Rol species. ex Rottb. essential oils species, and its interaction with commercial antifungals. The chemical composition of *N. megapotamica* and *N. lanceolata* essential oils was established by gas chromatography–mass spectrometry (GC–MS). The major compounds identified in *N. megapotamica* oil were bicyclogermacrene (33.4%) and germacrene D (16.8%), while *N. lanceolata* were identified β-caryophyllene (32.5%), bicyclogermacrene (27.8%) and spathulenol (11.8%). The oils presented antidermatophytic effect and affinity by ergosterol indicating a possible involvement in fungal membrane. The combination of the essential oil from *N. lanceolata* and ciclopirox showed synergistic and additive effect on most isolates (75%) reducing the active concentration of the antifungal agents when in combination. The oils were also tested for their ability to inhibit leukocyte migration *in vitro* stimulated by *Escherichia coli* lipopolysaccharide and showed antichemotactic effect with reduction in migration in the range of 30.7–96.7%, suggesting that it could act in acute stage of inflammatory process. Antioxidant concentration-dependent activity was observed on both samples. *N. lanceolata* showed 50% antioxidant effect in the highest concentration tested. The results of the combination between antifungals and *N. lanceolata* essential oil indicate this oil as a possible complement to conventional therapy for topical treatment of superficial infections caused by dermatophytes, acting in the chemosensitization of the fungal cell and resulting in antifungal effect improvement with the advantage of the anti-inflammatory effect associated.

1. Introduction

Natural products are considered an important source of the research and development of new drugs. In recent years, more than 50% of new approved drugs were derived from natural products or used as a basis for synthesis or semi-synthesis (Newman and Cragg, 2016). Several secondary metabolites produced by plants, including essential oils, are considered promising natural compounds in the research of new drugs for its broad array of reported biological activities (Miguel, 2010; Radulović et al., 2013; Llana-Ruiz-Cabello et al., 2015). Many of these metabolites are capable of inhibiting the growth of pathogens, becoming a great starting point in the search and development of new antimicrobial agents (Radulović et al., 2013; Raut and Karuppayil, 2014).

Combined with antimicrobial activity many oils presented anti-inflammatory and antioxidant properties (Amorati et al., 2013; Silva e Sá et al., 2015; Embuscado, 2015). A substance with these characteristics significantly aids in healing of injuries and has the advantage of limiting the symptoms related to fungal infections, especially cutaneous, such as dermatophytosis (Hube et al., 2015). This infectious process is caused by a group of filamentous fungi that infect keratinized tissues causing, in some cases,

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inflammation and oxidative damage in people and animals (Tabassum and Vidyasagar, 2013). In addition, the limited therapeutic arsenal, resistance to classical drugs and their toxicity, coupled with the high cost of treatment justifies the search for new strategies for alternative therapies or as a complement to conventional drug therapy (Ahmad et al., 2013; Scorzoni et al., 2016). Combinations of substances may affect multiple biochemical processes of a microorganism (Carrillo-Muñoz et al., 2014). The chemosentizing of pathogens using natural compounds has been shown to be a promising alternative, resulting in increased efficacy of classical antifungal therapy (Campbell et al., 2012). The use of chemosensitizing agents in conventional antifungal therapy has become a viable strategy to lack of new antimicrobials and to the increased numbers of cases of resistance (Carrillo-Muñoz et al., 2014). These substances are intended to improve the effect of the drug, however, without adding toxic or adverse effects (Campbell et al., 2012). Studies have demonstrated synergistic effects of essential oils and their derivatives with antifungal agents used in conventional therapy (Castro et al., 2015; Cardoso et al., 2016). Due to their hydrophobicity characteristic is supposed that the essential oils interact with the cell membrane of the microorganism, creating pores that facilitating entry of the drug inside the cell (Asdadi et al., 2015).

Lauraceae family includes about 52 genera and 3000 species, many of them known for the production of essential oils (Garcez et al., 2009). The *Nectranda* Rol. Ex Rottb. genus consists of about 43 native species, including *Nectandra megapotamica* (Spreng.) Mez and *Nectandra lanceolata* Ness, located in areas of Cerrado, Atlantic Forest and Pantanal (Quinet et al., 2016). Popularly known as cinnamon plants of this genus are traditionally used in the treatment of rheumatism, fevers, nervous disorders and pain relief (Silva-Filho et al., 2004). There are no reports in the literature on chemical composition and biological activity of *N. lanceolata*. However, the essential oil of the *N. megapotamica* species was related with antitumor effects, anti-inflammatory, anesthetic and hemolysis inhibitor induced pit viper venom (Apel et al., 2006; Tondolo et al., 2013; Torres et al., 2014).

Given the importance of identifying new substances with antifungal activity in order to provide effective therapeutic alternatives, the present study aims to determine the chemical composition and chemosensitizing effect of *N. megapotamica* and *N. lanceolata* essential oils against dermatophytes associated with antioxidant and antichemotactic activities. The effects of the interaction of the oil with commercial antifungals against species of *Trichophyton* and *Microsporum* and evaluation of toxicity *in vitro* model were also addressed.

2. Materials and methods

2.1. Plant material

Leaves of *N. megapotamica* and *N. lanceolata* were collected from native population in Southern Brazil, located in Barracão, Rio Grande do Sul State (27°43′57.6″S latitude and 51°22′18.9″W longitude; soil Yellow Latosol), during the winter. Both individuals were located at the border of a forest and for this reason they were partially exposed to solar illumination. The plant material was identified by the botanist Dr. Sérgio L. Bordignon and the voucher specimen was deposited in the Herbarium of the Federal University of Rio Grande do Sul (ICN-UFRGS: *N. lanceolata* – 192543 and *N. megapotamica* – 192542).

2.2. Obtaining and chemical analysis of essential oils

The essential oils were obtained from fresh material by a four-hour hydrodistillation, using a Clevenger-type apparatus (Farmacopeia Brasileira, 2010). The yield determination was performed as weight/volume (w/v). For chemical analysis, the essential oils were diluted in ethyl ether to a ratio 2:100 (v/v). The chemical composition was analyzed by gas chromatography–mass spectrometry (GC–MS) (Shimadzu QP5000). A capillary column of fused silica Durabond-DB5 (30 m × 0.25 mm × 0.25 μm) was used to separate constituents. The injector and detector temperature was set at 220 °C and 250 °C, respectively, and programming of column temperature was 60–300 °C at 3 °C/min, using helium as carrier gas at a flow rate of 1 mL/min. The identification of compounds was based on the comparison of retention indices calculated by linear interpolation relative to retention times of a series of *n*-alkenes, and their mass spectra with authentic samples and with data taken from the literature (Adams, 2009), or by comparison with mass spectra recorded in the database as NIST 62 and NIST 12 (National Institute of Technology and Standards). Relative amounts of the components were calculated based on GC peak areas.

2.3. Antifungal activity

The antifungal activity of *N. megapotamica* and *N. lanceolata* essential oils was evaluated against clinical isolates of *Candida albicans* (DEB14, CA04), *C. krusei* (CK01, CK02), *C. tropicalis* (CT56, ATCC750), *C. parapsilosis* (RL01, RL20), *C. glabrata* (CG185, CG40039), *Trichophyton rubrum* (TRU43, TRU51), *T. mentagrophytes* (TME16, TME40), *Microsporum canis* (MCA29, MCA01) and *M. gypseum* (MGY50, MGY42). All isolates are deposited in the Mycology Collection of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. To obtain viable cells for testing, the yeasts were grown in Sabouraud agar with chloramphenicol for 24 h at 35 °C, whereas the filamentous fungi were incubated at 32 °C for 5 days. Screening test was carried out using the concentration of 500 μg/mL. Minimum inhibitory concentration (MIC) was determined only in cases where antifungal activity was found in the screening. For this evaluation it was used the microdilution broth method in accordance with the standardized protocol M38-A2 by the Clinical Laboratory Standards Institute (CLSI, 2008) and essential oils samples were tested in the concentrations of 1.95; 3.90; 7.81; 15.62; 31.25; 62.5; 125; 250 and 500 μg/mL. The MIC was defined as the lowest concentration of substance at which the microorganism tested did no show visible growth. The experiments were performed in triplicate where terbinafine was used as positive control.

2.4. Antifungal mechanism of action

2.4.1. Sorbitol protection assay

The essential oils effect in the integrity of the fungal cell wall was determined by sorbitol protection assay. For this reason, the minimum inhibitory concentration (MIC) against dermatophyte species was measured in triplicate along 4 and 7 days in absence and presence of 0.8 mol/L sorbitol added to the medium as an osmotic support. Anidulafungin was used as positive control (Escalante et al., 2008).

2.4.2. Ergosterol effect

In order to determine the essential oils ability of complexing with ergosterol in fungal membranes, it was evaluated the MIC against dermatophytes strains (TRU43, TRU51, TME16, TME40, MCA29, MCA01, MGY50 and MGY42) in absence and presence of this sterol in concentrations of 50–250 μg/mL (Escalante et al.,

2008). The assay was performed in triplicate with amphotericin B as a positive control.

2.5. Checkerboard assay

The interaction of the essential oils with ciclopirox and terbinafine against eight dermatophytes strains was determined by checkerboard assay with slight modifications (Johnson et al., 2004). Both oils and antifungal agents were tested in combination at the following concentrations: MIC/4, MIC/2, MIC, MICx2 and MICx4, resulting in 25 different combinations between concentrations of the two substances analyzed. The interaction was defined quantitatively as fractional inhibitory concentration (FIC), calculated by the MIC of the essential oil in combination with antifungal, divided by MIC either oil or antifungal. The fractional inhibitory concentration index (FICI), obtained by adding both FICs was interpreted as synergism when ≤0.5, additivity when >0.5 and <1.0, indifference when ≥1.0 and ≤4.0, and antagonism when >4.0.

2.6. Antichemotactic assay

The evaluation of antichemotactic activity was performed according to the method of the modified Boyden chamber as described by Suyenaga et al. (2011). Prior to assay, neutrophils were treated with the essential oils dissolved in Hank's balanced salt solution (HBSS pH 7.4) in concentrations of 0.625–10 μg/mL, at 37 °C for 30 min. As negative control was used a neutrophils solution with no addition of antichemotactic agent. Indomethacin was used as positive control.

2.7. Antioxidant activity

The antioxidant capacity of the essential oils via free radical sequestration was determined by reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and thiobarbituric acid reactive substances (TBARS) in concentrations of 25–250 μg/mL. For DPPH assay the readings were performed at 5-min intervals during the total time of 30 min. It was determined scavenging activity of free radicals of DPPH (% Antioxidant Activity) using the equation: % AA = (Ac – As)/Ac × 100, where Ac refers to absorbance of DPPH and As refers to absorbance of the sample (Nascimento et al., 2011).

For TBARS assay the reaction medium was composed of 100 μL of the sample at the concentrations described in 0.5 M Tris buffer. Briefly, lipid peroxidation was initiated with the addition of $FeSO_4$ solution, ascorbate solution and 10% egg yolk solution. The subsequent step consisted of the addition of 12% trichloroacetic acid and 0.73% thiobarbituric acid. The supernatant obtained from this reaction was read at 523 nm in spectrophotometer and the protection against lipid peroxidation expressed in percentage of antioxidant activity (AA): %AA = 1 – (AS:Ac) × 100, where As refers to absorbance of sample and A refers to absorbance of the control (Külkamp et al., 2011). Rutin was used as positive control to both assays and all experiments were performed in triplicate.

2.8. Toxicity assays

The toxicity of essential oils was evaluated by cell viability and proliferation tests, comet assay and micronucleus rate, in the antifungal concentrations effective (MIC of 250 and 500 μg/mL) (Güez et al., 2012). Hydrogen peroxide solution (H_2O_2, 100 mmol/mL) and a suspension of leukocytes were used as positive and negative control, respectively. All determinations were performed in triplicate.

Table 1
Chemical composition (%) of *Nectandra* species leaves essential oils obtained by hydrodistillation.

Compound	RI	*N. megapotamica*	*N. lancelata*
Monoterpene hydrocarbons			
α-pinene	925	2.7	–
Sabinene	965	0.2	–
β-pinene	967	4.1	–
Myrcene	986	0.5	–
Limonene	1022	14.1	–
(*E*)-β-ocimene	1043	0.5	–
Sesquiterpene hydrocarbons			
α-copaene	1363	2.8	0.4
β-bourbonene	1371	0.1	1.3
β-cubebene	1377	0.4	–
β-elemene	1380	0.5	1.3
β-caryophyllene	1404	6.4	32.5
Aromadendrene	1424	0.3	0.7
α-humulene	1437	2.8	2.6
Allo-aromadendrene	1444	0.2	3.8
γ-muurolene	1462	0.3	0.3
Germacrene D	1467	16.8	5.1
β-selinene	1470	–	0.2
Bicyclogermacrene	1483	33.4	27.8
α-muurolene	1485	0.4	–
Germacrene A	1489	0.4	1.0
γ-cadinene	1501	0.2	0.1
δ-cadinene	1507	4.0	1.2
Germacrene B	1537	–	1.3
Oxigenated sesquiterpenes			
Elemol	1537	–	0.4
(*E*)-nerolidol	1547	1.7	–
Spathulenol	1558	1.9	11.8
Caryophyllene oxide	1561	0.2	1.5
Globulol	1564	1.1	1.8
epi-globulol	1571	0.2	0.4
Rosifoliol	1589	–	0.2
10-*epi*-γ-eudesmol	1609	–	0.2
isospathulenol	1626	–	0.5
α-muurolol	1630	0.3	1.7
τ-cadinol	1630	–	0.8
14-hydroxy-9-*epi*-(E)-caryophyllene	1660	–	0.1
Phenylpropanoids			
(*E*)-isoelemicin*	1644	2.6	–
Monoterpene hydrocarbons		22.1	–
Sesquiterpene hydrocarbons		69.0	79.6
Oxigenated sesquiterpenes		5.4	19.4
Phenylpropanoids		2.6	–
Total compounds		99.1	99.0

Compounds are listed in order of elution on DB5 column. RI, retention index. *Adams (2001). The chemical composition of the essential oils refers only to one analysis.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software, by ANOVA method followed by Tukey test, with data expressed as mean ± SD. The results for the toxicity assays were analyzed by ANOVA followed by Brown- Forsythe's test. Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Chemical composition of the essential oils

The essential oils obtained from leaves of *N. megapotamica* and *N. lanceolata* showed average yields of 0.3 ± 0.04% (14 extractions) and 0.2 ± 0.06% (22 extractions), respectively. Chemical analysis by GC–MS of the different species revealed the presence of 37 compounds, with predominance of sesquiterpenes, mainly hydrocarbon and absence of oxygenated monoterpenes.

Table 2
Minimum inhibitory concentration and fractional inhibitory concentration index of *N. megapotamica* and *N. lanceolata* essential oils in combination with terbinafine and ciclopirox against dermatophytes.

Essential oils	MIC (μg/mL)	*T. rubrum*		*T. mentagrophytes*		*M. canis*		*M. gypseum*	
		TRU43	TRU51	TME16	TME40	MCA29	MCA01	MGY50	MGY42
N. lanceolata	MIC oil	500	500	500	500	250	500	250	250
	MIC terbinafine	0.008	0.008	0.016	0.016	0.008	0.004	0.016	0.016
	MIC oil in combination	250	500	500	500	250	500	250	31.25
	MIC terbinafine in combination	0.001	0.001	0.008	0.016	0.001	0.001	0.002	0.008
	FICI	0.625	1.125	1.5	2.0	1.125	1.25	1.125	0.625
	Interpretation	ADD	IND	IND	IND	IND	IND	IND	ADD
	MIC oil	500	500	500	500	250	500	250	250
	MIC ciclopirox	2.0	4.0	2.0	4.0	4.0	2.0	2.0	4.0
	MIC oil in combination	62.5	250	31.25	31.25	31.25	250	62.5	31.25
	MIC ciclopirox in combination	0.5	2.0	1.0	2.0	1.0	1.0	1.0	2.0
	FICI	0.375	1.0	0.562	0.562	0.375	1.0	0.75	0.625
	Interpretation	SYN	IND	ADD	ADD	SYN	IND	ADD	ADD
N. megapotamica	MIC oil	250	250	500	500	250	500	500	500
	MIC terbinafine	0.008	0.008	0.016	0.016	0.008	0.016	0.016	0.016
	MIC oil in combination	250	250	500	500	31.25	500	500	250
	MIC terbinafine in combination	0.001	0.001	0.016	0.016	0.008	0.004	0.002	0.002
	FICI	1.125	1.125	2.0	2.0	1.125	1.25	1.125	0.625
	Interpretation	IND	IND	IND	IND	IND	IND	IND	ADD
	MIC oil	250	250	500	500	250	500	500	500
	MIC ciclopirox	2.0	4.0	2.0	4.0	4.0	2.0	2.0	4.0
	MIC oil in combination	125	250	125	250	31.25	250	250	125
	MIC ciclopirox in combination	2.0	0.5	1.0	2.0	2.0	1.0	1.0	2.0
	FICI	1.5	1.125	0.75	1.0	0.625	1.0	1.0	0.75
	Interpretation	IND	IND	ADD	IND	ADD	IND	IND	ADD

MIC: minimum inhibitory concentration. FICI: fractional inhibitory concentration index. SYN, synergism. ADD, additivity. IND, indifference.

For *N. megapotamica* 26 compounds were identified representing 99.1% of oil content (Table 1). The fraction of sesquiterpene hydrocarbons was predominant, with bicyclogermacrene as the most abundant compound (33.4%), followed by germacrene D (16.8%) and β-caryophyllene (6.4%). Monoterpenes represented only 22.1% of the total oil, highlighting limonene as major compound (14.1%). Among the minority, the phenylpropanoid (*E*)-isoelemicin was detected (2.6%). For *N. lanceolata* oil, only sesquiterpenes were identified (Table 1). Similar to *N. megapotamica*, the main compounds observed were β-caryophyllene (32.5%) and bicyclogermacrene (27.8%) that together with spathulenol (11.8%) constituted 70% of oil content. To our knowledge, this is the first chemical composition report of *N. lanceolata* essential oil. For the oil from *N. megapotamica*, similar results were obtained in the study of the Amaral et al. (2015) with predominantly hydrocarbon sesquiterpenes, and germacrene D and bicyclogermacrene among the major compounds. However, differences related to yield and chemical variability to oil of this species has also been observed. The oil obtained by Tondolo et al. (2013) characterized by the presence of bicyclogermacrene (46.5%), α-pinene (26.8%), β-pinene (7.9%) and germacrene D (9.6%). For the study of Romoff et al. (2010), α-bisabolol (64%) and δ-elemene (22%) were identified as major constituents. It is known that both

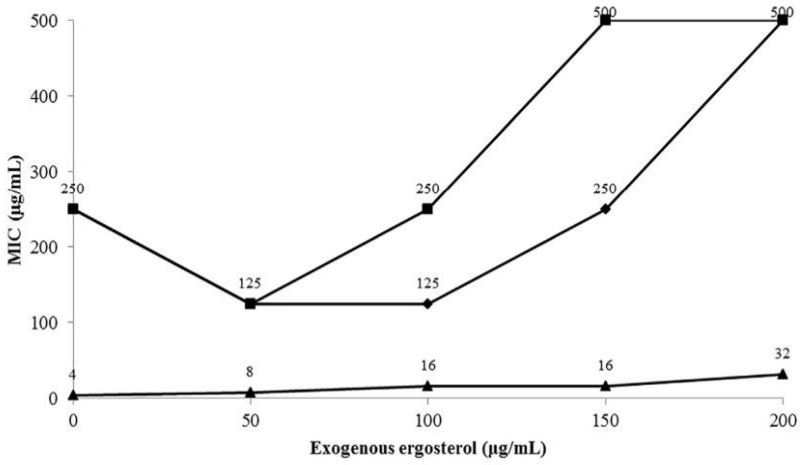

Fig. 1. Effect of exogenous ergosterol in minimum inhibitory concentration (MIC) of amphotericin B (▲) and essential oils of *N. megapotamica* (♦) and *N. lanceolata* (■) against *Microsporum canis* (MCA29).

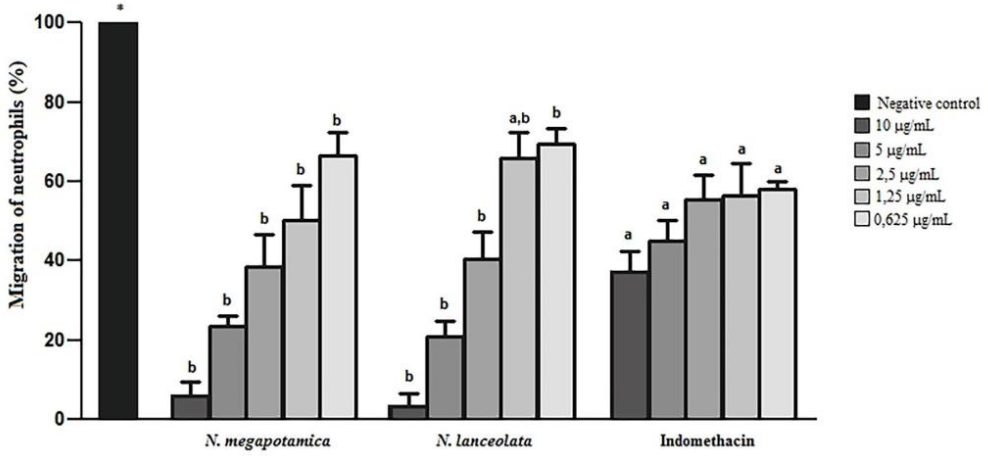

Fig. 2. *In vitro* effect of *N. megapotamica* and *N. lanceolata* essential oils and indomethacin in the neutrophil migration compared to negative control. *Significant inhibition in relation to all tested concentrations. $^{a,b,c}p < 0.05$ indicates significant difference between samples in the same concentrations (ANOVA followed by Tukey's test).

yield as essential oils chemical composition are associated with several factors, such as stage of plant developmental, chemotype and environmental conditions and those can vary within the same genus in accordance with the species characteristics (Almeida et al., 2016; Karimi et al., 2016).

3.2. Antifungal activity and mechanism of action

In the present study *in vitro* antifungal activity of *N. megapotamica* and *N. lanceolata* essential oils we evaluated against genus *Candida*, *Trichophyton* and *Microsporum*, agents of cutaneous and mucocutaneous infections. The screening showed that the essential oils inhibited selectively the growth of dermatophytes, showing no activity against *Candida* species at the concentration tested (500 μg/mL). The results demonstrate inhibition of fungal growth on both oils in MICs of 250 and 500 μg/mL. The terbinafine and ciclopirox results showed a MIC ranged from 0.004 to 0.016 μg/mL and 1 to 4 μg/mL, respectively (Table 2). Relative to sorbitol protection assay, MICs values of anidulafungin, exhibited an increase in presence of sorbitol. However, after ten days of incubation, no changes were observed related to the MICs of *N. megapotamica* and *N. lanceolata* oils, suggesting that these substances do not interfere in cell wall (Escalante et al., 2008). The results obtained in ergosterol assay affinity demonstrate increase in the MIC values of both oils and amphotericin B when different concentrations of exogenous ergosterol were added to the medium (Fig. 1). The addition of 50–250 μg/mL ergosterol caused change in the MICs of the oils to values higher than 500 μg/mL. Amphotericin B results showed MIC values increasing by eight times in presence of exogenous ergosterol, of 4–32 μg/mL. These results indicate that the essential oils of *N. megapotamica* and *N. lanceolata* may exert their effect through interaction with ergosterol present in the fungal membranes.

Different modes of action are involved in the mechanism by which the essential oils inhibit the microorganisms growth, but they are generally related with its hydrophobicity property (Viuda-Martos et al., 2011; Raut and Karuppayil, 2014). The main hypothesis refers to the interaction with the cell membrane by complexing with ergosterol, leading to change in its integrity with a

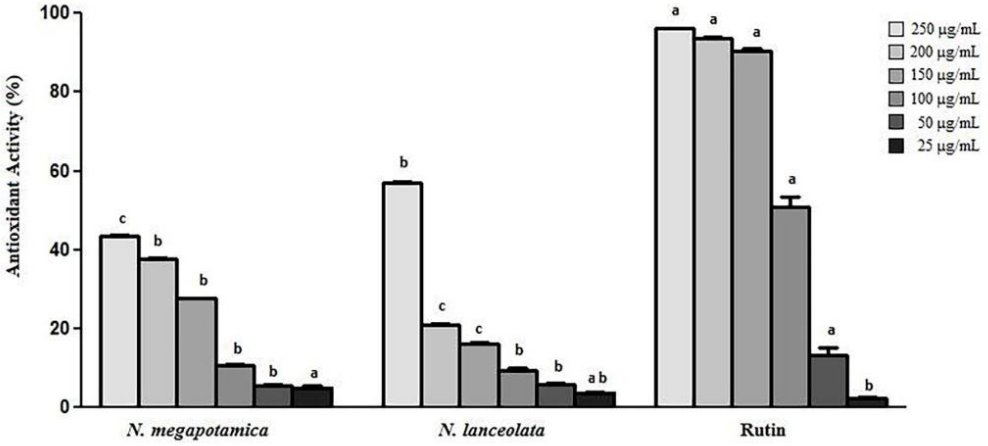

Fig. 3. Antioxidant activity of the *N. megapotamica* and *N. lanceolata* essential oils and rutin, via DPPH radical sequestration. $^{a,b,c}p < 0.05$ indicates significant difference between samples in the same concentrations (ANOVA followed by Tukey's test).

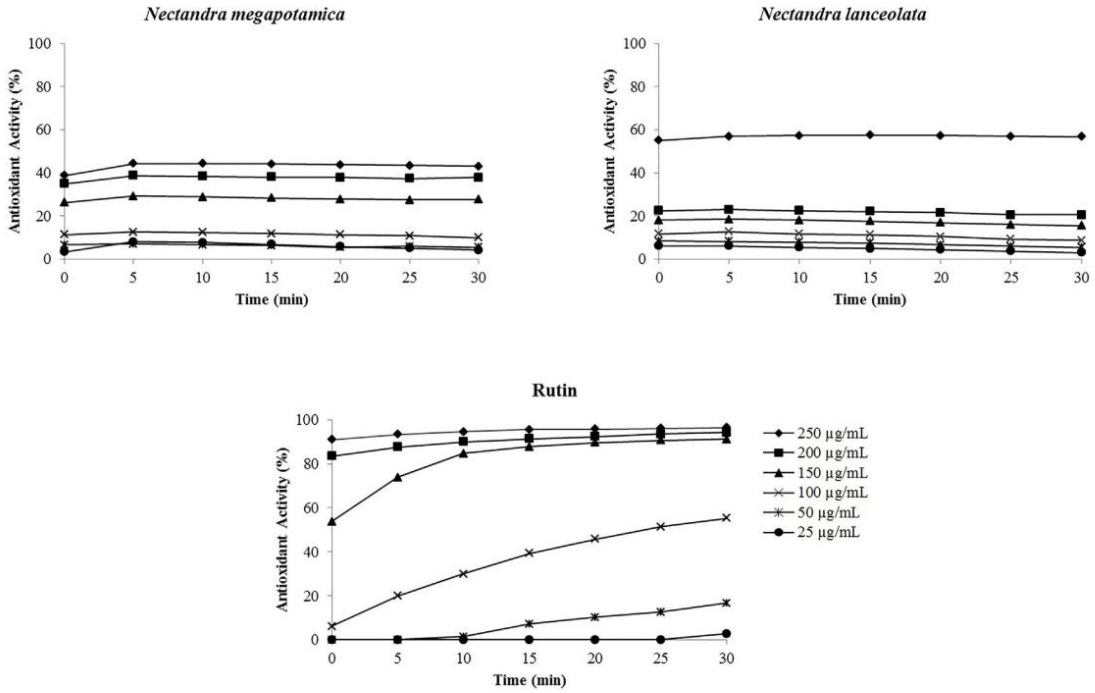

Fig. 4. Kinetic behaviour of DPPH radical scavenging activity for essential oils and rutin.

consequent increase in permeability to ions and leakage of intracellular content (Asdadi et al., 2015), confirming the results obtained in this study. This effect is related to the presence of oxygenates compounds, such as alcohols and phenolic substances (Lang and Buchbauer, 2012).

3.3. Checkerboard assay

FICI values of essential oils associated with antifungal drugs were calculated to determine possible interactions of these combinations against dermatophytes. The results showed synergistic interactions, additive and indifference against the different isolates (Table 2). Antagonism effect was not observed. The combinations produced FICIs values in the range of 0.375–2.0. FICIs observed by combination of both oils with ciclopirox were generally smaller in relation to FICIs obtained by combination with terbinafine. Combinations involving terbinafine resulted in 81.25% of indifference. *N. lanceolata* oil and ciclopirox exhibited synergistic effect (FICI 0.375) for two isolates: *T. rubrum* (TRU43) and *M. canis* (MCA29). Synergism was neither observed between *N. megapotamica* oil and antifungals drugs nor between *N. lanceolata* and terbinafine. To ciclopirox was also observed additive effect in 37.5 and 50% by combination of *N. megapotamica* and *N. lanceolata* oils, respectively. An additive interaction reduces mildly the effective concentration of the antifungal agent, which is especially relevant when it comes to drugs having significant side effects and toxicity.

Synergistic effect resulting of the combination of antifungal and essential oils from various species, or its single constituents, has been recently reported (Campbell et al., 2012; Castro et al., 2015; Cardoso et al., 2016). A synergistic effect occurs mainly due to multiple modes of action (Carrillo-Muñoz et al., 2014). Terbinafine acts by blocking the biosynthesis of ergosterol, an essential component of fungal membrane, through inhibition of squalene epoxidase enzyme (Dias et al., 2013). Such as terbinafine the essential oils evaluated in this study also act on the fungal cell membrane. Thus, the action mode similarity of these antifungal agents can explain the results of indifference obtained in checkerboard assay by this combination.

On the other hand, ciclopirox acts by binding trivalent cations, such as Fe^{3+}, inhibiting a variety of metal-dependent enzymes and affecting the energy production of mitochondrial process electron transport, reduces catalase and peroxidase, and commits nucleic acids and the synthesis of associated proteins (Hube et al., 2015). The possible interaction of *Nectandra* oil with fungal plasma membranes causing permeability changes, thus favoring the entry of ciclopirox inside the cell is considered a hypothesis to the synergistic and additive results from the combination.

3.4. Antichemotactic and antioxidant activities

The chemotaxis, characterized by the migration and accumulation of inflammatory cells to the site of injury is one of the first and major steps in inflammatory reactions (Medzhitov, 2008). Thus, the ability of the essential oils to inhibit leukocyte migration was determined by antichemotactic assay by the Boyden chamber method. The results are shown in Fig. 2, expressed as percentage of neutrophil migration relative to the negative control. Both oils in all tested concentrations (0.625–10 µg/mL) inhibited significantly the leukocyte migration toward the chemoattractant LPS. Indomethacin used as a positive control inhibited 62.9% of migration in the highest concentration tested (10 µg/mL). Polymorphonuclear leukocytes treated with 0.625–10 µg/mL of *N. megapotamica* oil showed a reduction in migration of 34.5–94.1%, compared to the negative control. *N. lanceolata* showed similar results, with inhibition percentage of 30.7–96.7% to same concentrations. The comparison of the effect of the oils in the same

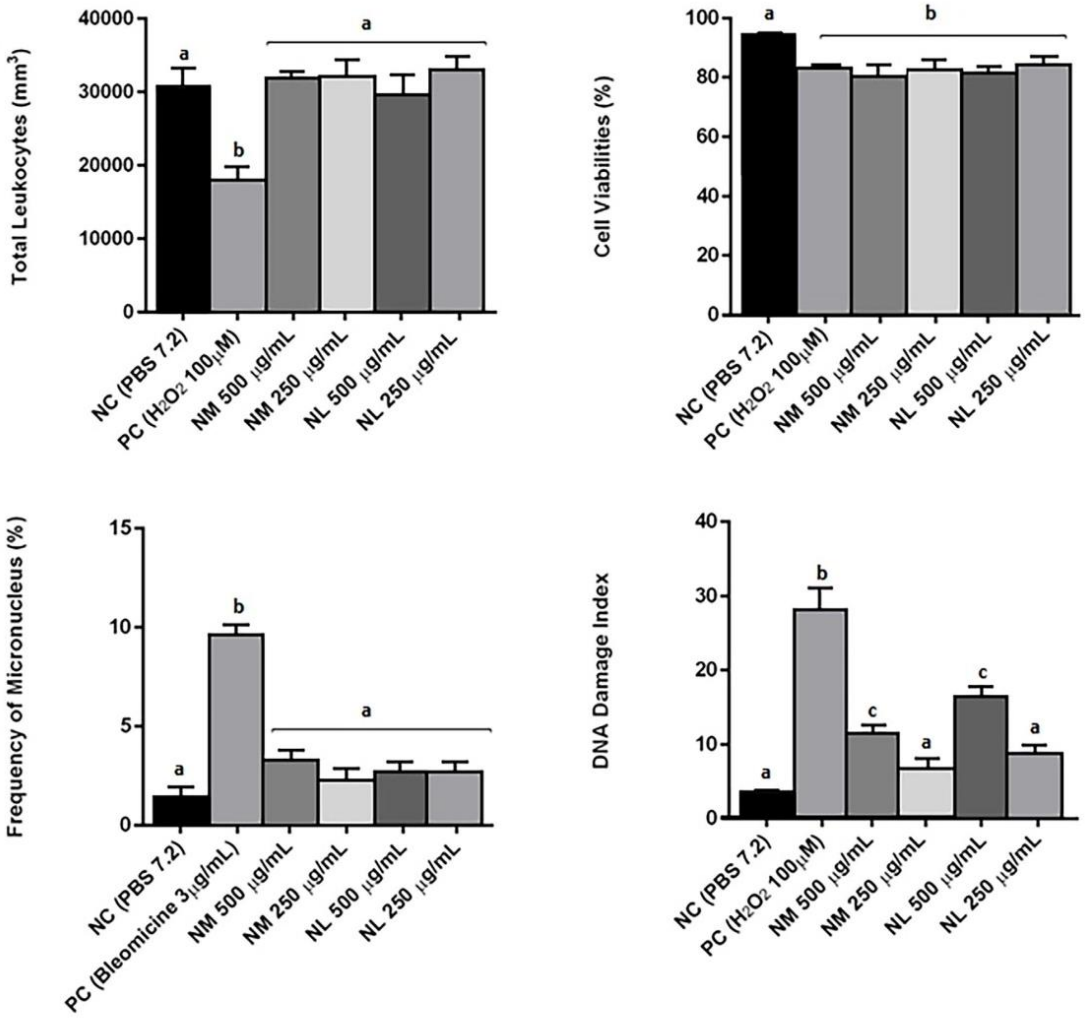

Fig. 5. Effect of *N. megapotamica* (NM) and *N. lanceolata* (NL) essential oils in leukocyte proliferation, cell viability, micronucleus rate and DNA damage. [a,b,c]$p < 0.05$ indicates significant difference between the controls and essential oils (ANOVA followed by Brown-Forsythe's test).

concentrations showed no statistically significant differences. To *N. megapotamica* all concentrations had a significantly higher leukocyte inhibition compared to the same concentrations tested for indomethacin indicating a potential inhibitory activity. The inhibitory effect obtained in this study corroborates the results of antichemotactic activity observed by Apel et al. (2006) for oil this species.

Studies have reported anti-inflammatory properties for essential oils or their compounds isolated by several mechanisms including inhibition of leukocyte migration (Kummer et al., 2013; Danielli et al., 2016). This property may be related to signaling cascade involving cytokines and transcription factors and the expression of pro-inflammatory genes (Miguel, 2010). The migration of leukocytes to the site of injury is considered one of the major early stages of an inflammatory process (Medzhitov, 2008). Therefore, this suggests that the essential oils from *Nectandra* act in response to an acute inflammatory process.

The essential oils ability to scavenge free radicals was evaluated by DPPH method. The results obtained at 30 min indicate scavenging activity of DPPH concentration-dependent. Only the concentration of 250 μg/mL of *N. lanceolata* oil showed antioxidant activity higher than 50% (Fig. 3). Activity significantly greater was observed for rutin in relation to oils ($p < 0.05$), except in the concentration of 25 μg/mL. The moderate reactivity by the DPPH test can be explained by the absence of phenolic compounds in the essential oils considering that most authors report that these molecules feature high capacity to react with hydroxyl radicals in the form of transfer of hydrogen atoms (Amorati et al., 2013). The assessing of kinetic effect indicated slight scavenging free radicals at the initial time (0–10 min) with subsequent decrease in relation to time, for all concentrations of both oils. The opposite was observed for the positive control, rutin, where the percentage of radical scavenging activity is directly proportional to the reaction time (Fig. 4). The kinetic assay DPPH antioxidant indicates mechanism of action of the substance. When a reaction is fast, equivalent to femtoseconds,

it suggested that free radical stabilization occurred from electron transfer. However, reactions considered slow in the range of seconds or minutes indicate activity by the transfer mechanism of hydrogen atoms (Xie and Schaich, 2014), as observed by oils from *N. megapotamica* and *N. lanceolata*. In relation to the TBARS assay, the oils had no effect on the protection of lipid peroxidation at the concentrations tested.

Dermatophytes and its metabolites induce an inflammatory response to the host tissue (Peres et al., 2010). The keratinolytic enzymes production causes damage to the host tissues inducing thus an inflammatory reaction at the site of infection responsible to attract cells of the immune system with the purpose of combating the pathogen (Peres et al., 2010; Hube et al., 2015). During the phagocytosis process occurs the formation of free radicals that neutralize the microorganism. However, the exacerbated formation of these radicals can unbalance the antioxidant system of the organism causing oxidative damage at inflammatory site (Boudiaf et al., 2016). In some cases, accented inflammatory responses are also associated with increased severity of fungal infection development and chronicity (Romani, 2011). Therefore, a substance with anti-inflammatory and antioxidant properties associated with the antifungal effect will contribute to more effective antifungal action (Cabral et al., 2015; Hube et al., 2015).

3.5. Cell toxicity assays

The effect of *N. megapotamica* and *N. lanceolata* essential oils in leukocyte proliferation, cell viability, micronucleus frequency and DNA damage, evaluated in concentration of MICs are shown in Fig. 5. For the leukocyte proliferation and frequency of micronuclei were not observed statistically significant differences compared to the negative control. The cell viability assay indicated about 80% viable cells to oils at both concentrations tested, similar to that observed for the positive control H_2O_2. Damage to DNA was observed in both species in the concentration of 500 μg/mL, but it remains significantly different from the positive control. According Campbell et al. (2012) a chemosensitizing agent must improve the activity of the drug, with no additional toxic effects or with minimum presence of these effects, which can be seen in the results obtained in this study. With respect to DNA damage and cell viability it is believed that the results demonstrated in the concentration of 500 μg/mL by the isolated oils probably will be minimized or extinguished when in combination, considering that in this situation there is a reduction in the active concentration. The essential oil samples showed antifungal activity against dermatophytes which are mainly characterized by infections on skin, hair and nails, thus, dermal toxicity test and hypoallergenicity are needed to confirm its possible use in the topical treatment these infections, besides toxicity tests of the combinations between oils and antifungals.

In order to address the lack of new antimicrobial agents and combat resistance to conventional treatment, including as possible reversion resistance, combination therapy becomes a viable strategy with multi-target (Ahmad et al., 2013; Zhang et al., 2014). In addition to reducing the cost of the treatment, this approach proposes obtain the lowest effective dose in order to reduce the incidence of adverse effects of drugs (Zhang et al., 2014), as observed in this study.

4. Conclusion

The combination of the essential oil from *N. lanceolata* and ciclopirox showed synergistic and additive effect on most isolates reducing the active concentration of the antifungal agents when in combination. The selective antifungal activity against dermatophytes, presumably by complexing with ergosterol of fungal membrane assumes its sensitizing activity of the fungal cell resulting in synergistic and additive effect when in combination with ciclopirox. So, the results indicate the essential oil from *N. lanceolata* as a possible complement to conventional antifungal therapy with the advantage of the combination of anti-inflammatory and antioxidant effect which can accelerate the relief of symptoms, to facilitate healing and prevent the dissemination of infection.

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CAPÍTULO IV – Mecanismo de ação antifúngico do óleo volátil de *Schinus lensticifolius* Marchand e seu efeito sinérgico *in vitro* com terbinafina e ciclopirox

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Manuscript Draft

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Article Type: Full length article

Section/Category: Chemistry and Bioactive Products

Keywords: Schinus lentiscifolius; Anacardiaceae; essential oil; antifungals; mechanism of action; synergism; dermatophytes

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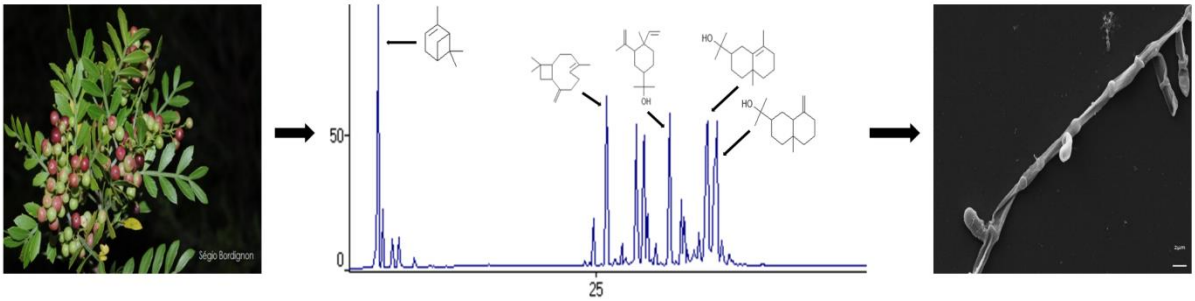
Abstract: Antifungal agent's combination with natural products is an important strategy being investigated to the development of new therapies, considering the limited therapeutic arsenal available, associated with high levels of toxicity and increase of development of resistance to conventional treatment. So, we evaluated the antifungal, antichemotactic and antioxidant activities of Schinus lentiscifolius essential oil, as well as its combined effect with terbinafine and ciclopirox against dermatophytes. The oil from the fresh leaves was obtained by hydrodistillation and the chemical analysis perfomed by GC-MS revealed the presence of 33 compounds being γ-eudesmol (12.8%), elemol (10.5%), β-eudesmol (10.2%) and β-caryophyllene (10.0%) as majors. The oil presented 97.4% inhibition of leukocyte migration and 37.9% of scavenging activity of DPPH radical. Antifungal screening showed effect against dermatophytes with MIC of 125 and 250 µg/ml. The increase of up to 8-fold in the MIC value verified in the sorbitol assay and structural damage in the hyphae observed by SEM, indicate that the oil can acts on the wall and fungal cell membrane. Synergistic interactions were observed from the combination with antifungals, mainly terbinafine. This effect is probably related to simultaneous action of these substances at different targets. Furthermore, no epidermal lesions and irritative potential were observed. So, the results indicate that S. lentiscifolius essential oil acts as chemosensitizer of the fungal cell resulting in antifungal effect improvement and that can be an alternative for the topical treatment of dermatophytosis, with the advantage of an associate anti-inflammatory effect.

Graphical Abstract



Antifungal mechanism of action from *Schinus lentiscifolius* Marchand essential oil and its synergistic effect *in vitro* with terbinafine and ciclopirox against dermatophytes

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Abstract

Antifungal agents combination with natural products is an important strategy being investigated to the development of new therapies, considering the limited therapeutic arsenal available, associated with high levels of toxicity and increase of development of resistance to conventional treatment. So, we evaluated the antifungal, antichemotactic and antioxidant activities of *Schinus lentiscifolius* essential oil, as well as its combined effect with terbinafine and ciclopirox against dermatophytes. The oil from the fresh leaves was obtained by hydrodistillation and the chemical analysis perfomed by GC-MS revealed the presence of 33 compounds being γ-eudesmol (12.8%), elemol (10.5%), β-eudesmol (10.2%) and β-caryophyllene (10%) as majors. The oil presented 97.4% inhibition of leukocyte migration and 37.9% of scavenging activity of DPPH radical. Antifungal screening showed effect against dermatophytes with MIC of 125 and 250 µg/mL. The increase of up to 8-fold in the MIC value verified in the sorbitol assay and the structural damage in the hyphae observed by SEM indicate that the oil acts on the wall and fungal cell membrane. Synergistic interactions were observed from the combination with antifungals, mainly terbinafine. This effect is probably related to simultaneous action of these substances at different targets. Furthermore, no epidermal lesions and irritative potential were observed. So, the results indicate that *S. lentiscifolius* essential oil acts as chemosensitizer of the fungal cell resulting in antifungal effect improvement and that can be an alternative for the topical treatment of dermatophytosis, with the advantage of an associate anti-inflammatory effect.

Keywords: *Schinus lentiscifolius*; Anacardiaceae; essential oil; antifungals; mechanism of action; synergism; dermatophytes.

1. Introduction

Dermatophytosis consist in an infectious process caused by filamentous fungi specialized in invade and infect keratinized tissues in humans and animals (Hube et al., 2015). These infections in general are superficial but considered unwieldy. Spontaneous healing is very unlikely and the therapy is considered complex, costly and long besides being characterized by recurrent episodes (Peres et al., 2010; Gupta et al., 2013). Furthermore, the restricted available therapeutic arsenal associated with long treatment and the development of resistance by microorganisms render this type of infection a worldwide problem (Scorzoni et al., 2016).

In this context, many researchs seeking new active substances as alternative to the conventional therapy or its complementation have been conducted (Soares et al., 2013; Raut; Karuppayil, 2014). As multi-target approach, the combination therapy has been employed as a strategy for the lack of new antimicrobial drugs (Johnson et al., 2004; Carrillo-Muñoz et al., 2014). Therefore, the identification of synergistic associations has been the focus of many studies evaluating antifungal combination therapy (Campbell et al., 2012; Zhang et al., 2014). Besides to antifungal effect, substances that have anti-inflammatory or antioxidant properties can be promising in the treatment of cutaneous mycosis. The sum of these features can significantly help in wound healing caused by dermatophytes infections once they trigger an inflammatory process as a response to the cutaneous infection (Boudiaf et al., 2016; Orhan et al., 2016).

A combination of conventional antifungals with natural compounds can also minimize adverse effects of these drugs in view of reducing the dose required to obtain the same effect (Campbell et al., 2012; Zhang et al., 2014). Thus, the challenge is the search for new substances capable of complement conventional therapy acting synergistically with available drugs in order to maximize its effect or reverte resistance (Chen et al., 2010). The use of

chemosensitizers agents of pathogen has been shown as a promising alternative to the lack of new antimicrobials and to the increasing resistance to conventional antifungal therapy, however, without adding toxic or adverse effects (Campbell et al., 2012; Carrillo-Muñoz et al., 2014). In this context, studies have shown that chemosensitization of microorganisms by essential oils or their isolated compounds improved the efficacy of commercial antifungal agents (Campbell et al., 2012; Castro et al., 2015; Cardoso et al., 2016). Essential oils are an important group of plant secondary metabolites employed on a large scale in the cosmetic, food and pharmaceutical industry due to related biological effects (Amorati et al., 2013; Raut; Karuppayil, 2014; Carvalho et al., 2016). Therefore, these substances can be an alternative to use in combination with antifungals agents, since they are associated with antimicrobial, anti-inflammatory and antioxidant activities (Amorati et al., 2013; Asdadi et al., 2015; Sá et al., 2015).

Schinus lentiscifolius Marchand is a tree belonging to the family Anacardiaceae (Cole et al., 2014). Several activities, as antimicrobial, anticancer, antioxidant, repellant and inseticide have been described to essential oils from different species of this genus (Bendaoud et al., 2010; Cole et al., 2014; Pratti et al., 2015). Considering the essential oils as a source of potential antimicrobial substances, this study evaluated the antifungal, antioxidant and antichemotactic activities from *S. lentiscifolius* essential oil, as well as its antifungal mechanism of action and toxicity. It was also evaluated, the effect of the essential oil in combination with terbinafine and ciclopirox against dermatophytes.

2. Results

2.1. Chemical composition of the essential oil

The essential oil obtained from leaves of *S. lentiscifolius* presented yield of 0.6%. Chemical analysis allowed the identification of 33 compounds, which represents 96.2% of the total oil (Table 1). Sesquiterpene fraction was identified as being predominant with oxygenates compounds representing 50.7% of the total content of oil. Among the sesquiterpenes hydrocarbons, β-caryophyllene (9.9%), germacrene D (8.2%) and bicyclogermacrene (7.9%) were identified as the predominant compounds. For the oxygenated fraction, elemol (10.5%) and three compounds of the eudesmane route presented themselves as majority: γ-eudesmol (12.8%), β-eudesmol (10.2%) and α-eudesmol (9.2%). Only hydrocarbon compounds were observed in the monoterpene fraction, highlighting α-pinene as main constituent (8.2%).

2.2. Antifungal activity and mechanism of action

The screening of essential oil from *S. lentiscifolius* showed selective inhibition against dermatophytes, exhibiting no activity against *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* at the tested concentration (500 μg/mL). About the MIC, the oil inhibited the growth of *T. rubrum, M. gypseum* and *M. canis* at concentrations of 125 and 250 μg/mL, whereas to *T. mentagrophytes* showed MIC single of 250 μg/mL. For terbinafine and ciclopirox, MIC values ranged from 0.004 to 0.016 μg/mL and 1 to 4 μg/mL, respectively (Table 2).

The results in Table 3 show increase of two to eight times in the oil MIC values through the addition of sorbitol to all strains except TME43 and MCA01. Similarly, anidulafungin MIC values were changed at the presence of the osmotic protector. The addition of ergosterol to the medium resulted in dose-dependent increase of two to three times in oil MIC values, with inhibitory concentrations exceeding 500 μg/mL (Fig. 1). Concentrations of exogenous

ergosterol equal or higher than 50 μg/mL increased amphotericin B MIC of in 8-fold (4 μg/mL to 32 μg/mL).

Time kill assay showed reduction of viability of *T. rubrum* and *M. canis* treated with oil, confirming the results obtained in the susceptibility testing (Fig. 2). The sample presented time-dependent fungicidal effect for the two strains. At the concentration of 500 μg/mL the oil presented fungicidal effect after 12-h treatment and for 250 μg/mL the same effect was obtained only after 24 h. For *M. canis* the fungicidal effect was observed after 24 h.

The effect of subinhibitory concentrations of ciclopirox, terbinafine and combinations with oil was observed by SEM. In control specimens, mycelial cells showed long strands of hyphae with smooth and intact cell walls (Fig. 3A). In a hyphal specimen treated with oil only, the ultrastructural changes indicated shrivelling and lysis cell (Fig. 3B, C). No predominant structural changes were observed in hyphae when treated with subinhibitory concentrations of cyclopirox and terbinafine (3D, 3F). However, when the antifungals were combined with the essential oil the same cells presented narrowing similar to the observed on treatment with oil only (3E, 3G).

2.3. Checkerboard assay

The FICI values results from the combination between essential oil and antifungals indicated synergistic, additive and indifferent interactions (Table 2). Antagonism was not observed. For the combinations generated with terbinafine the FICI values ranged from 0.375 to 1.0625 and synergistic effect was demonstrated in 62.5% of the strains. For strains of *T. mentagrophytes* the combination was additive or indifferent. The association between oil and ciclopirox showed synergistic effect for only two strains of *Microsporum* genus, while additivity was observed as predominant effect present in 62.5% of the strains (FICI 0.375 to 1.25). Only one *T. rubrum* strain demonstrated indifference to this combination.

2.4. Antichemotactic and antioxidant evaluations

Fig. 4 demonstrates the percentage of leukocyte migration in relation to the negative control. In all concentrations tested, the oil reduced significantly ($p < 0.05$) the migration toward the chemoattractant with inhibition in the ranged from 27.4 to 97.4%. Indomethacin, used as postitive control, showed significantly lower effect in relation to oil ($p < 0.05$) for concentrations from 1.25 to 10 μg/mL, being that the last one demonstrated antichemotactic activity of only 62.9%.

In the DPPH assay, the oil exhibited ability to sequester free radicals from 12.1 to 37.9% in the tested concentrations showing significant lower effect in relation to rutin in the same concentrations (55.3 to 96.4%) ($p < 0.05$) (Fig. 5). The kinetic assay effect of the oil in scavenging of the DPPH radical showed activity in the initial reaction time (0 to 5 minutes) with subsequent decrease in relation to time for all concentrations tested (Fig. 6). The opposite was observed for rutin, which scavenging activity increased in relation to time.

2.5. Cytotoxicity, genotoxicity and mutagenicity assays

The MIC and MICx2 of the essential oil were not differents to negative control ($p < 0.05$) for the both genotoxic and mutagenic parameters (Fig. 7). The oil decreased the cellular viability similarly to the positive control (Fig. 7). However, the samples were not tested at the active concentrations obtained from the combination with the antifungals. When the oil is employed in association its concentrations are reduced by up to four times, so it is believed that the effect in these trials would also be decreased.

2.6. Histopathology and HET-CAM test

The histopathological evaluation results indicated no microscopic lesions in the tested oil and antifungals concentrations, as well as in combinations thereof (Fig. 8). Fig. 9 shows the relationship between the irritation score and the logarithms of the relative concentrations of the antifungal agents, oil and combinations. According to methodology used all the samples tested were classified as non-irritating.

3. Discussion

Studies have shown the chemosentizing of pathogens and synergistic interactions between essential oils and their active compounds with conventional antifungal drugs (Khan; Ahmad, 2011; Campbell et al., 2012; Castro et al., 2015; Cardoso et al., 2016). In the current study, the antifungal effect of *S. lentiscifolius* essential oil and its combination with commercial antifungals were evaluated. Synergistic interactions of essential oil with terbinafine and ciclopirox were observed and this effect may be related to the simultaneous effect of these substances at different targets. Terbinafine acts by inhibiting squalene epoxidase, an enzyme involved in the ergosterol biosynthesis (Baddley; Pappas, 2005), while ciclopirox, by having high affinity for trivalent cations, such as Fe^{3+}, inhibits metal-dependent enzymes, affects the energy production in the electron transport chains in mitochondria, nutrient uptake and synthesis of proteins and nucleic acids (Gupta et al., 2013). The fungal cell wall and membran are considered the target site for the oils and their isolated compounds (Khan; Ahmad, 2011; Cardoso et al., 2016). An effect on the cell wall of the microorganism caused by the action of these substances can be related to an increase in the amount of the drug inside the cell and available to exert its effect, causing a rapid death of the cell (Khan; Ahmad, 2011). This hypothesis corroborates the results obtained in this study for the antifungal mechanism of *S. lentiscifolius* oil action and it can justify the synergistic interactions resulting from the combination with terbinafine and ciclopirox.

The increase in oil MIC values observed in sorbitol protection test suggests its possible role in the cell wall level. Whereas the effect was not found for two of the strains, it can not be affirmed that the wall is the main target, being likely that other mechanisms are also involved. Similarly, changes in MIC values observed in ergosterol protection assay indicate that the oil may act interacting with the membrane sterol of fungi. Due to their hydrophobic character, the main hypothesis reported in the literature rests on the interaction of essential oils with the lipid bilayer of the fungal membrane changing integrity and permeability through the formation of transmembrane channels that result in extravasation of vital intracellular constituents, besides affecting the cellular respiration and other enzyme systems (Khan; Ahmad, 2011; Viuda-Martos et al., 2011). This hypothesis corroborates the results obtained in this study, indicating that the *S. lentiscifolius* oil exerts its effect by complexing ergosterol of fungal cell membrane altering its permeability. Moreover, its ability to act on cell wall observed in the test with sorbitol and associated to presence of alterations in hyphal morphology as shrivelling and lysis can intensify this effect.

The time kill assay indicates fungicidal effect of oil at 12 and 24 h of treatment for *T. rubrum* and *M. canis*, respectively. Fungicide substances are considered clinically important considering that the use of fungistatic drugs is strongly associated with increased resistence (Tangarife-Castaño et al., 2011). Infections requiring prolonged treatment as dermatophytosis are often associated with poor adhesion to therapy and consequently with recurrence increase (Peres et al., 2010). Thus, substances that demonstrate fungicidal character are highly relevant in infections caused by dermatophytes.

By accessing the host tissue, dermatophytes induce an immune response by keratinocytes, triggering an inflammatory process (Peres et al., 2010). It is important to note that, despite being a typical and fundamental immune response as a defense factor of the organism, inflammation when in sharp and prolonged response is related to a wide range of

chronic and autoimmune diseases, and in some cases compromising the host's ability to face the pathogen (Romani, 2011; Kim et al., 2015). One of the fundamental stages of the inflammatory process involves leukocyte migration directly to the site of injury, known as chemotaxis, representing the first line of host defense (Boudiaf et al., 2016).The inhibition of this process may be related to some of the inflammatory response control mechanism. The evaluation of the antichemotactic activity performed in this study demonstrated inhibition of leukocyte migration significantly higher by the *S. lentiscifolius* essential oil as compared with indomethacin. These results suggest that this substance can act on the modulation of the acute inflammatory response.

Also in relation to the infection process, an imbalance in the redox system of the organism caused by excessive production of free radicals from the defense cells at the moment the phagocytosis of microorganism can cause damage to the injured tissue and its surrounding (Boudiaf et al., 2016; Orhan et al., 2016). Antioxidant activity is characterized as one of the main bioactivities associated to essential oils (Viuda-Martos et al., 2011; Amorati et al., 2013; Raut; Karuppayil, 2014). Although the studied oil did not show any effect on the inhibition of lipid peroxidation, by TBARS assay, it demonstrated free radical sequestration activity characterized by slow reactions to DPPH (in the range of seconds to minutes), representative of substances with hydrogen donation capacity (Schaich et al., 2015).

Thus, the combination of anti-inflammatory and antioxidant effect with antifungal activity observed on *S. lentiscifolius* make this oil a possible option to complement antifungal therapy, in order to accelerate the relief of symptoms, promoting healing and preventing the spread of infection, besides avoiding the development of chronicity (Hube et al., 2015). Moreover, the absence of injury and irritating potential observed on the oil and its combinations with the antifungals indicate the possibility to apply this substance in a

formulation for topical level, since dermatophyte infections are considered superficial reaching keratinized tissues such as skin, hair and nails.

4. Conclusion

S. lentiscifolius essential oil was investigated for its biological properties and *in vitro* interactions with commercial antifungal agents. To our knowledge, this is the first study on the antioxidant and anti-inflammatory effect, and the combination of the essential oil of this species with antifungal agents. The oil showed selective activity against dermatophytes and the evaluation of the antifungal mechanism of action indicated effect on the wall and fungal cell membrane, confirmed by structural damage observed in the hyphae. Besides exhibiting antioxidant activity by free radical sequestration and important antichemotactic effect. The substance that presents antioxidant, anti-inflammatory and antifungal properties associated has the advantage of promoting rapid relief of symptoms of the infection and accelerating the healing process. The synergistic interaction results from the combination with terbinafine showed that the essential oil acted in the chemosensitization of the fungal cell resulting in an antifungal effect improvement. The results obtained associated with the absence of epidermal injury and no irritation potential indicates that this oil can be used as a complementary way to the conventional therapy in the topical treatment of superficial infections caused by dermatophytes.

5. Experimental

5.1. Plant material

Leaves of *S. lentiscifolius* were collected from native population in Southern Brazil and identified by the botanist Dr. Sérgio L. Bordignon. A voucher specimen was deposited in the Herbarium of the Federal University of Rio Grande do Sul (UFRGS) (ICN-UFRGS: 179855).

5.2. Obtaining and chemical analysis of essential oil

Fresh leaves were submitted to four-hour hydrodistillation using a Clevenger-type apparatus to obtain the essencial oil (Farmacopeia Brasileira, 2010). The essential oil was obtained from fresh material by hydrodistillation for 4 hours, using a Clevenger-type apparatus, according to the procedure described in the Brazilian Pharmacopeia (2010). The yield determination was performed as weight/volume (w/v). For chemical analysis, the essential oil was diluted in ethyl ether to a ratio 2:100 (v/v). The chemical composition was analyzed by gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP5000) with a column DB-5 fused silica capillary with a (5% phenyl)-methyl poly siloxane stationary phase, film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. Helium was used as carrier gas in a flow rate of 1 mL/min. Injector and detector temperatures were set at 220 °C and 250 °C, respectively, and GC oven was programmed to 60 to 300 °C at a rate of 3 °C/min. The identification of compounds was based on the comparison of retention indices, calculated by linear interpolation relative to retention times of a series of n-alkenes, and their mass spectra, with authentic samples and with data taken from the literature (Adams, 2009), or by comparison with mass spectra recorded in the database as NIST 62 and NIST 12 (National Institute of Technology and Standards). Relative amounts of the components were calculated based on GC peak areas.

5.3. Antifungal activity

5.3.1. Screening and minimum inhibitory concentration (MIC)

The antifungal activity of *S. lentiscifolius* oil was evaluated against clinical strains of *Candida* spp., *Trichophyton* spp. and *Microsporum* spp. deposited in the Mycology Collection of the UFRGS, Brazil. Screening test was carried in the concentration of 500 µg/mL and MIC (tested in range of 1.95 at 500 µg/mL) was determined by microdilution broth method (CLSI, 2008) in cases where antifungal activity was observed in the screening. The MIC was defined as the lowest concentration of substance at which the microorganism tested did not show visible growth. Terbinafine was used as positive control. All the experiments were performed in triplicate.

5.3.2. Time kill assay

The time kill assay was performed with exposure of *Trichophyton rubrum* TRU51 and *Microsporum canis* MCA29 to MIC and MICx2 oil, and the procedure was conducted as previously described by Ghannoum et al. (2013). Aliquots were serially diluted (10^{-1}, 10^{-2} and 10^{-3}) with sterile water at times of 0, 3, 6, 12 and 24 h plated and incubated to determine the number of CFU/mL. The assay was performed in triplicate and untreated fungal inoculum was used as control. The curves were constructed by plotting $\log_{10}$ CFU/mL in relation to the exposure time of fungal cells to the different oil concentrations. Fungicidal effect was considered when there was a decrease $\geq$ 99.9% in $\log_{10}$ of the number of CFU/mL compared with the starting inoculum.

5.3.3. Sorbitol protection assay and ergosterol effect

In order to determine the oil effect on the cell wall integrity and the ability of complexing with ergosterol in fungal membranes, it was evaluated the MIC against dermatophytes strains (TRU43, TRU51, TME16, TME40, MCA29, MCA01, MGY50 and MGY42) in absence and presence of sorbitol (0.8 mol/L) and ergosterol (concentrations of 50

298 to 200 µg/mL), respectively (Escalante et al., 2008). For the sorbitol assay the MIC was
299 measured along 4 and 7 days and anidulafungin was used as positive control. While for the
300 ergosterol effect assay the MIC was mensuared after 5 days and amphotericin B was
301 employed as positive control.

302 *5.3.4. Scanning electron microscopy (SEM)*

303 Scanning electron microscopy was perfomed as described by Joubert et al. (2015) with
304 few modifications. An isolate of *M. canis* (MCA29) treated with subinhibitory concentrations
305 of ciclopirox, terbinafine and combinations thereof with the essential oil was used in the
306 assay. Inhibitory and subinhibitory concentrations of oil were tested singly. In this assay,
307 aliquots of each sample were used in the respective concentration obtained from the
308 checkerboard test. Briefly, the samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate
309 pH 7.4 for 2 h, 3x washed with post fixative (0.1 M cacodylate pH 7.4 + 0.2 M sucrose and 2
310 mM magnesium chloride) and added in coverslips previously functionalized with poly-L-
311 lysine for 30 minutes. Dehydration was carried out with ethanol 30, 50, 70, 95 (5 min) and
312 100% (10 min), and then the samples were submitted to critical point immediately after
313 dehydration, mounted on metallic stubs, sputter-coated with a 15-20 nm gold-palladium layer
314 and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10, Oberkochen,
315 Germany), operating at 10kV.

316 *5.4. Checkerboard assay*

317 The checkerboard assay with few modifications was employed to determine the
318 interaction of the oil with ciclopirox and terbinafine against eight dermatophyte strains
319 (Johnson et al., 2004). The results obtained lead to 25 different combinations between oil and
320 antifungal agents in concentrations of the MIC/4, MIC/2, MIC, MICx2 and MICx4. For this

assay 125 µg/mL was considered the MIC of oil. The interaction was defined quantitatively as fractional inhibitory concentration index (FICI), obtained by adding both FICs and it was interpreted as synergism when ≤ 0.5, additivity when > 0.5 and < 1.0, indifference when ≥ 1.0 and ≤ 4.0, and antagonism when > 4.0.

5.5. Antichemotactic assay

The capacity of oil to inhibit leukocyte migration was evaluated by modified Boyden chamber method (Suyenaga et al., 2011). The neutrophils were treated with the oil in concentrations of 0.3125 to 10 µg/mL, at 37 °C for 30 minutes. A neutrophil solution without the addition of antichemotactic agent was used as negative control. Indomethacin was used as positive control.

5.6. Antioxidant evaluation

The reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to evaluate the capacity of the oil to sequester in free radical sequestration in the concentrations of 100 to 250 µg/mL. In this assay the reading absorbance (A) was performed at 5-minute intervals during the total time of 30 minutes and the results expressed in percentage of antioxidant activity (Nascimento et al., 2011). Rutin was used as control and the experiments were performed in triplicate. Thiobarbituric acid reactive substances (TBARS) assay was performed to evaluate the capacity of the oil to inhibit lipid peroxidation (Külkamp et al., 2011), however the oil did not present activity by this method.

5.7. Toxicity assays

5.7.1. Cytotoxicity, genotoxicity and mutagenicity assays

The toxicity of essential oil was evaluated by cell viability and proliferation tests, comet assay and micronucleus rate, in the effective antifungal concentrations (MIC of 250 and 500 µg/mL) (Güez et al., 2012). Hydrogen peroxide solution (H_2O_2, 100 mmol/mL) and a suspension of leukocytes were used as positive and negative control, respectively. All determinations were performed in triplicate.

5.7.2. Histopathological evaluation

Tissue samples (ear skin) from adult male pigs were used to evaluate the formation of tissue damage due to the action of the essential oil. The oil, antifungals and combinations thereof were tested at MIC concentrations. The epidermal side of the skin was exposed to samples, PBS pH 7.0 (negative control) and 0.1 M NaOH solution (positive control) for a 6-hour period. Fragments of these tissues were harvested, fixed in 10% neutral-buffered formalin, processed routinely and stained with hematoxylin and eosin, and examined under light microscopy. The experiments were performed in triplicate.

5.7.3. Hen´s Egg Test on the Chorioallantoic Membrane (HET-CAM)

White fertile eggs fresh Lohmann were kept under optimized incubation conditions (temperature between 38 to 39 °C and humidity between 55 and 60% for 10 days) and they were used in HET-CAM test. On the 10^{th} day the egg shell around the air space was carefully removed. Afterwards, it was added 0.3 ml of each substance in each egg: 0.9% saline solution (negative control), 0.1 M NaOH (positive control), oil, antifungals and combinations thereof. The irritant effect was observed at 30 seconds, 2 minutes and 5 minutes after the application of each substance. The result of the irritation score (IS) was given according to the equation 1, on a scale from 0 to 4.9 which denoted nonirritant (or practically no irritation) and 5.0 to 21 which denoted irritant (moderate/severe or extreme irritation) (Equation 1) (ICCVMA, 2010).

5.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software, by ANOVA method followed by Tukey test, with data expressed as mean ± SD. The results for the toxicity assays were analyzed by ANOVA followed by Brown-Forsythe's test. Differences were considered statistically significant when $p < 0.05$.

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Figures and legends

Fig. 1. Effect of diferent concentrations of exogenous ergosterol (50 - 200 µg/ml) on the minimum inhibitory concentration (MIC) of both amphotericin B (■) and *Schinus lentiscifolius* essential oil (♦) against *Microsporum canis* (MCA01).

Fig. 2. Kinetics of dermatophyte death in the presence of *Schinus lentiscifolius* essential oil. Plots of mean values for $\log_{10}$ CFU/ml versus time for *T. rubrum* (TRU51) (A) and *M. canis* (MCA29) (B) at concentrations of 250 µg/ml (■) and 500 µg/ml (▲) of essential oil (concentrations of MIC and MICx2, respectively); control (♦).

Fig. 3. Scanning electron microscopy of hyphae of *Microsporum canis* exposed to treatment. (A) control, (B) essential oil (125 µg/ml), (C) essential oil (250 µg/ml), (D) terbinafine (0.004 µg/ml), (E) combination of essential oil and terbinafine (31.25 µg/ml + 0.001 µg/ml), (F) ciclopirox (2 µg/ml) and (G) combination of essential oil and ciclopirox (31.25 µg/ml + 0.5 µg/ml). *M. canis* (MCA29). Note alterations in hyphal morphology including hyphal shrivelling and lysis (arrows) in samples treated with oil. Scale bar: 2 µm.

Fig. 4. *In vitro* effect of the *Schinus lentiscifolius* oil and indomethacin (positive control) in the neutrophil migration compared to negative control. *Significant inhibition in relation to all tested concentrations. ^{a,b} $p < 0.05$ indicates significant difference between samples in the same concentrations (ANOVA followed by Tukey's test).

Fig. 5. Radical DPPH scavenging activity of the *Schinus lentiscifolius* essential oil and rutin, via DPPH radical sequestration. ^{a,b} $p < 0.05$ indicates significant difference between samples in the same concentrations (ANOVA followed by Tukey's test).

Fig. 6. Kinetic behaviour of DPPH radical scavenging by rutin (A) and *Schinus lentiscifolius* essential oil (B). Concentrations of (♦) 250 μg/ml, (-) 200 μg/ml, (▲) 150 μg/ml and (■) 100 μg/ml.

Fig. 7. Effect of *Schinus lentiscifolius* essential oil in leukocyte proliferation, cell viability, micronucleus rate and DNA damage. ^{a,b,c} $p < 0.05$ indicates significant difference between the controls and essential oil (ANOVA followed by Brown-Forsythe's test). Positive control (PBS 7.2), negative control (H_2O_2 100 μM). For frequency of micronucleus assay was used bleomicine 3 μg/ml as positive control.

Fig. 8. Histopathological evaluation of porcine epidermal cells treated with *Schinus lentiscifolius* essential oil, terbinafine, ciclopirox and combinations thereof. Magnification times of 100 (1) and 400 (2). Porcine epidermal cells treated with (A) PBS pH 7.0 (negative control), (B) terbinafine (0.0016 μg/ml), (C) essential oil (250 μg/ml), (D) essential oil (500 μg/ml), (E) combination of essential oil and terbinafine (62.5 μg/ml + 0.002 μg/ml), (F) ciclopirox (4 μg/ml), (G) combination of essential oil and ciclopirox (62.5 μg/ml + 0.5 μg/ml).

Fig. 9. Dose-response relationship for antifungal agents and the *Schinus lentiscifolius* essential oil and combinations thereof. Eggs treated with (♦) 0.9% NaCl (negative control), (■) 0.1 NaOH (positive control), (▲) terbinafine (0.016 μg/ml), (X) essential oil (250 μg/ml), (X) essential oil (500 μg/ml), (+) combination of terbinafine and essential oil (62.5 μg/ml + 0.002 μg/ml), (●) ciclopirox (4 μg/ml) and (-) combination of ciclopirox and essential oil (62.5 μg/ml + 0.5 μg/ml). Each point represents an experiment (n = three eggs).

Formulae

Equation 1

$$IS = \left(\left(\frac{(301 - Hemorrhage\ Time)}{300}\right)x5\right) + \left(\left(\frac{(301 - Lysis\ Time)}{300}\right)x7\right) + \left(\left(\frac{(301 - Coagulation\ Time)}{300}\right)x9\right) \quad (1)$$

Table 1 - Chemical composition of essential oil obtained from leaves of *Schinus lentiscifolius* by hydrodistillation.

RT	RI	Compound	%
Monoterpene hydrocarbons			
5.48	926	Tricyclene	2.3
5.61	930	α-pinene	8.2
5.91	941	α-fenchene	0.3
6.00	943	Camphene	1.4
6.80	970	Sabinene	0.3
6.88	973	β-pinene	0.8
7.42	991	Myrcene	1.0
8.81	1028	Limonene	0.2
Sesquiterpene hydrocarbons			
24.05	1366	α-copaene	0.1
24.80	1408	β-caryophyllene	9.9
26.75	1425	Aromadendrene	0.3
27.36	1438	α-humulene	0.7
27.66	1445	Allo-aromadendrene	0.1
28.66	1467	Germacrene D	8.2
28.80	1470	β-selinene	0.3
29.31	1482	Bicyclogermacrene	7.9
29.47	1485	α-muurolene	0.2
29.61	1488	Germacrene A	2.0
29.84	1493	β-bisabolene	0.1
29.99	1497	γ-cadinene	0.2
30.35	1505	δ-cadinene	1.0
Oxigenated sesquiterpenes			
31.64	1540	Elemol	10.5
32.65	1567	Spahtulenol	2.8
32.75	1569	Caryophyllene oxide	0.3
32.88	1573	Globulol	2.4
33.16	1581	*Epi*-globulol	0.5
33.77	1597	5-*epi*-7-*epi*-α-eudesmol	0.4
34.23	1609	10-*epi*-γ-eudesmol	1.0
34.64	1619	Eremoligenol	0.3
34.96	1627	γ-eudesmol	12.8
35.65	1645	β-eudesmol	10.2
35.83	1649	α-eudesmol	9.2
36.91	1677	Eudesma-4(15),7-dien-1-β-ol	0.3
Monoterpene hydrocarbons			14.5
Sesquiterpene hydrocarbons			31.0
Oxigenated sesquiterpenes			50.7
Total of compounds identified			**96.2**

Compounds are listed in order of elution on DB5 column; RT, retention time; RI, retention index; percentage of peak area relative to total peak area.

Table 2 - Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) of *Schinus lentiscifolius* (SL) essential oil in combination with terbinafine (TRB) and ciclopirox (CIC) against dermatophytes.

	MIC (µg/ml)			**MIC (µg/ml) in combination**							
	TRB	**CIC**	**SL**	**TRB**	**SL**	**FICI**	**INT**	**CIC**	**SL**	**FICI**	**INT**
Trichophyton rubrum											
TRU43	0.008	2	125	0.001	31.25	0.375	SYN	0.5	125	1.25	IND
TRU51	0.008	4	250	0.001	62.5	0.375	SYN	1	31.25	0.625	ADD
Trichophyton mentagrophytes											
TME16	0.016	2	250	0.008	62.5	0.75	ADD	0.5	125	0.625	ADD
TME40	0.016	4	250	0.001	250	1.0625	IND	0.5	125	0.625	ADD
Microsporum canis											
MCA29	0.008	4	250	0.001	62.5	0.375	SYN	1	31.25	0.375	SYN
MCA01	0.004	2	125	0.001	62.5	0.75	ADD	1	31.25	0.75	ADD
Microsporum gypseum											
MGY50	0.016	2	125	0.004	31.25	0.5	SYN	1	31.25	0.75	ADD
MGY42	0.016	4	250	0.002	62.5	0.375	SYN	0.5	62.5	0.375	SYN

INT, interpretation; SYN, synergism; ADD, additivity; IND, indifference.

Table 3 - Minimum inhibitory concentration (MIC) of the *Schinus lentiscifolius* essential oil and minimum effective concentration (MEC) of anidulafungin in the presence and absence of osmotic protector against dermatophytes.

	MEC Anidulafungin				**MIC *Schinus lentiscifolius***			
	4 days		**7 days**		**4 days**		**7 days**	
	S (-)	**S (+)**	**S (-)**	**S (+)**	**S (-)**	**S (+)**	**S (-)**	**S (+)**
Trichophyton rubrum								
TRU50	1	2	2	16	1.95	31.25	7.8	62.5
TRU43	2	4	2	32	15.6	15.6	31.25	125
Trichophyton mentagrophytes								
TME16	1	2	1	16	15.62	125	62.5	250
TME43	1	4	2	32	>500	>500	>500	>500
Microsporum canis								
MCA40	1	2	1	16	1.95	31.25	125	250
MCA01	2	4	2	16	62.5	62.5	125	125
Microsporum gypseum								
MGY42	1	4	2	16	31.25	62.5	62.5	250
MGY50	1	2	2	32	31.25	62.5	125	125

S (+), MEC and MIC values with addition to the medium of 0.8 mol/L of sorbitol; S (-), MEC and MIC values without addition of the osmotic protector; all values are expressed in μg/ml.

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Figure 1

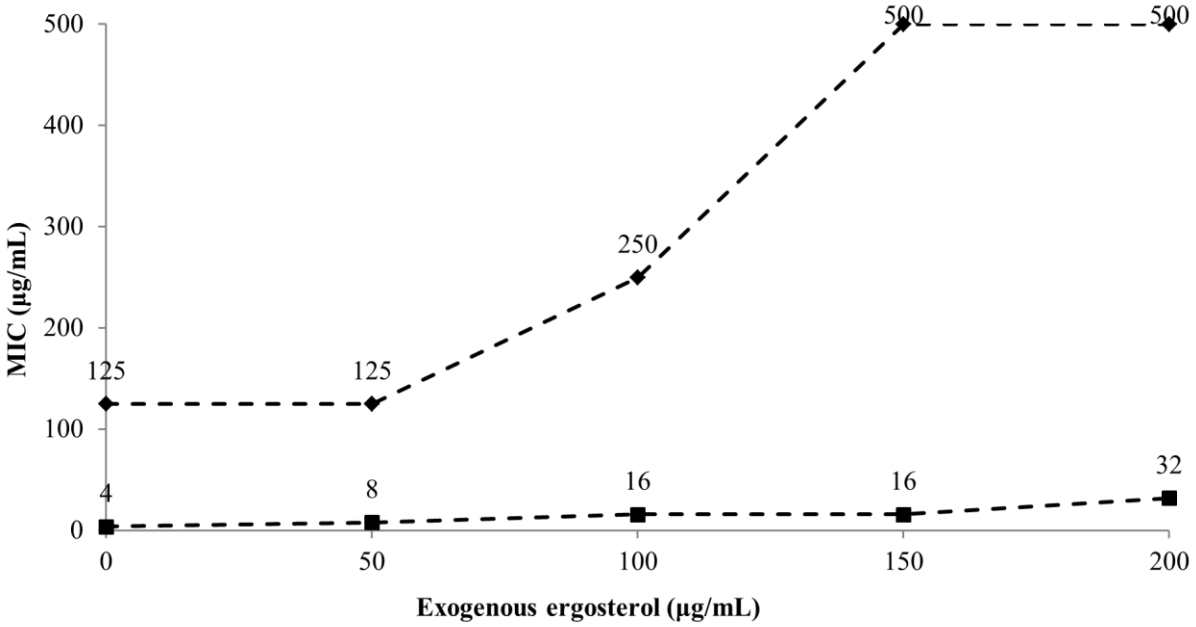


Figure 2

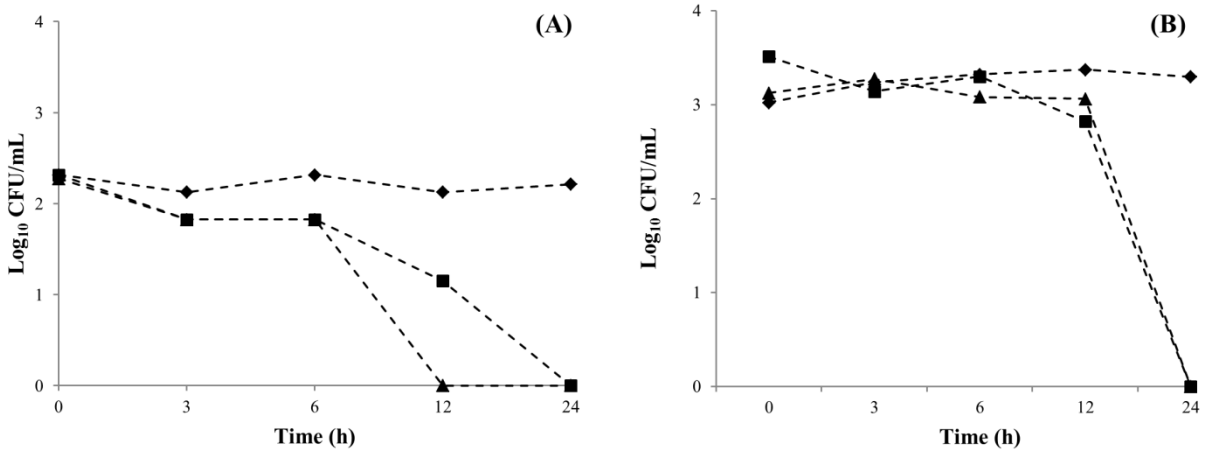


Figure 3

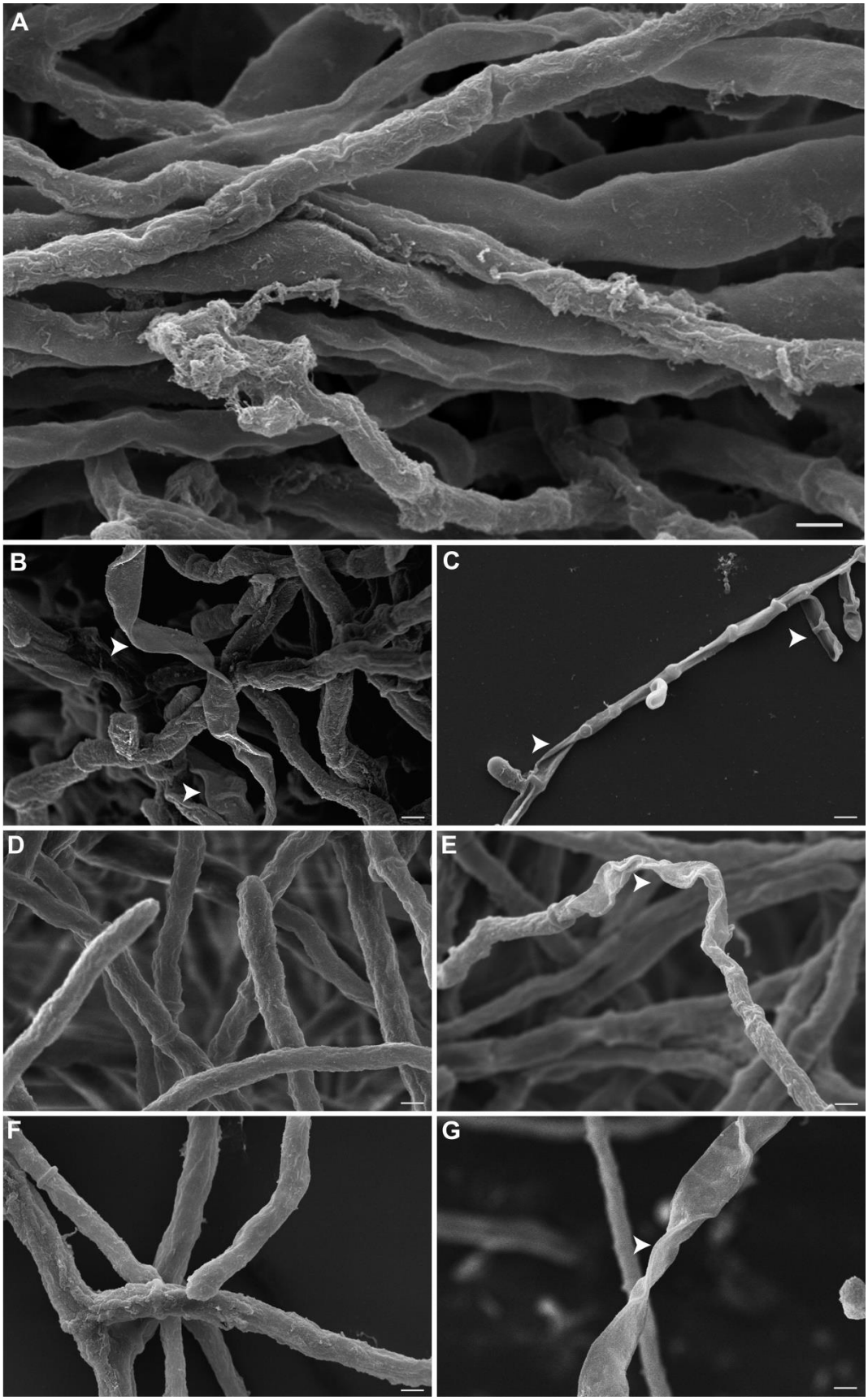


Figure 4

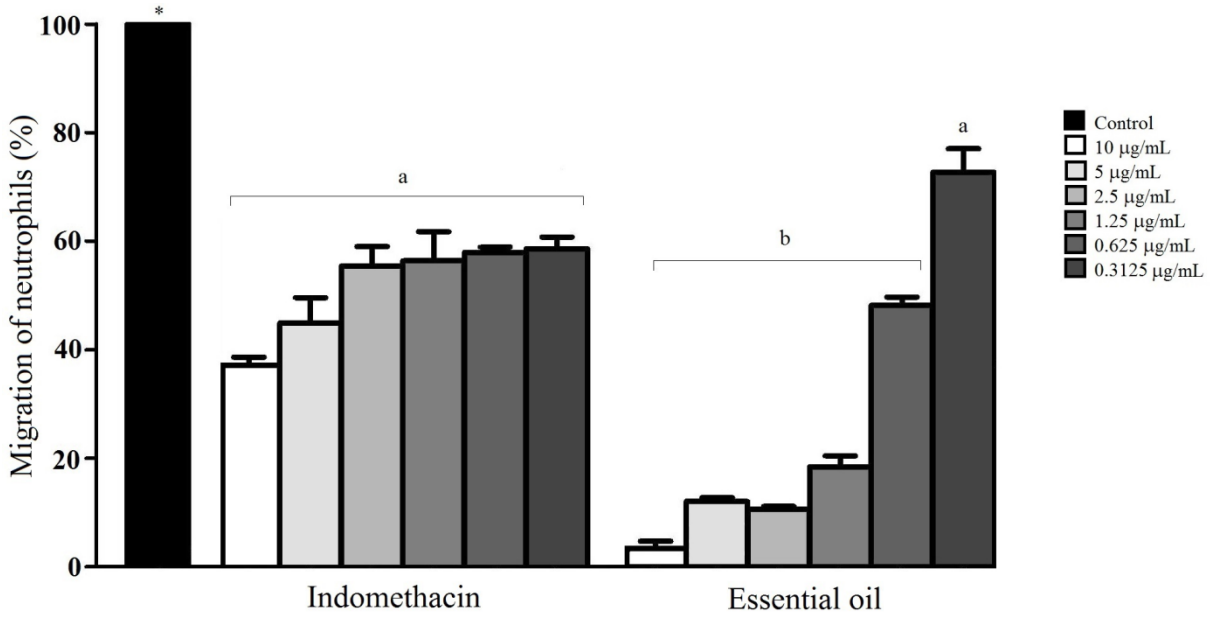


Figure 5

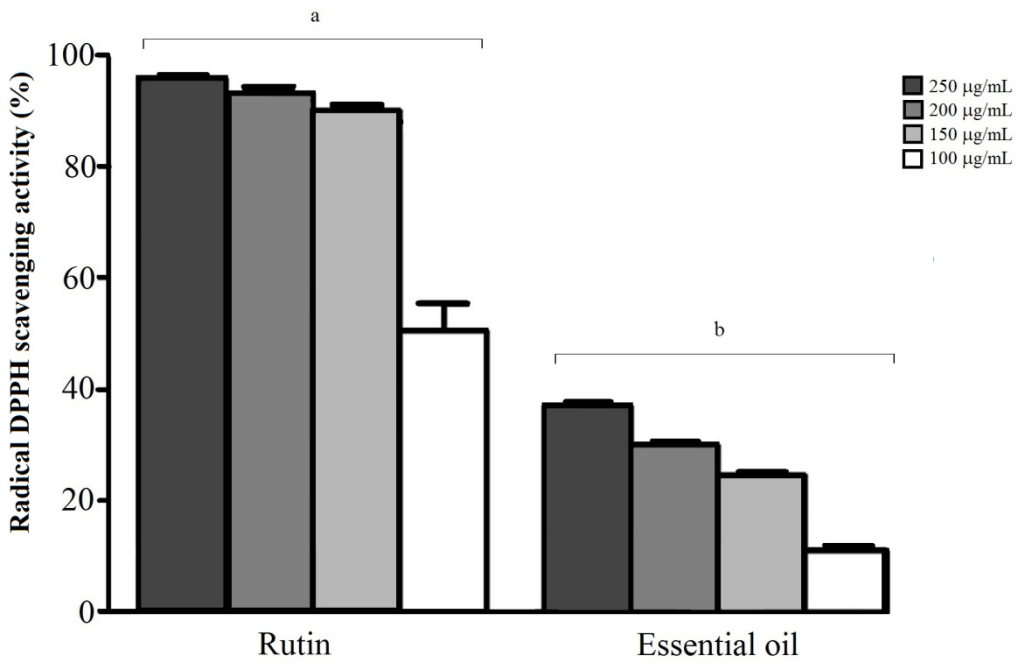


Figure 6

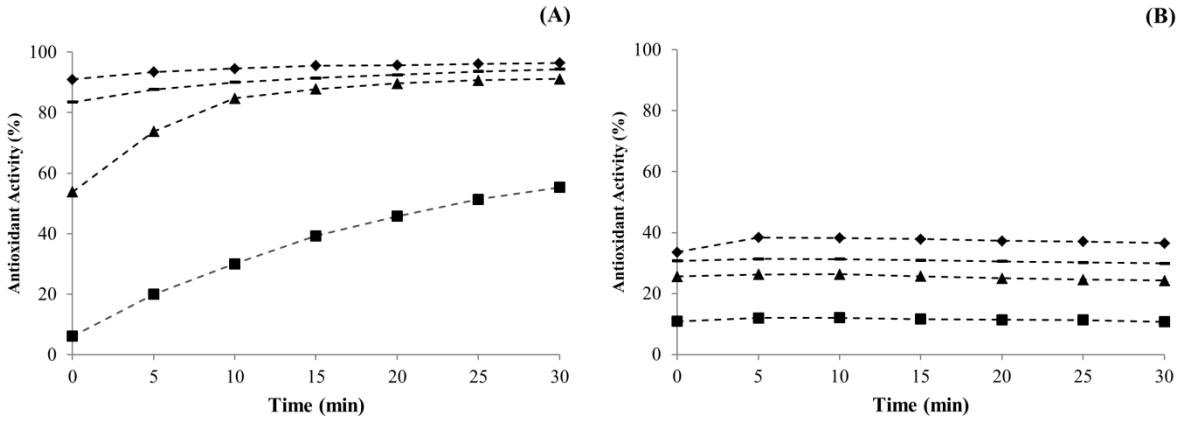


Figure 7

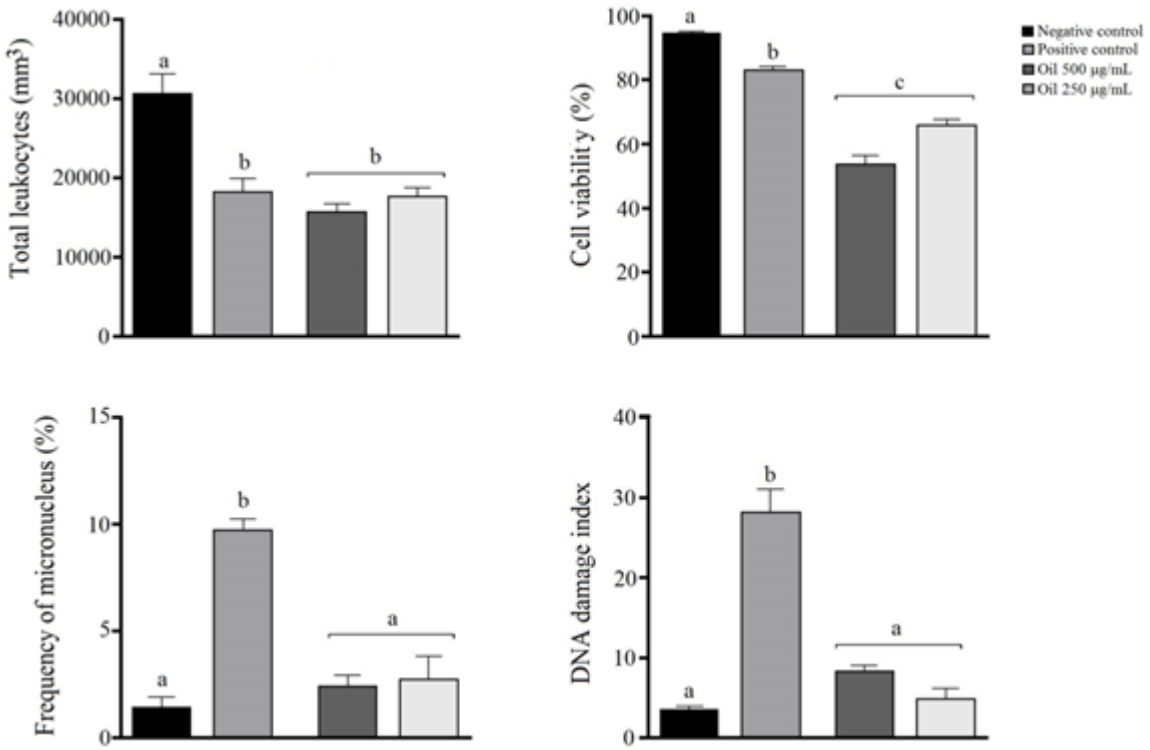


Figure 8

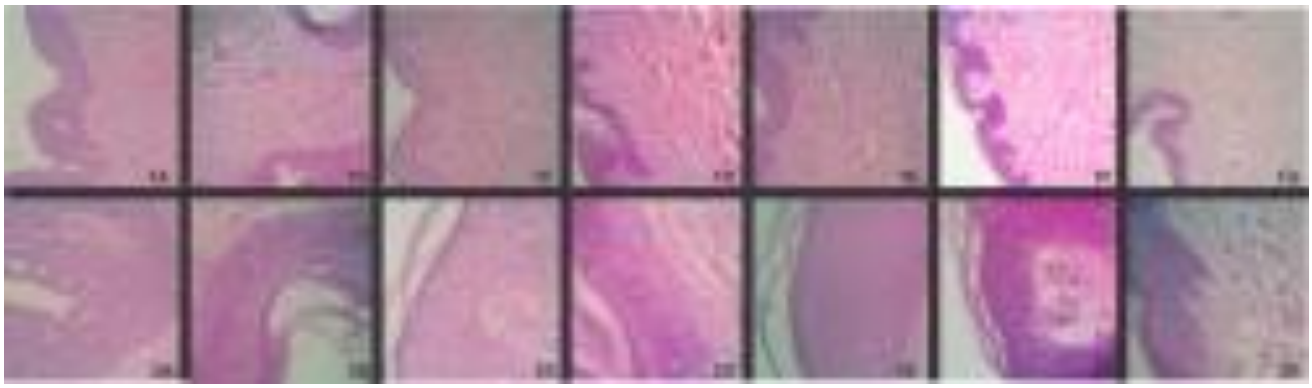
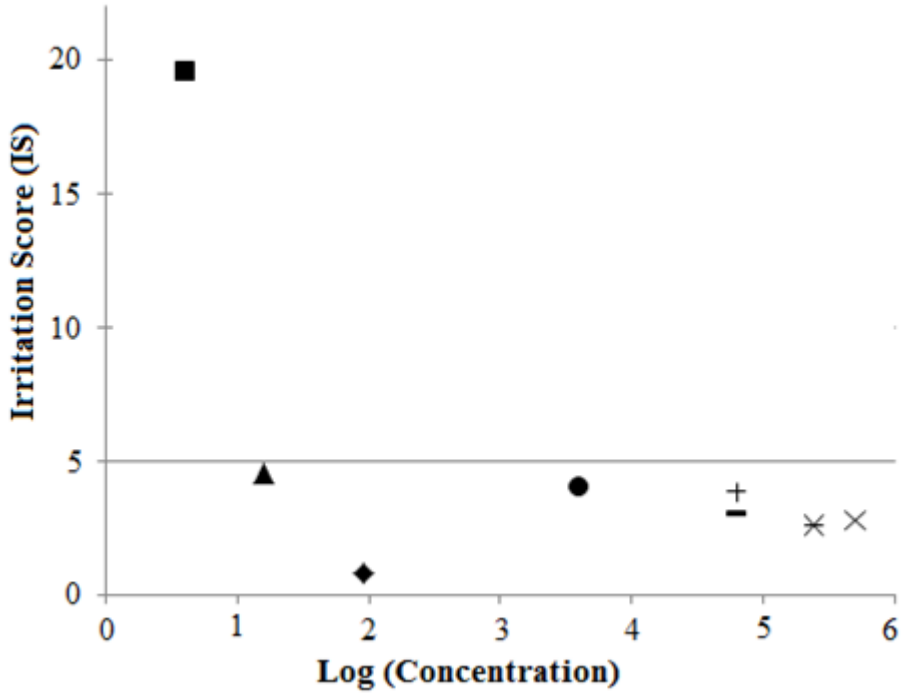


Figure 9



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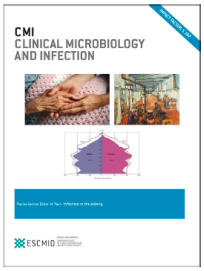

Biofilm formation by *Microsporum canis*

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Biofilm formation by *Microsporum canis*

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Manuscript category: Picture of a Microorganism

Keywords: Biofilm, Dermatophytes, *Microsporum canis*, SEM, Tinea capitis.

Text

Biofilm formation is an important cause of failures in antimicrobial therapy usually associated to the increasing of resistance to antimicrobial agents and to host immune response. The ability to form such structures has been described for several fungal species however the biofilm formation by dermatophytes was reported only for *Trichophyton* spp. [1]. In this study, we described for the first time the biofilm formation by *Microsporum canis*, considered as one of the main etiological agents of tinea capitis and characterized by a poor therapeutic response. Biofilm was formed in glass coverslips after 120 hours of culturing and observed by scanning electron microscopy (SEM) from a clinical sample of *M. canis.*

The images obtained by SEM demonstrate the presence of a highly structured three-dimensional mycelium characteristic of biofilm (Fig. 1 A-B) with expansion in the form of a network of hyphae growing in all directions (Fig. 1 C-D). Moreover, polysaccharide extracellular matrix that links one hyphae to another is observed surrounding some areas of this mycelium structure. The presence of polysaccharide was confirmed by reaction with Periodic Acid-Schiff (PAS) (Fig. 1 E-F). The extracellular matrix presents compact regions, but usually with a porou s and thin consistency, concentrated in the superficial region of the mycelium. Formation of biofilm by dermatophytes can be considered as an important factor of fungal virulence, which may be involved with the persistence of the infection. So, this finding contribute perspectives for effective therapy to eradicate infections related to biofilm formation by dermatophytes.

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Transparency declaration

The authors report no conflicts of interest.

Author's contribution

L.J. Danielli - drafting the manuscript, literature review and laboratory work.

W. Lopes - drafting the manuscript, literature review and laboratory work.

M.H. Vainstein - drafting the manuscript and literature review.

A.M. Fuentefria - drafting the manuscript and literature review.

M.A. Apel - drafting the manuscript and literature review.

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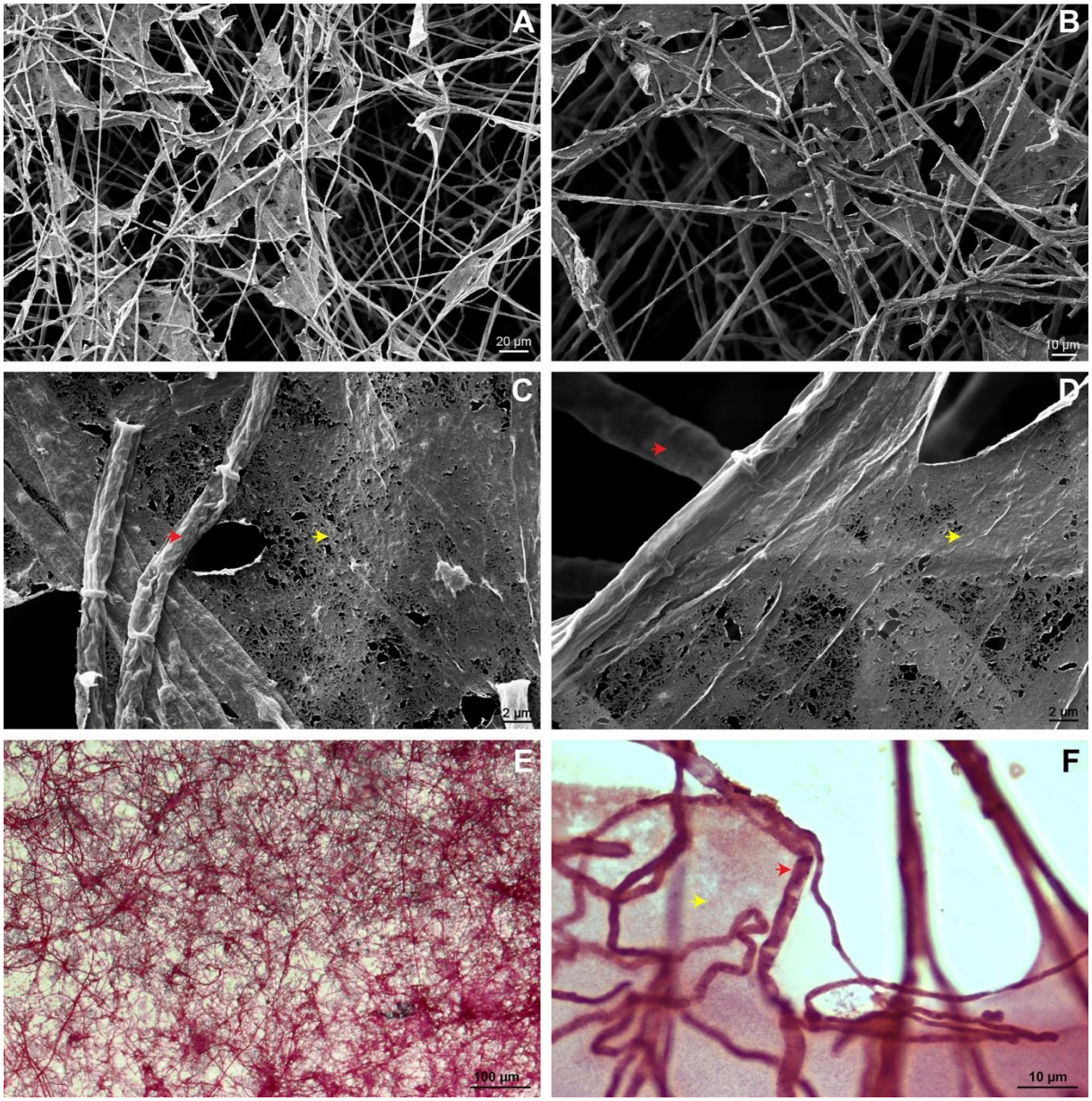

Fig. 1 – SEM of *Microsporum canis* biofilm illustrating biofilm characteristics. (A-B) General view of hyphal-layering networks covered by ECM at the top of the mycelium. (C-D) Higher magnification showing hyphae (red arrows) associated with thin layers of ECM (yellow arrows). (E-F) Presence of polysaccharides in the extracellular matrix confirmed by PAS technique.

Schinus lentiscifolius Marchand essential oil inhibits biofilm formation by *Microsporum canis*

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Abstract

Biofilms are closely related to increased resistance and failures in antifungal treatment. Essential oils and their isolated compounds are considered to be promising to complement conventional therapy. The effect of *Schinus lentiscifolius* oil in the biofilm formation by *Microsporum canis* was investigated. Scanning electron microscopy images demonstrate the presence of hyphae with high structural arrangement in all directions surrounded by extracellular matrix connecting one hyphae to another, characteristic of a mature biofilm. The oil presented reduction of extracellular matrix and complete inhibition of the biofilm formation in the subinhibitory and minimum inhibitory concentrations, respectively. The biofilm formation capacity is considered an important factor of fungal virulence. For *M. canis*, this virulence is related to human cases of tinea corporis and tinea capitis, characterized by an inadequate therapeutic response pointing out the importance of the results obtained as a way to assist in therapy these infections.

Keywords: biofilm; dermatophytes; essential oil; *Microsporum canis*; *Schinus lentiscifolius.*

Introduction

The biofilm formation is an important cause of failures in antimicrobial therapy (Acker et al. 2014) and it is estimated that is related to 95 % of the microorganisms found in nature (Sardi et al. 2014). A biofilm can be defined as a complex community of microorganisms linked to a biotic or abiotic surface surrounded by a matrix of polysaccharides highly resistant to antimicrobials (Donlan and Costerton 2002; Percival et al. 2012). Its formation is a multistage process in which microbial cells adhere to the surface with subsequent production of extracellular matrix responsible for the adhesion (Harding et al. 2009).

In the clinical field, infections related to the formation of biofilm are highly important and often neglected or not adequately detected (Ramage et al. 2014).

According to Sardi et al. (2014), microscopic and molecular techniques have demonstrated biofilms as the natural and preferred form of fungal growth. It is estimated that 80% of all microbial infections are biofilm-related, justifying the large number of treatment failures (Davies 2003). In such cases it is necessary the administration of high doses or combination of therapy in order to improve drug penetration through the extracellular matrix, considering the reports that this structure acts as an antifungal drug sponge (Ramage et al. 2014).

A large part of the studies related to fungal biofilms are associated with *Candida albicans*, although the involvement of other *Candida* spp. as well as other genera of yeasts such as *Cryptococcus* spp. and *Trichosporon* spp. have been reported (Desai et al. 2014; Sardi et al. 2014; Pierce et al. 2015). The topic of filamentous fungi forming biofilm is relatively new (Kvasničková et al. 2016), although *Aspergillus* spp., *Fusarium* spp. and *Trichophyton* spp. have already been described presenting this characteristic (Costa-Orlandi et al. 2014; Peiqian et al. 2014; González-Ramírez et al. 2016). Most of the studies focus in *Aspergillus fumigatus*, considered as a model for the investigation of biofilms in this fungal class (Müller et al. 2011; Rajendran et al. 2013; Kaur and Singh 2014; González-Ramírez et al. 2016; Kvasničková et al. 2016). The capacity of biofilm formation by dermatophytes was reported for *Trichophyton rubrum* and *Trichophyton mentagrophytes* (Costa-Orlandi et al. 2014), and recently for *Microsporum canis* by our research group (Danielli et al. 2017).

Dermatophyte infections are among the most common causes of skin diseases worldwide and their prevalence is underestimated (Hayette and Sacheli 2015). These fungi are highly specialized in invading keratinized tissues, such as hair, skin and nails of humans and animals (Aala et al. 2010). Pathogenic disorders caused by these microorganisms require long and costly treatment, with important cases of recurrence, emergence of resistance and exacerbated adverse effects, considering the similarity of their cellular structure to the host cells (Lewis 2011). Tinea capitis infections of the ecto-endothrix type caused by *M. canis* are related to invasion of the medial part of the hair follicle by the hyphae with subsequent growth out covering the hair surface (Rebollo et al. 2008). Thus, the formation of a biofilm can be one of the main causes related to the difficulty of treating this type of infection.

Besides being considered important antimicrobial agents with broad action spectrum, essential oils and their compounds isolated have shown potential anti-biofilm effect (Cannas et al. 2014; Curvelo et al. 2014; Peixoto et al., 2017). Studies demonstrated such substances acting on the stability of the mature fungal biofilm and on the removal of adherent cells and extracellular polymer matrix, indicating an ability to cause disarrangement in the preformed fungal structures (Curvelo et al. 2014; Almeida et al. 2016).

The genus *Schinus* L. is characterized by several essential oil producing species with related biological activities, such as antioxidant, anticancer, antimicrobial and insecticide (Bendaoud et al. 2010; Cole et al. 2014; Pratti et al. 2015). Among these species, *Schinus lentiscifolius* Marchand was recently reported presenting selective activity against dermatophytes and important synergistic effect in the combination of the essential oil and terbinafine (Unpublished data). Thus, considering essential oils as a potential source of antimicrobial substances and given the importance of identifying new substances with anti-biofilm activity in order to provide effective therapeutic alternatives, the present study determined the effect of *S. lentiscifolius* essential oil in the biofilm formation by *M. canis*, a species of dermatophyte considered as one of the main etiological agents of tinea capitis and characterized by a poor therapeutic response.

Materials and methods

Plant material and obtaining of the essential oil

Leaves of *S. lentiscifolius* were collected from native populations in Southern Brazil. The plant material was identified by the botanist Dr. Sérgio L. Bordignon and the voucher specimen was deposited in the Herbarium of the Federal University of Rio Grande do Sul (ICN-UFRGS: 179855).

The essential oil was obtained from fresh material by hydrodistillation using a Clevenger-type apparatus and the chemical composition was analyzed by gas chromatography-mass spectrometry (CG-MS) (Shimadzu QP5000) (Danielli et al.

2016). The relative amounts of the components were calculated based on GC peak areas.

Microorganism

This study was performed with a strain of *M. canis* (MCA 29) deposited in the Mycology Collection of the Federal University of Rio Grande do Sul, Brazil. In order to obtain viable cells for the assay, the fungus was grown on Sabouraud agar (Kasvi®, Curitiba, BRA) at 32 °C for 5 days.

Biofilm formation assay

The fungal inoculum used in the assay was prepared by washing the cultures with 5 ml of sterile saline 0.85% and counting the conidia on a hemocytometer - final concentration adjusted in 1 x 10^7 conidia ml^{-1}. The assay consisted of the addition of inoculum (1 ml) to 12-well plates containing sterilized coverslips and 1 ml of the essential oil solution in RPMI 1640 (Gibco®, Waltham, USA) at concentrations of 250 and 125 μg ml^{-1}. These values corresponding to the minimum inhibitory concentration (MIC) and the subinhibitory concentration of the oil, respectively, previously determined by broth microdilution method for this strain (Unpublished data). The coverslips were previously functionalized with poly-L-lysine (Sigma-Aldrich®, St Louis, USA) for 30 minutes. The plates were incubated without agitation at 32 °C for 5 days. The experiments were performed in duplicate.

Scanning electron microscopy (SEM)

The biofilms formed in the coverslips were prepared for SEM as described by Joubert et al. (2015), with some modifications. Briefly, the biofilms were fixed in 5% glutaraldehyde in 0.2 M cacodylate for 20 minutes and subsequently they were washed 3X with post fixative solution (0.2 M cacodylate + 0.2 M sucrose and 2 mM magnesium sulfate) (all reagents were purchased from Sigma-Aldrich®, St Louis,

USA). Dehydration was carried out with ethanol (Merck®, Darmstadt, Germany) in concentrations of 30, 50, 70, 95% during 5 minutes and 100% during 10 minutes, with posterior subjected to critical point CO_2/ethanol.

Results and discussion

Biofilms are involved in the resistance and tolerance to treatment, including slow penetration of the antimicrobial agent through the fungal network, adaptive response to stress, changes in microenvironment chemistry and occurrence of tolerant cells (Stewart and Costerton 2001). Infections with presence of biofilm present a great challenge in medicine, considering its important resistance to the antimicrobial agents and to the immune system of the host (Jabra-Rizk et al. 2004; Acker et al. 2014; Sardi et al. 2014). Anti-biofilm property from essential oils and their isolated compounds has been reported against different fungi, including species with reduced sensitivity to azoles (Cannas et al. 2014; Curvelo et al. 2014; Manganyi et al. 2015; Peixoto et al., 2017).

In a previous study, the essential oil of *S. lentiscifolius* showed an important antifungal effect against filamentous fungi of the genus *Trichophyton* and *Microsporum* determined by the broth microdilution method, with MIC ranging from 125 to 250 μg ml^{-1} (Unpublished data). From these results the anti-biofilm activity was investigated at the subinhibitory concentration (125 μg ml^{-1}) and at the minimal inhibitory concentration (MIC) (250 μg ml^{-1}). Figure 1 shows the results obtained by MEV in the biofilm formation by *M. canis* from treatment with the essential oil. The images of the fungus without treatment indicate the presence of mature biofilm containing evident mycelial expansion, including a network of hyphae with high structural arrangement and presence of extracellular matrix bound from one hyphae to another (Figure 1A-B). It also possible to observe compact regions, concentrated in the superficial portion of the mycelium, possibly for the purpose of protecting the fungal structure. In relation to the mycelium, a three-dimensional complex with micellar expansion in the form of a coordinated network of hyphae is observed growing in all directions and intersecting, surrounded in some areas with polysaccharide extracellular

matrix. Mature biofilms formed by filamentous fungi are describe presenting a complex structure composed of layers of compacted hyphae, with hyphal-hyphal adhesion, high structural arrangement and presence of extracellular matrix, responsible for binding the cells and forming the biofilm structural base (González-Ramírez et al. 2016).

Treatment with the essential oil at sub-inhibitory concentration showed an important reduction in biofilm formation (Figure 1C-D). The structure formed presented extracellular matrix bound from one hyphae to another, however with a non-compact, extremely porous and thin aspect. At the minimum inhibitory concentration (250 μg ml-1) the oil totally inhibited biofilm formation. As can be observed in Figure 1E-F, only the presence of the hypha network characteristic of the fungal mycelium occurs. Biological properties of essential oils are closely related to their chemical composition. Antifungal activity is associated with presence of oxygenated substances, as alcohols and phenolic compounds (Burt 2004; Lang and Buchbauer 2012). *S. lentiscifolius* oil presented the fraction of oxygenated sesquiterpenes as predominant (50.7%) and γ-eudesmol (12.8%), elemol (10.5%) and β-eudemol (10.2%), as major compounds, that can justifyed their antifungal action (Unpublished data).

The main hypothesis of the antifungal mechanism of action of essential oils is related to the interaction with the lipid bilayer of the fungal membrane, leading to a change in the integrity and permeability of the cell (Khan and Ahmad, 2011; Viuda-Martos et al. 2011). In the case of *S. lentiscifolius* essential oil the effect seems to occur at cell wall level and fungal membrane, however, other targets may be involved (Data not yet published). The *Cymbopogon citratus* essential oil inhibited the biofilm formation of *C. albicans* and interfered in the cell membrane integrity causing shrinkage of cell surface and lysis of sessile cells (Khan and Ahmad. 2012). *Syzygium aromaticum* and *Thymus vulgaris* oils prevented cell attachment, biofilm development and caused total inhibition of biofilm formation by *Fusarium oxysporum* on contact lenses (Manganyi et al. 2015). According Khan and Ahmad (2012) the hydrophobic and volatile nature of oils assists in their increased uptake through extracellular matrix of biofilms that can enlarge their action on persister and sessile cells resulting a retarded and disorganized development of biofilm structure.

In clinical terms what is known about the formation of biofilms in dermatophytes is allusive to nail infections. The presence of a thick biomass surrounded by an extracellular matrix associated with factors such as solid adherence of the fungus to the nail plate, presence of fungal structures dormant and difficulty in eradicating the infection, suggest biofilm involvement in the pathogenesis of onychomycosis (Nusbaum et al. 2012). The same applies to dermatophytosis that affects the hair. Formation of biofilm can be considered a barrier to treatment, keeping hyphae protected from drug action, facilitating their adhesion to the hair follicle and the continuity of infection. In these cases, the ability of dermatophytes to form biofilms is considered a virulence factor that can be related to persistent infection and refractory to oral antifungal treatment (Harding et al. 2009; Gupta et al. 2016; Martinez-Rossi et al. 2016). The knowledge of fungal virulence factors are essential for the therapeutic determination and effective eradication of the infectious agent avoiding recurrence frames commonly associated with dermatophytoses.

Conclusion

Biofilm formation is closely related to failures in the treatment of infections due to its structural feature that denotes an increase in antimicrobial resistance and host immune response. Although there are many reports available regarding the antimicrobial properties of essential oils, few of these focus on the anti-biofilm activities. To our knowledge, this is the first study about effect of essential oils in biofilm formed by dermatophytes. The inhibition of the biofilm formation by *M. canis* by the *S. lentiscifolius* oil was observed by MEV. This microorganism is considerate one of the main causative agents of tinea capitis, including cases highly inflammatory and low response to conventional therapy which may be related with the presence of biofilm. The results of this study indicate that *S. lentiscifolius* oil as an alternative for the topical treatment of this dermatophyte agent.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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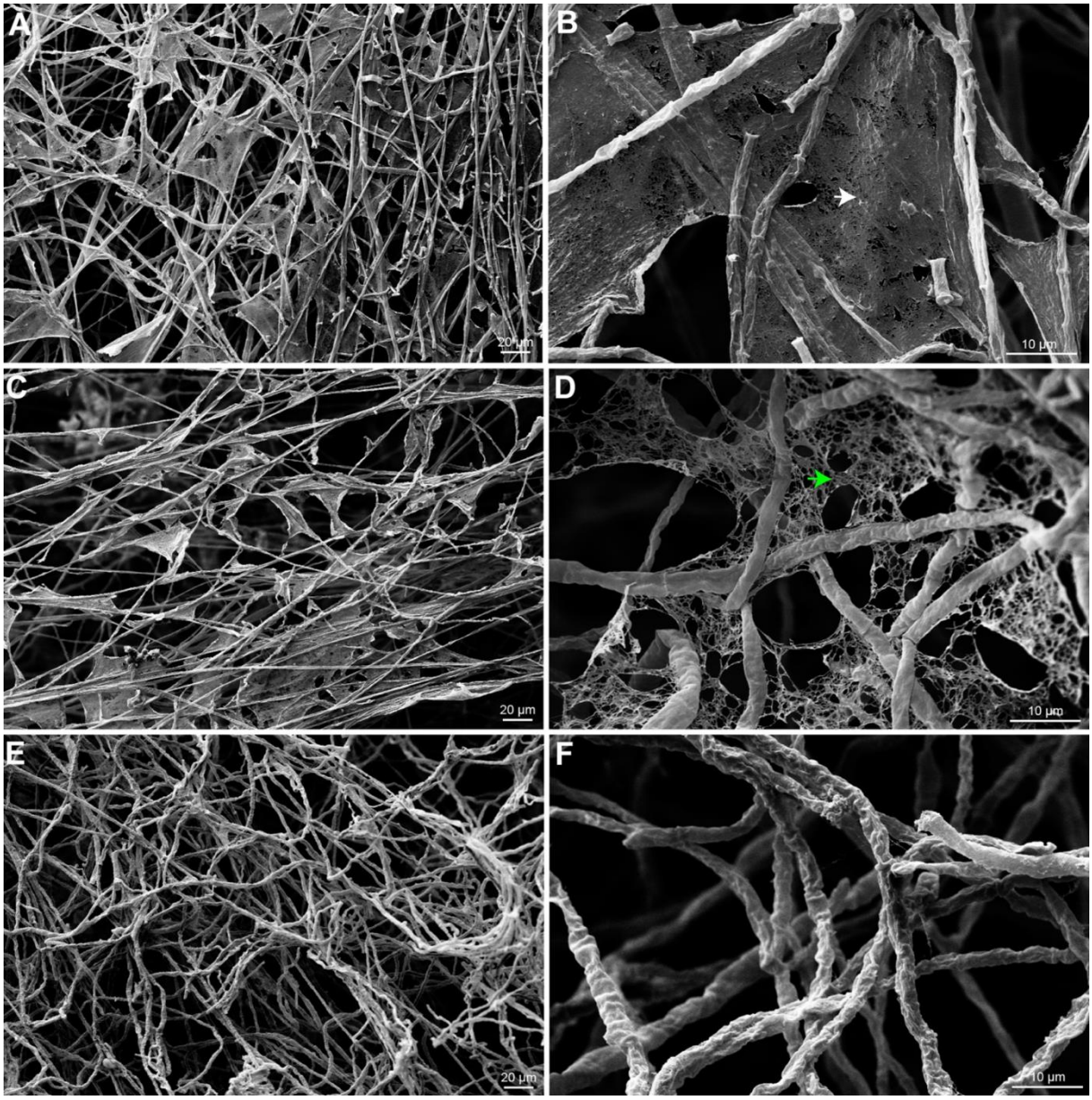

Figure 1. Scanning electron microscopy of *Microsporum canis* biofilm exposed to treatment with *Schinus lentiscifolius* essential oil. (A-B) Control (no treatment). (C-D) Essential oil in the subinhibitory concentration – 125 µg mL^{-1}. (E- F) Essential oil in the minimum inhibitory concentration – 250 µg mL^{-1}. Note the important reduction in the biofilm formation in the sample treated with sub-inhibitory concentration of oil and the absence of this structure when treated with minimum inhibitory concentration.

CAPÍTULO VII – Influência dos monoterpenos nas atividades biológicas do óleo volátil de *Nectandra megapotamica*

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Title: Influence of monoterpenes on antichemotactic, antioxidant and antifungal activities of Nectandra megapotamica (Spreng.) Mez essential oils

Article Type: Research Paper

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Keywords: Biological activities; essential oil; monoterpenes; Nectandra megapotamica; principal component analysis; seasonal variation.

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Abstract: Essential oils are a group of secondary metabolites characterized by important biological activities. However, genetic and environmental factors directly influence the production of these substances. Thus, research relating the influence of seasonal variation, plant development phase, environmental and agronomic conditions in biological activities are important for pharmacological studies and metabolic engineering. Therefore, this study determined the variation of the chemical composition of the essential oil obtained from Nectandra megapotamica (Spreng.) Mez leaves, collected in the productive rest, end of the productive rest, pre-blooming, blooming and fruiting phases, as well as its influence on the radical DPPH scavenging, antichemotactic and antifungal activities. Thirty-eight compounds were identified, accounting for 97.0 to 99.2% of the chemical composition of the oils. Bicyclogermacrene (22.0-36.7%) and germacrene D (10.9 - 19.2%) were the predominant compounds in all collections. In addition to this, limonene (14.1%), β-pinene (15.5%), α-pinene (14.8%) and spathulenol (9.1%) were identified as majorities in the flowering and fruiting phases, respectively. The essential oils significantly inhibited the leukocyte migration in relation to the control at all the concentrations evaluated. In relation to the antioxidant activity, significant differences of effect between the samples were observed with scavenging of DPPH radical rangin from 18.1 to 43.4% at the concentration of 250 µg/mL. Variations were also observed in the antifungal activity, in which the samples referring to the pre-blooming and fruiting phases inhibited the growth of the largest number of isolates (60 and 53.3%, respectively), with MICs ranging from 125 to 500 µg/mL. Principal component analysis revealed clustering of samples due to biological effect profile similarity, furthermore indicate correlation between the oil obtained in the pre-blooming phase and all the biological activities evaluated. These results

Influence of monoterpenes on antichemotactic, antioxidant and antifungal activities of *Nectandra megapotamica* (Spreng.) Mez essential oils

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Abstract

Essential oils are a group of secondary metabolites characterized by important biological activities. However, genetic and environmental factors directly influence the production of these substances. Thus, research relating the influence of seasonal variation, plant development phase, environmental and agronomic conditions in biological activities are important for pharmacological studies and metabolic engineering. Therefore, this study determined the variation of the chemical composition of the essential oil obtained from

Nectandra megapotamica (Spreng.) Mez leaves, collected in the productive rest, end of the productive rest, pre-blooming, blooming and fruiting phases, as well as its influence on the radical DPPH scavenging, antichemotactic and antifungal activities. Thirty-eight compounds were identified, accounting for 97.0 to 99.2% of the chemical composition of the oils. Bicyclogermacrene (22.0-36.7%) and germacrene D (10.9 - 19.2%) were the predominant compounds in all collections. In addition to this, limonene (14.1%), β-pinene (15.5%), α-pinene (14.8%) and spathulenol (9.1%) were identified as majorities in the flowering and fruiting phases, respectively. The essential oils significantly inhibited the leukocyte migration in relation to the control at all the concentrations evaluated. In relation to the antioxidant activity, significant differences of effect between the samples were observed with scavenging of DPPH radical ranging from 18.1 to 43.4% at the concentration of 250 μg/mL. Variations were also observed in the antifungal activity, in which the samples referring to the pre-blooming and fruiting phases inhibited the growth of the largest number of isolates (60 and 53.3%, respectively), with MICs ranging from 125 to 500 μg/mL. Principal component analysis revealed clustering of samples due to biological effect profile similarity, furthermore indicate correlation between the oil obtained in the pre-blooming phase and all the biological activities evaluated. These results demonstrate the influence of monoterpenes, mainly limonene, α-pinene and β-pinene on the bioactivities of *N. megapotamica* essential oil. The sample collected in the productive rest phase presented as promising, mainly in relation to antichemotactic effect and higher yield obtained in the extraction of the essential oil.

Keywords: biological activities; essential oil; monoterpenes; *Nectandra megapotamica*; principal component analysis; seasonal variation.

1 Introduction

Essential oils are an important group of secondary plant metabolites used in large scale in the cosmetics, food and pharmaceutical industries due to their correlated biological effects (Lesage-Meessen et al., 2015; Llana-Ruiz-Cabello et al., 2015; Carvalho et al., 2016; Low et al., 2016; Tiwari, 2016). Among several reported activities, the oils and their isolated compounds stand out for their antimicrobial, antioxidant and anti-inflammatory properties, and recently for the chemosensitizing effect (Amorati et al., 2013; Raut; Karuppayil, 2014; Sá et al., 2014; Perricone et al., 2015; Sá et al., 2015; Uliana et al., 2016).

These secondary metabolites are characterized as complex mixtures of low molecular weight substances, chemically characterized by the predominant presence of terpenoide and phenylpropanoids compounds (Bassolé; Juliani, 2012; Rehman et al., 2016). Due to their repellent or attractive properties, these metabolites are implicated in several ecological and biological functions, and different chemical profiles are observed throughout plant development in response to biotic or abiotic environmental factors (Grulova et al., 2015). The chemical composition of medicinal and aromatic plants as well as their biological effects are influenced by genetic and environmental factors (Furtado et al., 2014; Lemos et al., 2015; Dhouioui et al., 2016). Such variations can be correlated with the plant collection site (Verma et al., 2014; Zhang et al., 2015; Karimi et al., 2016), developmental phase (Amaral et al., 2015a; Karimi et al., 2016), agronomic and environmental conditions (Olmedo et al., 2015; Almeida et al., 2016), in addition to the method of extraction (Danh et al., 2013). The knowledge of the changes in the chemical composition of an essential oil caused by seasonal variations is closely related to the evaluation of its pharmacological properties and also has important applications in the area of metabolic engineering, as in attempts to improve the yield and to facilitate the accessibility of a particular compound (Lange; Ahkami, 2013; Lange; Turner, 2013; Almeida et al., 2016; Karimi et al., 2016; Tiwari, 2016).

Nectandra megapotamica (Spreng.) Mez (Lauraceae) is popularly known in Brazil as "canela-preta", "canela-do-mato" or "canela-imbuia" (Silva-Filho et al., 2004a; Alves et al., 2008; Rego, 2009). Species of this genus have been popularly used as antifungal, antidiarrheal, antirheumatic and analgesic (Romoff et al., 2010). Previous studies of this species have revealed the presence of neolignans, phenylpropanoids and lignans tetrahydrofurans with cytotoxic activity against leukemic cells, as well as pro-oxidant, antifungal, antileishmanial, antimalarial, trypanocidal, analgesic and anti-inflammatory activities (Silva-Filho et al, 2004a; Silva-Filho et al, 2004b; Silva-Filho et al., 2008; Garcez et al., 2009; Ponci et al., 2015), confirming the properties indicated by traditional medicine. Its essential oils were described as exerting anti-inflammatory and antitumoral activities, anesthetic effect in *Centropomus parallelus* and neutralizing the effect induced by the venom of *Bothrops diporus* (Apel et al., 2006; Tondolo et al., 2013; Torres et al., 2014).

In addition, multivariate analysis tools, such as exploratory analyzes using HCA (Zerzucha, Walczak, 2012) and PCA (Bro; Smilde, 2014), have allowed to elucidate which chemical structures or classes of compounds are representative in the differentiation of samples based on the present metabolites (Grasel et al. 2016).

In this context, considering that the knowledge of the influence of seasonal variation on the chemical composition of volatile oils is essential for the optimization of the production of biologically active substances, this study evaluated the antichemotactic, antioxidant and antifungal effect of the essential oil of *N. megapotamica* obtained in different stages of development of the plant. An attempt to correlate the chemical composition with biological activities by multivariate analysis was also performed.

2 Materials and methods

2.1 Plant material

Leaves of *N. megapotamica* were collected from native populations in Southern Brazil during the following phases: productive rest (March), end of productive rest (April), pre-blooming (August), blooming (August) and fruiting (December). The plant material was identified by a botanist Dr. Sérgio L. Bordignon, and an exsicata was deposited in the Herbarium of the Federal University of Rio Grande do Sul (ICN-UFRGS - 192542).

2.2 Essential oil isolation

The essential oils were obtained from fresh leaves by hydrodistillation for 4 hours using a Clevenger-type apparatus (Farmacopeia Brasileira, 2010). After distillation, the oils were dried over anhydrous sodium sulfate and stored at 4 °C. The yield determination was performed as weight/volume (w/v).

2.3 Gas chromatography-mass spectrometry (GC-MS) analysis

The qualitative and quantitative analyzes were performed by gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP5000), using a capillary column of fused silica Durabond-DB-5 (John Wiley & Sons Scientific, 30 m x 0.25 mm and coated with a 0.25 μm thick film of polydimethyldiphenylsiloxane containing 5% of phenyl groups) for separation of the constituents. The injector and detector temperatures were set at 200 °C and 250 °C, respectively, and the column temperature was programmed with a heating ramp of 60 °C to 300 °C with variation of 3 °C/min, with helium as carrier gas at 80 kPa and flow rate of 1 mL/min. For the chromatographic analysis, the oils were diluted 2% in ethyl ether (v/v).

Identification of compounds was based on the comparison of their retention indices (calculated by linear interpolation relative to retention time of a series of n-alkenes), and mass spectra with those of authentic samples and with data taken from the literature (Adams, 2009), or by comparison with mass spectra recorded in the database as NIST 62 and NIST 12 (National Institute of Technology and Standards). Relative amounts of the components were calculated based on GC peak areas.

2.4 Antifungal activity

The antifungal activity of essential oils was determined against strains deposited in the Applied Mycology Laboratory of the Faculty of Pharmacy of the Federal University of Rio Grande do Sul: *Trichophyton rubrum* (TRU43, TRU51, TRU50, TRU48), *T. mentagrophytes* (TME16, TME40, TME32, TME46), *Microsporum canis* (MCA29, MCA01, MCA33, MCA40) and *M. gypseum* (MGY50, MGY42, MGY58, MGY42). To obtain viable cells used in the assay, the filamentous fungi were incubated on potato dextrose agar at 32 °C for 5 days. The minimum inhibitory concentration (MIC) was determined from the broth microdilution method according to the M38-A2 protocol standardized by the Clinical Laboratory Standard Institute (CLSI, 2008). The experiments were conducted in RPMI-MOPS culture medium and samples of oils were tested in the concentration range from 1.95 to 500 μg/mL. MIC was defined as the lowest concentration of the substance in which the microorganism tested did not show visible growth. The experiments were performed in triplicate using terbinafine as positive control.

2.5 Radical DPPH scavenging activity

The sequestering capacity of free radicals by the oils was determined by the reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) in concentrations of 25 to 250 μg/mL. Basically,

the samples at their respective concentrations were added to the 0.004% DPPH methanolic solution and the absorbance was read in spectrophotometer (517 nm) in 30 minutes of reaction. Rutin was used as positive control and all experiments performed in triplicate. The percentage of activity was determined by formula $\%AA = (A_{DPPH} - A_{sample})/A_{DPPH} \times 100$ (Nascimento et al., 2011).

2.6 Antichemotactic assay

The evaluation of the antichemotactic activity was performed according to modified Boyden chamber method described by Suyenaga et al. (2011). Prior to the assay, leukocytes were treated with essential oils and positive control solubilized in Hank's balanced salt solution (HBSS pH 7.4) at concentrations of 0.5 and 5 μg/mL at 37 °C for 30 min. As negative control a solution of neutrophils without addition of antichemotactic agent was employed. The treated leukocytes were added to the upper wells of the chamber, separated by a nitrocellulose filter (8.0 μm) of the chemotactic factor (lipopolysaccharide from *Escherichia coli* - LPS) present in the lower compartment. The leukocyte migration was determined by the distance measured in micrometers between the upper plane of the filter and the lower plane containing two cells in ten microscopic fields. Indomethacin was used as positive control.

2.7 Multivariate analysis

The percentage areas of the peaks obtained in the chromatographic analysis were tabulated and missing data were replaced by 0.001. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) per performed using Chemostat® software (Helfer et al., 2015). Data sets referring to chemical analysis and radical DPPH scavenging activity were preprocessed through autoscaling, whereas for the antifungal activity were used centering data.

159 *2.8 Statistical analysis*

160 Statistical analysis was performed using GraphPad Prism 5.0 software, by ANOVA
161 method followed by Tukey test, with data expressed as mean ± SD. Differences were
162 considered statistically significant when $p < 0.05$.

163 **3 Results and discussion**

164 *3.1 Chemical composition and yield of essential oils*

165 Samples of *N. megapotamica* collected at different developmental stages were
166 investigated in relation to the yield and chemical composition of the essential oil.
167 Informations relative to climate during the collection period as well as yield of oil are
168 described in Table 1. The yield of the essential oil obtained from fresh leaves collected from
169 the same tree ranged from 0.3 to 0.5%. The highest yield was observed during the austral
170 summer (0.5%), corresponding to productive rest phase of the plant, and characterized by
171 high temperatures and total precipitation of 150 - 200 mm. The opposite was observed in the
172 pre-blooming stage, which presented lower yield (0.3%) and collection period with lower
173 maximum and minimum temperatures, in addition to a higher volume of total precipitation
174 (250 - 300 mm). The oil yield observed in the sample collect during the austral winter is
175 similar the one reported in the study by Amaral et al. (2015a), where the oil of *N.*
176 *megapotamica* obtained from young leaves was reported to have average yields of 0.35, 0.59
177 and 0.28%, respectively for the the winter, the spring and the autumn seasons, while aged
178 leaves showed averages of 0.28, 0.30 and 0.21% in the same seasons.

179 The impact of environmental factors on the yield of essential oils was also described for
180 species of *Tetradenia riparia* (Gazim et al., 2010), *Porcelia macrocarpa* (Silva et al., 2013),
181 *Mentha* x *piperita* (Grulova et al., 2015), *Rosmarinus officinalis* (Lemos et al., 2015) and

Copaifera langsdorffii (Almeida et al., 2016). Differences were related to climatic and geographic influences, such as temperature, ultraviolet radiation, atmospheric pollution, altitude and water and nutrient availability, as well as development all stages of the plant and genetic factors (Lemos et al., 2015; Dhouioui et al., 2016).

The comparison of the results of chemical analysis shows considerable quantitative variations between collections. A total of 38 compounds were identified by CG-MS, representing 97.0 to 99.2% of the total composition of the oils (Table 2). The sesquiterpene hydrocarbon fraction was predominant in all samples (55.3 - 79.4%), followed by monoterpene hydrocarbons (13.8 - 37.1%) and oxigenated sesquiterpenes (5.3 - 15.2 %). Bicyclogermacrene (22.0 - 36.7%) and germacrene D (10.9 - 19.2%) were identified as major compounds for all collections. Although predominant in all phases, bicyclogermacrene showed higher relative abundance during the productive rest (32.1 and 36.7%) and pre-blooming (33.4%) stages. Similar variations were observed in germacrene D, with 18.7, 19.2 and 16.8%, respectively for the mentioned phases. Fig. 1 shows a comparison between the major compounds of the essential oils from leaves of *N. megapotamica* in different collections.

The main differences were observed in the monoterpenic fraction. This fraction gradually increased from productive rest to blooming phase (5.1 – 37.1%, respectively), with a slight reduction in the fruiting phase (20.7%). This variation is predominantly related to the quantities of α- and β-pinene and limonene in different samples. The productive rest phase was characterized by ausence of α-pinene, with the identification of β-pinene only as a minor compound (0.2%) and limonene (4.6%) being the main compound in this fraction. At the end of this phase α- and β- pinene were observed as major compounds (4.3 and 6.6%, repectively) and the abundance of limonene have been reduced (2.7%). In the pre-blooming period limonene was predominant (14.1%), followed by β-pinene (4.1%) and α-pinene (2.7%). The

opposite was observed in the blooming phase, with the presence of both pinenes as main compounds (14.8 and 15.5%, respectively for α- and β-pinene) and limonene with only 5.3% of relative abundance. Similarly to the productive rest and pre-blooming phases, in the fruiting phase the percentage of pinenes was reduced (5.0 and 6.4%), while limonene was slightly predominant (8.6%).

Concerning the hydrocarbon sesquiterpene fraction, bicyclogermacrene and germacrene D were predominant in all collections, with varying amounts in each one (22.0 to 36.7% and 10.9 to 19.2%, respectively). Besides, β-caryophyllene (3.9 - 6.4%), δ-cadinene (3.9 - 6.8%), α-copaene (2.8 - 4.5%) and α-humulene (2.7 - 3.9%) were present as main compounds with smaller variation between the collections. Among the oxygenated sesquiterpenes identified in the different samples, spathulenol was the main compound present in all collections (1.9 - 3.3%) and the major in the fruiting phase, with relative abundance of 9.1%. This may be related to the fact that the oil obtained from this phase presented the lowest percentage of germacrene D (10.9%) and also a reduced amount of bicyclogermacrene (22.8%) in relation to the other collections. Compounds of the phenylpropanoid class (elemycin and *(E)*-isoelemicin) were observed as minor compounds, except for the oil from the productive rest phase which presented 9.4% of *(E)*-isoelemicin.

A qualitative similar chemical composition was observed in the essential oil from aged leaves of *N. megapotamica* collected in southern Brazil, however, with high importance. In this study, Amaral et al. (2015b) reported predominance of the hydrocarbon monoterpene fraction in all seasons, with α-pinene and β-pinene identified as major compounds for all collections, especially in the austral spring and summer. Limonene exhibited variations during the spring (2.4 - 10%) and it was a minor compound in the winter (3.1 - 4.6%). For samples of this species collected in the summer and the winter, in southeastern Brazil, δ-elemene (8.2 - 22.6%) and α-bisabolol (62.3 - 69.4%) were reported as main compounds in all collections.

Besides that, α-pinene, β-pinene, limonene, germacrene D, bicyclogermacrene and spathulenol were identified among the minor constituents, ranging from 0.1 to 4.2% (Romoff et al., 2010). Gobbo-Neto and Lopes (2007) consider the collection period as one of the most important factors affecting the nature and quantity of the constituents, which are variable during the year. In addition, the plant developmental phase can considerably influence the relative proportions of the components.

As observed in our study, Verma et al. (2014) verified considerable quantitative variations, related to the collection season, between the monoterpenes identified in the essential oil of *Agle marmelos*, including limonene, (*E*)-β-ocimene and α-pinene. The effect of several environmental factors stimulating its biosynthesis was considered responsible for the changes observed in the monoterpene fraction of *Clinopodium macrostemum* var. *laevigatum* essential oil (Villa-Ruano et al., 2015). The main oxygenated and non-oxygenated sesquiterpenes identified in *Copaifera langsdorffii* oil showed variable abundances in relation to light intensity and precipitation (Almeida et al., 2016). Grulova et al. (2015) reported increase in limonene content for *Mentha* x *piperita* oil, related to low temperatures.

The chemical data of essential oils obtained at different stages of plant development has submitted multivariate techniques, HCA and PCA, to investigate possible groupings between compounds and between samples due to similarity in the chemical profile. This evaluation considers all the constituents identified in the different samples. The dendrogram produced by HCA (Figure 2) indicated different chemical profiles among the collected samples, influenced by minor compounds. It is possible to observe the formation of a cluster containing the samples of the productive rest and pre-blooming phases associated with the final productive rest phase. This grouping (productive rest and pre-blooming phases) is related to a greater similarity of the quantitative and qualitative composition of the sesquiterpene fraction among the samples. The same occurs in the final productive rest phase. The blooming and fruiting

samples are distinguished from the others by the quantitative differences in the fractions of monoterpene hydrocarbons and oxygenated sesquiterpenes, respectively.

The results obtained through the HCA were confirmed by PCA. In this analysis the formation of four main components explained 99.99% of the total variation. Figure 3 shows a biplot graph (scores and loadings) of the first two components (PC1 and PC2), which together account for 62.63% of the variance. This graph distinguished two groups of plant development phases characterized by PC1, which corresponds to 32.45% of total variance and is positively correlated with the compounds *(E)*-nerolidol, α-humulene (15), β-caryophyllene (12), globulol (29), aromadendrene (14), sabinene (3), α-copaene (8), β-elemene (11), β-cubebene (10), *epi*-globulol (30), α-muurolone (20), δ-muurolene (17), humulene epoxide II (31), spathulenol (27), elemicin (37), β-gurjunene (13), germacrene B (25), germacrene A (21), caryophyllene oxide (28) and β-pinene (4). This group corresponds to the oils obtained in the fruiting and final productive rest phases. The second group observed for the left of the PCA chart presents negative scores on the PC1 scale to the compounds *(E)*-isoelemicin (38), δ-cadinene (23), bicyclogermacrene (19), τ-cadinol (35), 1-*epi*-globulol (32), cadina-1,4-diene (24), germacrene D (18), (*E*)-β-ocimene (7), limonene (6), α-muurolol (34), γ-cadinene (22), myrcene (5), camphene (2), *iso*-spathulenol (33), cubenol (36), β-bourbonene (9), *allo*-aromadendrene (16) and α-pinene (1), and corresponds to the oils obtained in the productive rest, pre-blooming and blooming phases.

Morever, from the Figure 3 a strong correlation was observed between the compounds globulol (29), aromadendrene (14), sabinene (3) and α-copaene (8) with sample collected in the final productive rest phase, while β-elemene (11), β-cubebene (10), humulene epoxide II (31), spathulenol (27) and elemicin (37) were correlationed with the fruitifing phase. In the productive rest phase this correlation can be observed to cadina-1,4-diene (24), bicyclogermacrene (19), *τ*-cadinol (35) and 1-epi-cubenol (32), mainly. And finally, the

282 blooming phase is correlated to the compounds camphene (2), *iso*-spathulenol (33), cubenol
283 (36) and β-bourbonene (9).

284 The Figure 4 shows the HCA results of the 38 compounds identified in the different
285 essential oil samples. The obtained dendrogram indicates the formation of two large clusters.
286 The first group consists only of sesquiterpenes germacrene D (18) and bicyclogermacrene
287 (19), considered as major compounds in all collections (together they represent 32 - 50% of
288 the chemical composition of the samples). In the second group, four subgroups were formed:
289 I) β-pinene (4), α-pinene (1) and limonene (6) compounds: both major compounds of the
290 monoterpene class; II) compounds δ-cadinene (23), β-caryophyllene (12), γ-humulene (15), α-
291 copaene (8), (*E*)-isoelemicin (38) and spathulenol (27): all compounds were considered major,
292 with percentages above 2% in all collections, except for (*E*)-isoelemicin (38), which was not
293 identified in all samples. Also in this group, the separation of compounds by class is observed,
294 where compounds 23, 12, 15 and 8 are sesquiterpene hidrocarbons, while the compound 27
295 belongs to the class of oxygenated sesquiterpenes and 38 is a phenylpropanoid; III) globulol
296 (29) and (*E*)-nerolidol (26): oxygenated sesquiterpenes compounds with relative abundance of
297 up to 1.8 and 3.5%, respectively, in some collections; IV) compounds considered minority,
298 regardless of chemical class.

299 *3.2 Antifungal activity*

300 In relation to the antifungal activity, the essential oils were evaluated at concentrations
301 of 1.95 to 500 μg/mL against strains of *Trichophyton* and *Microsporum* genus. Values of MIC
302 in the range of 125 to 500 μg/mL were observed for samples of the pre-blooming, blooming
303 and fruiting phases (Table 3). HCA and PCA were performed in an attempt to correlate this
304 biological activity (MIC values) to the chemical composition of the samples. The dendrogram
305 obtained (Figure 5A) indicated the formation of two clusters: I) oils from productive rest and

306 final productive rest phase; II) oils from pre-blooming, blooming and fruiting phases, with the
307 presence of a subgroup formed by the last two samples. PCA (Figure 5B) shows 91.29% of
308 the total variation in the first two axes (PC1 and PC2) and confirms the groups formed by
309 HCA, presenting the pre-blooming phase with a distinct profile in relation to other samples.
310 The oil from the productive rest and final productive rest phases are related to MIC values
311 greater than 500 μg/mL for all strains tested, except for TRU48 that presented a MIC of 500
312 μg/mL.

313 To second group observed in the PCA, pre-blooming, blooming and fruiting samples,
314 exhibited antifungal effect for a greater number of microorganisms with MICs from 125 to
315 500 μg/mL. The oil of pre-blooming phase, containing limonene among the major
316 compounds, showed inhibition of 60% of the strains tested, with MICs ranging from 250 to
317 500 μg/mL. A similar result was observed for the oil of fruiting phase, inhibiting 53.3% of the
318 strains and exhibiting MIC of 125 μg/mL. Besides limonene, the oil obtained at this phase
319 contained spathulenol, an oxygenated sesquiterpene, in higher amounts. The lower content of
320 limonene and higher contents of α- and β-pinene in the sample collected during the blooming
321 phase correlated with a reduced antifungal effect, inhibiting only 33.3% of the strains. The
322 presence of the monoterpene limonene in higher amount in the oil obtained from the pre-
323 blooming phase indicated a direct relation of this compound to the inhibition of the growth of
324 dermatophytes, considering that the sample of the end of the productive rest phase contained
325 only 13.8% of monoterpenoid compounds and inhibited the growth of only one isolate.
326 Absence of antifungal effect was also observed for the oil from productive rest phase,
327 containing β-pinene and limonene as minor compounds.

328 In the present study, differences observed in antimicrobial activity among the evaluated
329 oils in relation to the seasonal variation can be attributed to the differences in the chemical
330 composition (Furtado et al., 2014; Lemos et al., 2015; Dhouioui et al., 2016). In general, this

activity is attributed to higher content of oxygen-containing compounds, such as oxygenated sequiterpenes (Burt, 2004; Lang, Buchbauer, 2012). This hypothesis is corroborated by the reduced MIC values (125 μg/mL) observed for the oil of fruiting phase containing spathulenol (9.1%) among the major compounds. However, Dhouioui et al. (2016) observed that different samples of *Aristolochia longa* L. ssp. *paucinervis* oil, containing smaller fractions of oxygenated compounds, were more effective against the tested microorganisms, in the same way as α-pinene and limonene presented higher antifungal activity than *Wedelia prostate* total oil with MICs in the range of 62.5 to 125 μg mL (Dai et al., 2013). These findings may justify the improvement in the oil activity from blooming and pre-blooming phases in relation to the productive rest phases, since former contains α- and β-pinene and limonene as major compounds.

3.3 Radical DPPH scavenging activity

The samples ability to scavenge free radicals measured by the DPPH method is demonstrated in Table 4. The essential oils obtained from different collections showed a DPPH radical scavenging ability significantly lower in relation to rutin at all the concentrations tested, except for 25 μg/mL ($p < 0.05$). The oil obtained in the pre-blooming phase showed a superior effect in relation to the other samples at concentrations of 150 to 250 μg/mL ($p < 0.05$), with scavenging rates varying from 27.6 to 43.4%. Except in the concentration of 250 μg/mL, in which the final productive rest, blooming and productive rest phases showed antioxidant activities of 22.3, 26.3 and 35.9%, respectively, all the other concentrations tested showed effect around or lower than 20%.

Figure 6 presents HCA and PCA results for the different essential oil samples in relation to the DPPH radical sequestration effect. Similar to the antifungal activity, the dendrogram (Figure 6A) indicates the formation of two main clusters, confirmed by PCA. PC1 and PC2

355 explained 93.52% of the total variance (Figure 6B), grouping the obtained oils in the
356 productive rest and blooming phases, which are related to the oil of the pre-blooming phase.
357 This is due to the fact that both samples presented antioxidant activity, especially the oil of the
358 pre-blooming phase, with higher percentages of DPPH radical scavenging effect. The fruiting
359 and final productive rest phases formed a second group, exerting lower no activity at the
360 lowest concentrations (25, 50 and 100 μg/mL).

361 Limonene was identified as one of the major compounds in the oil obtained in the pre-
362 blooming phase, which demonstrated stronger free radical scavenging activity. The presence
363 of this compound in significant amounts may have contributed to its action, since the
364 antioxidant activity measured by this same method, has been previously correlated to a
365 fraction of hydrocarbon monoterpenes in *Citrus x limon* oil which had limonene as the main
366 compound (Loizzo et al., 2016). There are also reports of antioxidant activity comparable to
367 BHT for this compound (Yang et al., 2010). In the study of Dai et al. (2013), limonene was
368 more active than α-pinene, although moderate antioxidant effect was reported for both
369 compounds. The compounds α-pinene and β-pinene tested alone in DPPH assay did not
370 present free radical scavenging ability (Tepe et al., 2005; Emami et al., 2011), indicating that
371 the antioxidant effect observed in this study for the samples of productive rest and blooming
372 phases can be attributed to a synergistic interaction between different compounds present in
373 the oil.

374 Essential oils can exert antioxidant activity by several mechanisms besides free radical
375 scavenging, including inhibition of lipid peroxidation, hydrogen donation and metal chelation
376 (Miguel, 2010; Amorati et al., 2013). The antioxidant effect evaluated in this study occurs
377 when a substance has hydrogen donation or electron transference capacity, thus being able to
378 stabilize the radical DPPH (Sarikurkcu et al., 2009; Shao et al., 2014). For essential oils, this
379 effect is attributed to the presence of phenols, alcohols and other oxygenated compounds, due

to their high hydrogen donation capacity (Ruberto; Barata, 2000; Luís et al., 2016) and may have contributed to the antioxidant effect of the productive rest phase oil, wich contais *(E)*-isoelemicin as the major compound (9,4%). Notwithstanding the foregoing, it is also common to attribute this activity to the synergistic interaction between major and minor compounds of different chemical classes (Riahi et al., 2015).

3.4 Antichemotactic activity

The ability of essential oils to inhibit leukocyte migration was assessed by the Boyden method. All oils samples, in the tested concentrations, exerted statistically significant reduction in leukocyte migration in relation to the negative control ($p < 0.05$) (Fig. 7). Percentage inhibition varied from 34.6 to 100% for the samples, whereas indomethacin inhibited only 33 and 58.5%, respectively at the concentrations of 0.5 and 5 μg/mL. Significant differences were observed between the oils on each concentration. In the concentration of 5 μg/mL, the oils from final productive rest, productive rest, pre-blooming and fruiting phases showed a significantly higher antichemotactic effect ($p < 0.05$) than the blooming phase, with inhibition percentages of 77.5 to 100%. These results indicated the presence of monoterpenes in the affects the activity, considering that the effect increased while the monoterpene fraction decreased. The oil obtained from the productive rest phase presented 100% inhibition of leukocyte migration and presence of a fraction of monoterpenes of only 5% of the total oil content, whereas the fraction of sesquisterpene hydrocarbons was predominant (73.9%). On the other hand, the blooming phase oil showed lower antichemotactic effect and higher percentage of monoterpenes, including limonene, α- and β-pinene as main compounds. The antichemotactic activity observed for the oil from productive rest phase can be related with presence of (*E*)-isoelemicin, one of the main compounds

indentified in this sample, considering several reports in the literature of anti-inflammatory activity of phenylpropanoids (Sá et al., 2014).

Several studies indicated anti-inflammatory activity of essential oils and their isolated compounds through the inhibition of the migration of polymorphonuclear leukocytes to the site of injury (Medeiros et al., 2007; Melo et al., 2011a,b; Kummer et al., 2013; Danielli et al., 2016), however, this effect is not attributed exclusively to this mechanism (Miguel, 2010). Studies have reported that α-pinene exerts an anti-inflammatory effect through suppression of protein kinase and nuclear factor κ-B (Kim et al., 2015), while limonene inhibits leukocyte migration in concentrations of 1, 3 and 10 μg/ mL (Hirota et al., 2010; Kummer et al., 2013). The fact that α-pinene acts through an anti-inflammatory mechanism differently from the one investigated in this study may explain the lower inhibitory effect of the oil obtained from blooming phase in relation to other samples.

4 Conclusions

This study reports differences in yield and chemical composition of the essential oil from leaves of *N. megapotamica* collected in productive rest, end of productive rest, pre-blooming, blooming and fruiting phases. Although the major compounds identified in the oils are common among the samples, they exhibited important quantitative differences. Such chemical variation, mainly in the content of monoterpenes, limonene, α-pinene and β-pinene, and of the sesquiterpene spathulenol, resulted in important differences in the antichemotactic effect, antifungal and DPPH radical scavenging activity. Multivariate exploratory analysis by HCA and PCA revealed the formation of groupings of samples due to their similarity in the profile of biological effects, indicating also a correlation of the oil obtained in the pre-blooming phase with all biological activities evaluated. This variability points to the importance of qualitative and quantitative chemical characterization of *N. megapotamica* oil

in relation to the seasonal variations, as well as its influence on the biological effects. In summary, highlights the productive rest phase, characterized by lower content of monoterpenes and with important antichemotactic effect, as well as a moderate antioxidant activity, associated to the best yield obtained in the extraction of the essential oil. The oil collected in this phase is considered promising for in-depth studies of anti-inflammatory activity. Studies that relate variations in the chemical composition of an essential oil may offer a strategy for the production of a compound or group of compounds of interest to the industry.

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Table 1 - Climatic characteristics and yield of the *Nectandra megapotamica* essential oils in relation to the collected period.

	Productive rest	**End productive rest**	**Pre-blooming**	**Blooming**	**Fruiting**
Collected period	Summer	Autumn	Winter	Winter	Spring
Total precipitation (mm)	150 - 200	100 - 150	250 - 300	150 - 200	100 - 150
Maximum temperature (°C)	24 - 26	22 - 24	14 - 18	20 - 22	24 - 26
Minimum temperature (°C)	14 - 16	14 - 16	6 - 10	10 - 12	14 - 16
Yield (%)	0.5	0.4	0.3	0.4	0.4

Information referring the month of collection. Source: National Institute of Space Research, Brazil.

Table 2 - Variability of chemical composition of essential oils from leaves of *Nectandra megapotamica* collected in different phases of the development of the plant.

ID	Compounds	RI	Relative peak area (%)				
			Productive rest	**End productive rest**	**Pre-blooming**	**Blooming**	**Fruiting**
Monoterpene hydrocarbons							
1	α-pinene	925	-	4.3	2.7	14.8	5.0
2	Camphene	939	-	-	-	0.5	-
3	Sabinene	965	-	0.2	0.2	Tr	0.7
4	β-pinene	967	0.2	6.6	4.1	15.5	6.4
5	Myrcene	986	Tr	-	0.5	0.7	-
6	Limonene	1022	4.6	2.7	14.1	5.3	8.6
7	(*E*)-β-ocimene	1043	0.3	-	0.5	0.3	-
Sesquiterpene hydrocarbons							
8	α-copaene	1363	4.0	4.5	2.8	3.8	4.3
9	β-bourbonene	1371	0.1	Tr	0.1	0.3	0.1
10	β-cubebene	1377	0.5	0.5	0.4	0.5	0.6
11	β-elemene	1380	0.6	0.7	0.5	0.5	0.9
12	β-caryophyllene	1404	6.2	5.3	6.4	3.9	6.0
13	β-gurjunene	1414	0.1	-	-	0.1	0.2
14	Aromadendrene	1424	0.4	0.4	0.3	0.3	0.5
15	α-humulene	1437	3.9	3.8	2.8	2.7	3.2
16	Allo-aromadendrene	1444	0.2	1.0	0.2	0.9	0.3
17	γ-muurolene	1462	-	-	0.3	-	0.5
18	Germacrene D	1467	18.7	19.2	16.8	15.0	10.9
19	Bicyclogermacrene	1483	32.1	36.7	33.4	22.0	22.8
20	α-muurolene	1485	0.5	Tr	0.4	0.4	0.6
21	Germacrene A	1489	-	0.5	0.4	0.3	0.6
22	γ-cadinene	1501	0.3	Tr	0.2	0.4	0.1
23	δ-cadinene	1507	6.1	6.8	4.0	4.1	3.9
24	Cadina-1,4-diene	1513	0.2	-	-	-	-
25	Germacrene B	1537	-	-	-	0.1	0.3
Oxigenated sesquiterpenes							
26	(*E*)-nerolidol	1547	3.5	0.9	1.7	0.6	2.4
27	Spathulenol	1558	2.4	3.0	1.9	3.3	9.1
28	Caryophyllene oxide	1561	0.2	0.5	0.2	0.6	0.9
29	Globulol	1564	1.5	-	1.0	0.9	1.8
30	*Epi*-globulol	1571	-	1.3	0.2	0.2	0.8
31	Humulene epoxide II	1596	-	-	-	-	0.2
32	1-*epi*-cubenol	1617	0.2	-	-	-	-
33	*Iso*-spathulenol	1626	-	-	-	0.2	-
34	α-muurolol	1629	0.2	-	0.3	0.2	-
35	τ-cadinol	1632	0.7	-	-	-	-
36	Cubenol	1643	-	-	-	0.6	-
Phenylpropanoids							
37	Elemicin	1544	0.1	0.3	-	0.2	0.7
38	(*E*)-isoelemicin*	1644	9.4	-	2.6	-	4.6
Monoterpene hydrocarbons			5.1	13.8	22.1	37.1	20.7
Sesquiterpene hydrocarbons			73.9	79.4	69.0	55.3	55.8
Oxigenated sesquiterpenes			8.7	5.7	5.3	6.6	15.2
Phenylpropanoids			9.5	0.3	2.6	0.2	5.3
Total identified			**97.2**	**99.2**	**99.0**	**99.2**	**97.0**

Compounds are listed in order of elution on DB5 column; ID. Identification; RI. retentionRetention index; Tr. Traces.* identification based on comparison of retention index with those of published data (Adams, 2001).

Table 3 - Minimum inhibitory concentration (MIC) of essential oils from leaves of *Nectandra megapotamica* collected in different phases of the plant development against dermatophytes.

	Minimum inhibitory concentration (µg/mL)					
	Productive rest	**End productive rest**	**Pre-blooming**	**Blooming**	**Fruiting**	**Terbinafine**
Trichophyton rubrum						
TRU 48	>500	500	500	250	125	0.03
TRU50	>500	>500	>500	>500	>500	0.06
TRU51	>500	>500	250	>500	500	0.008
TRU55	>500	>500	500	500	125	-
Trichophyton mentagrophytes						
TME16	>500	>500	500	>500	>500	0.016
TME33	>500	>500	>500	>500	>500	0.03
TME40	>500	>500	500	>500	>500	0.016
TME46	>500	>500	>500	>500	500	0.13
Microsporum canis						
MCA01	>500	>500	500	250	250	0.004
MCA29	>500	>500	250	>500	>500	0.008
MCA33	>500	>500	>500	>500	>500	-
MCA40	>500	>500	>500	>500	>500	1.00
Microsporum gypseum						
MGY42	>500	>500	500	250	125	0.016
MGY52	>500	>500	>500	>500	500	0.13
MGY58	>500	>500	500	500	125	2.00

(-) not tested.

Table 4 - Radical DPPH scavenging activity of essential oils from leaves of *Nectandra megapotamica* collected in different phases of the plant development.

Sample	DPPH scavenging activity (%)					
	250 µg/mL	**200 µg/mL**	**150 µg/mL**	**100 µg/mL**	**50 µg/mL**	**25 µg/mL**
Productive rest	35.9 ± 0.3^{c}	21.7 ± 0.5^{c}	16.3 ± 0.6^{c}	11.4 ± 1.6^{b}	7.8 ± 0.2^{b}	4.1 ± 0.3^{a}
End productive rest	22.3 ± 2.4^{e}	9.9 ± 0.5^{e}	6.3 ± 0.2^{f}	4.0 ± 0.0^{c}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
Pre-blooming	43.4 ± 0.4^{b}	37.7 ± 0.3^{b}	27.6 ± 0.2^{b}	10.7 ± 0.7^{b}	5.6 ± 0.3^{bc}	5.0 ± 0.8^{a}
Blooming	26.3 ± 0.2^{d}	20.4 ± 0.2^{c}	13.6 ± 0.3^{d}	12.3 ± 0.3^{b}	3.4 ± 0.3^{cd}	2.0 ± 0.3^{b}
Fruiting	18.1 ± 0.2^{f}	14.7 ± 0.2^{d}	9.4 ± 0.2^{e}	3.8 ± 0.4^{c}	2.8 ± 0.4^{cd}	0.0 ± 0.0^{c}
Rutin	96.1 ± 0.3^{a}	93.5 ± 0.7^{a}	90.5 ± 0.6^{a}	50.8 ± 3.9^{a}	13.2 ± 2.6^{a}	2.3 ± 0.4^{b}

Rutin, positive control. Different letters indicates significant difference between samples in the same concentrations ($p < 0.05$) (ANOVA followed by Tukey's test).

Captions to illustrations

Figure 1. Comparison between the major compounds identified in the essential oils from leaves of *Nectandra megapotamica* collected in different phases of the development of the plant. α-PIN, α-pinene; β-PIN, β-pinene; LIM, limonene; α-COP, α-copaene; β-CAR, β-caryophyllene; GER, germacrene D; BIC, bicyclogermacrene; δ-CAD, δ-cadinene; SPA, spathulenol; (E)-ELE, (*E*)-isoelemicin.

Figure 2. Dendrogram obtained by HCA of samples of essential oils from leaves of *Nectandra megapotamica* collected in different phases of the development of the plant.

Figure 3. Biplot based in the first two principal components of samples of essential oils from leaves of *Nectandra megapotamica* collected in different phases of the development of the plant.

Figure 4. Dendrogram obtained by HCA of the different compounds identified in the samples of essential oils from *Nectandra megapotamica*.

Figure 5. Variation in the antifungal activity of *Nectandra megapotamica* essential oil estimated from cluster analysis and principal component analysis. (A) Dendrogram based on hierarchical cluster analysis and (B) PCA of the first two principal components of oil samples collected at different plant development phases related to minimum inhibitory concentration (MIC).

Figure 6. Radical DPPH scavenging activity of *Nectandra megapotamica* essential oil estimated from cluster analysis and principal component analysis. (A) Dendrogram based on HCA and (B) PCA of the first two principal components of oil samples collected at different plant development phases.

Figure 7. *In vitro* effect of the different essential oils from *Nectandra megapotamica* and indomethacin in the neutrophil migration compared to negative control. *Significant inhibition to all tested concentrations. Different letters indicates significant difference between samples in the same concentrations ($p < 0.05$) (ANOVA followed by Tukey's test).

Figure 1

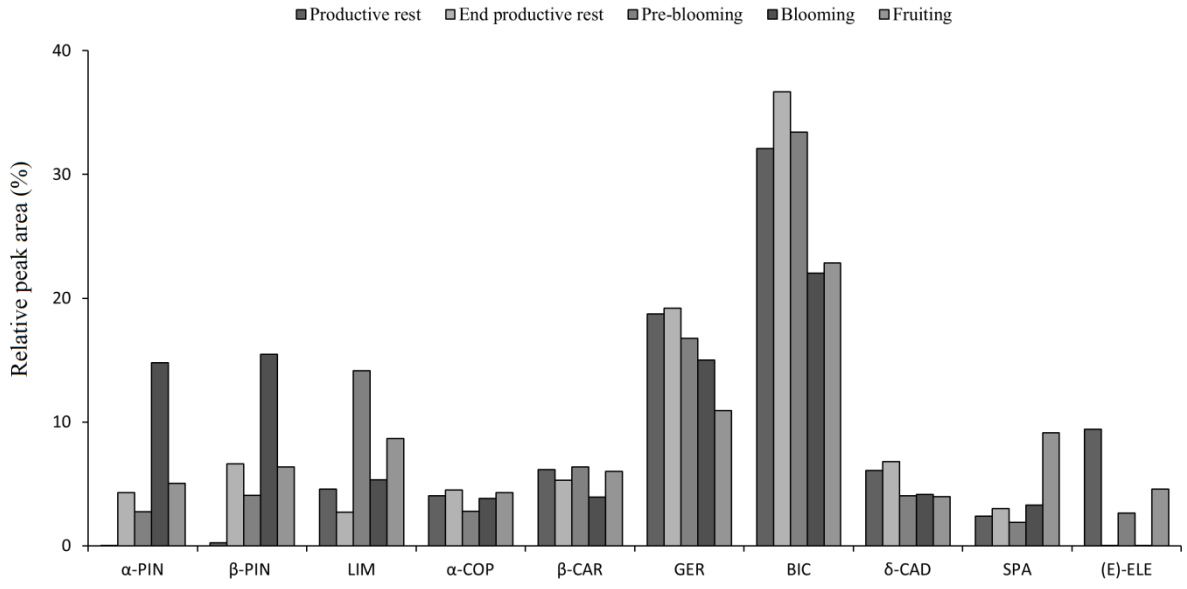

Productive rest
End productive rest
Pre-blooming
Blooming
Fruiting
Relative peak area (%)
40
30
20
10
0
α-PIN
β-PIN
LIM
α-COP
β-CAR
GER
BIC
δ-CAD
SPA
(E)-ELE

Figure 2

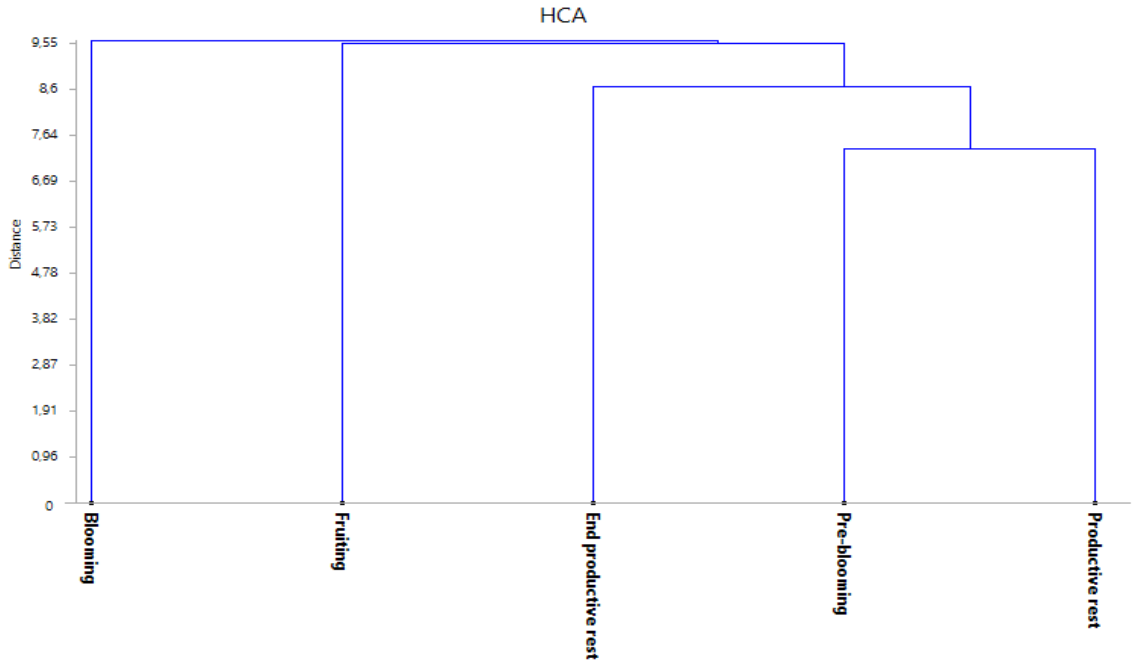


Figure 3

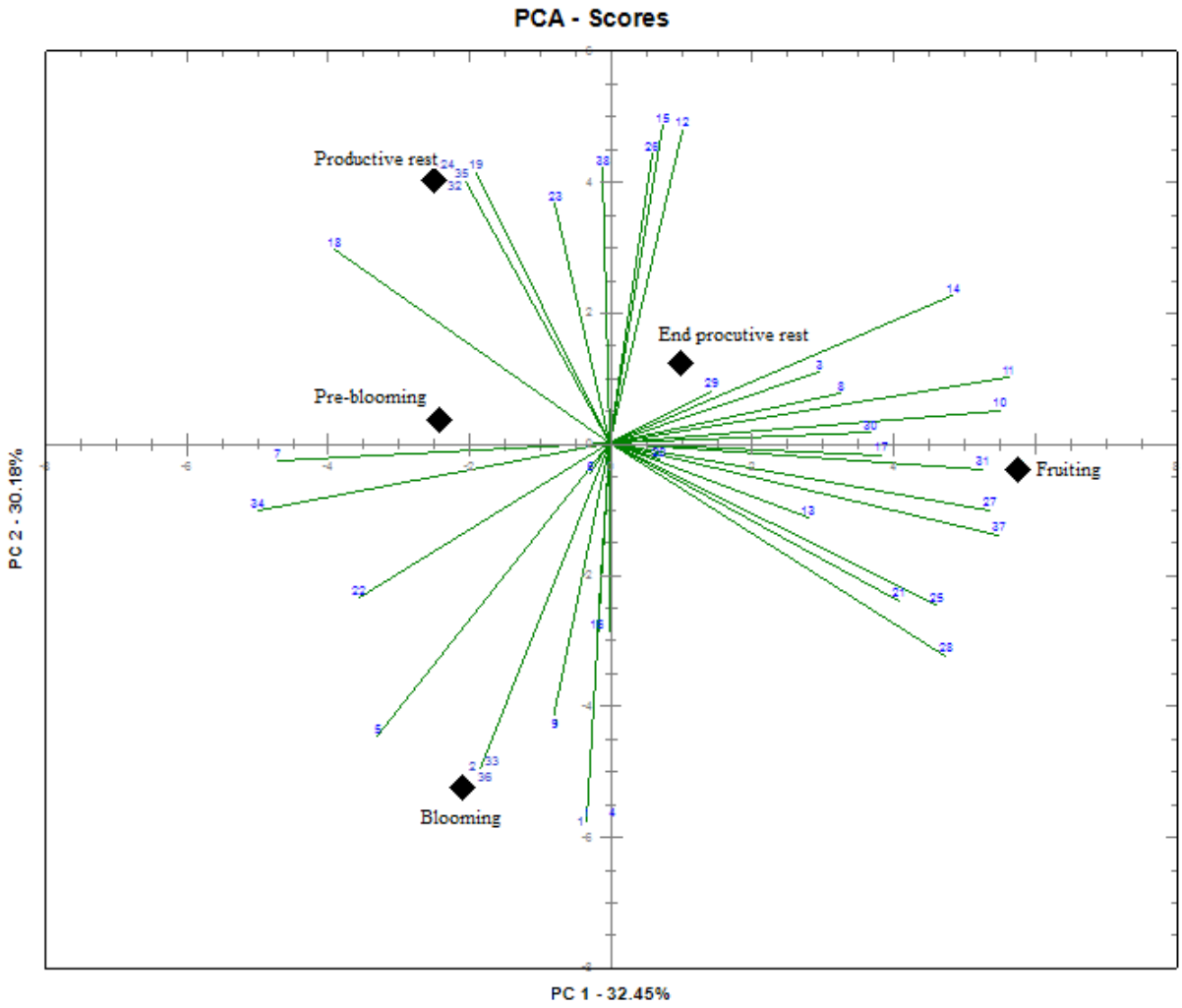


Figure 4

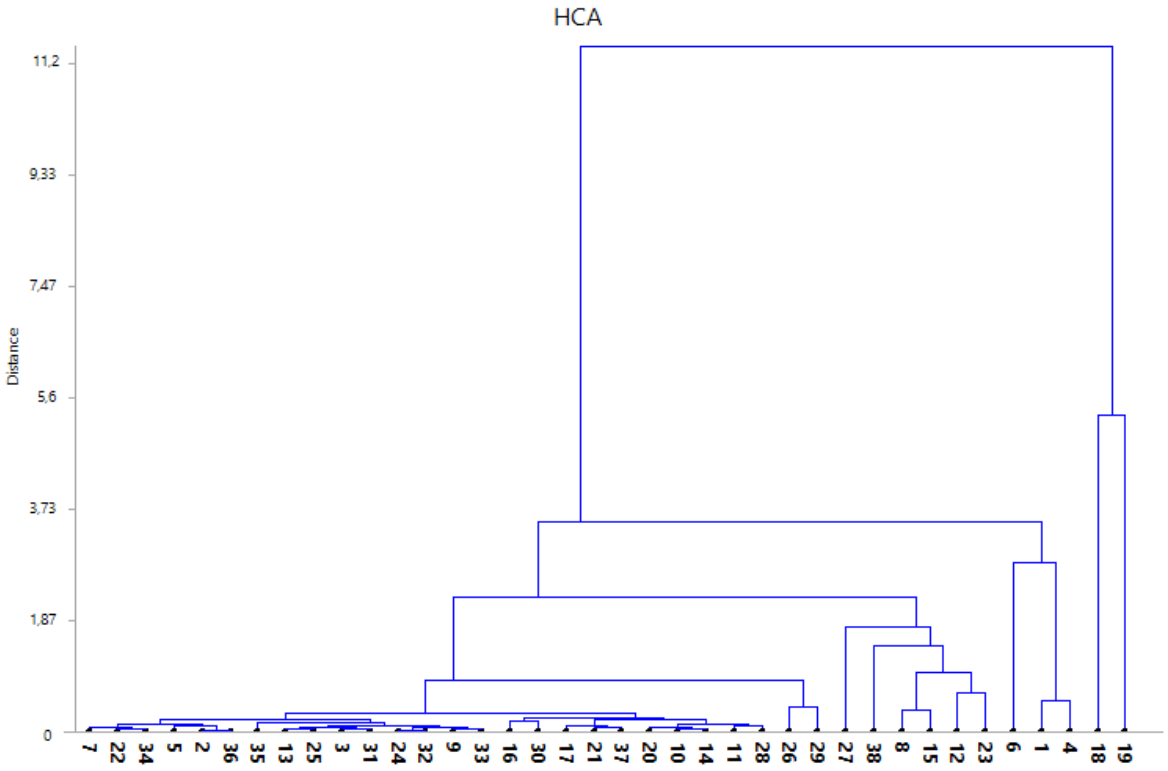

HCA
Distance
11,2
9,33
7,47
5,6
3,73
1,87
0
7 22 34 5 2 36 35 13 25 3 31 24 32 9 33 16 30 17 21 37 20 10 14 11 28 26 29 27 38 8 15 12 23 6 1 4 18 19

Figure 5

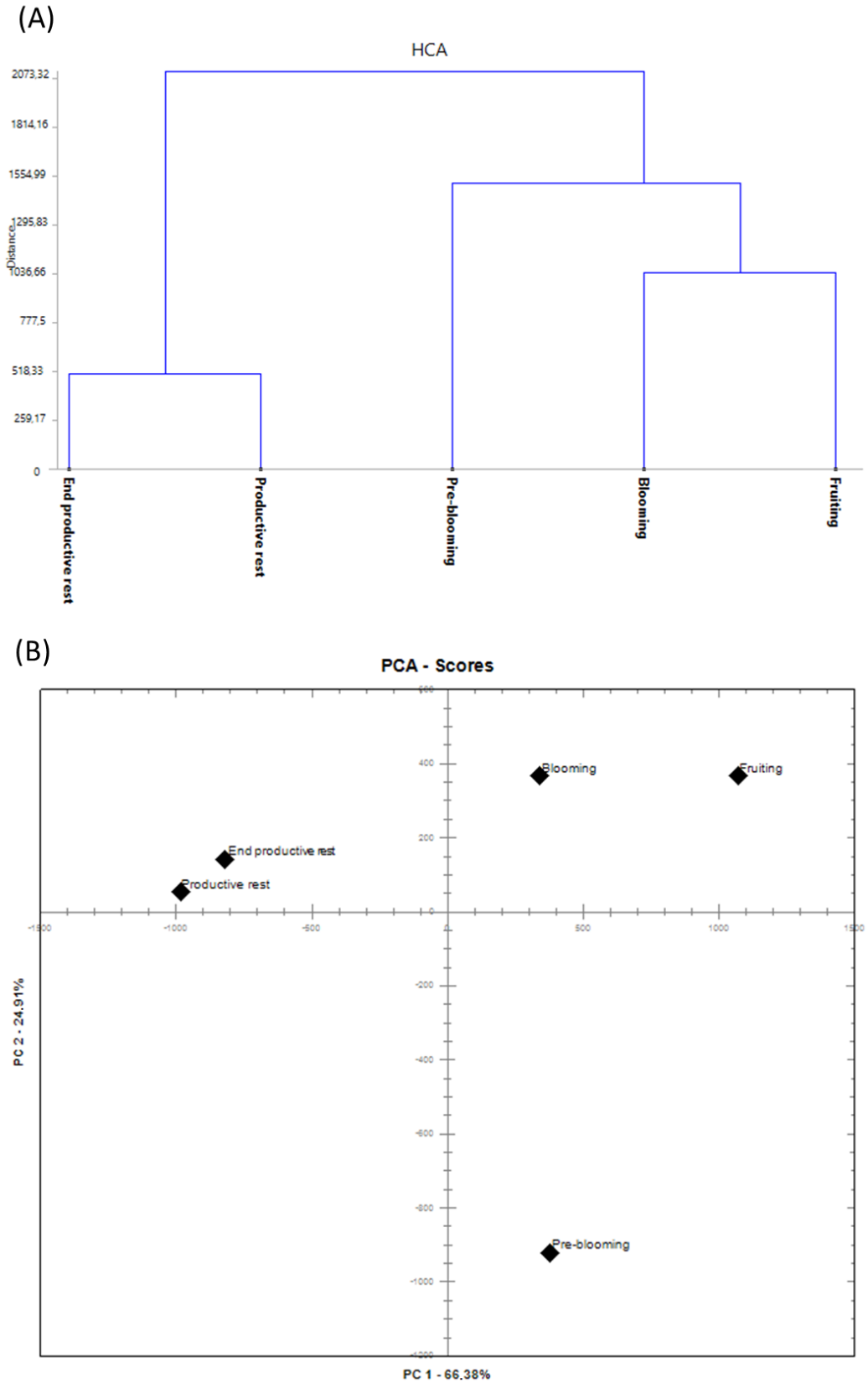

(A)
HCA
2073,32
1814,16
1554,99
1295,83
1036,66
777,5
518,33
259,17
0
Distance
End productive rest
Productive rest
Pre-blooming
Blooming
Fruiting
(B)
PCA - Scores
Blooming
Fruiting
End productive rest
Productive rest
Pre-blooming
PC 2 - 24,91%
PC 1 - 66,38%

Figure 6

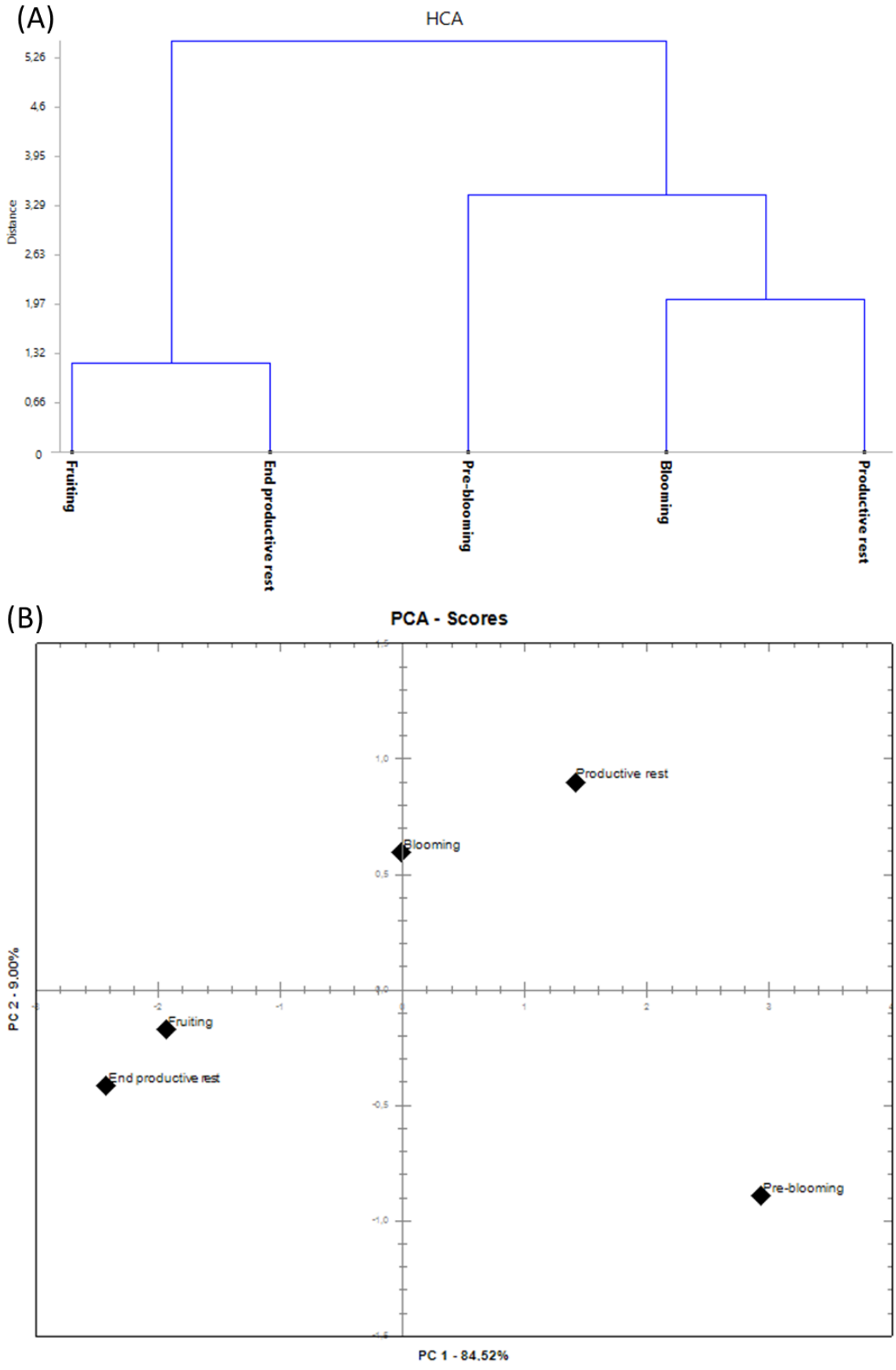
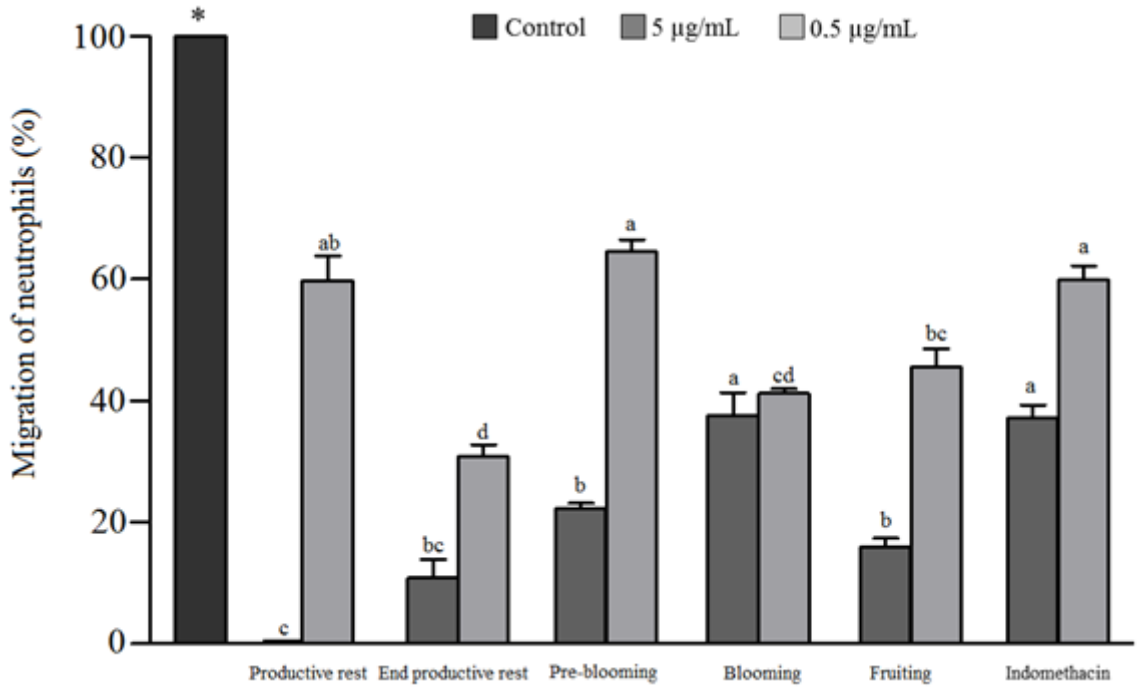

(A)
HCA
Distance
5,26
4,6
3,95
3,29
2,63
1,97
1,32
0,66
0
Fruiting
End productive rest
Pre-blooming
Blooming
Productive rest
(B)
PCA - Scores
Productive rest
Blooming
Fruiting
End productive rest
Pre-blooming
PC 2 - 9,00%
PC 1 - 84,52%

Figure 7


Control
5 µg/mL
0.5 µg/mL
Migration of neutrophils (%)
100
80
60
40
20
0
*
c
ab
bc
d
b
a
a
cd
b
bc
a
a
Productive rest
End productive rest
Pre-blooming
Blooming
Fruiting
Indomethacin

Fatores como aumento significativo na incidência e severidade das infecções fúngicas, especialmente em pacientes com sistema imune debilitado; emergência de microrganismos oportunistas, com destaque àqueles com sensibilidade reduzida ou intrinsicamente resistentes aos agentes antifúngicos e o restrito arsenal terapêutico disponível, caracterizado por agentes com importantes efeitos adversos, têm contribuído para o agravamento nas taxas de morbidade e mortalidade dos pacientes (SOARES et al. 2013; NEGRI et al., 2014; PIANALTO et al., 2016) e instigado pesquisadores na busca por novas opções terapêuticas. Entre as estratégias investigadas, a terapia combinada a partir de uma abordagem multialvo, apresenta-se como uma alternativa capaz de minimizar a lacuna de novos agentes antimicrobianos, com a vantagem de redução na concentração ativa e nos efeitos adversos, consequentemente (CHEN et al., 2010; ZHANG et al., 2014). Outro fator importante a ser considerado nesta abordagem refere-se à redução ou retardo no surgimento de resistência, e principalmente a reversão desta condição (CARRILLO-MUÑOZ et al., 2014; MERTAS et al., 2015).

Nesse contexto, diversos estudos têm investigado os efeitos da combinação de fármacos antifúngicos com produtos naturais, incluindo óleos voláteis e seus compostos isolados (SAMBER et al., 2015; CARDOSO et al., 2016; DANIELLI et al., 2017b). O fato de que esta classe de metabólitos secundários apresenta função de proteção do vegetal contra o ataque de patógenos é considerado um indicativo da presença de substâncias com potencial antimicrobiano (RAUT; KARUPPAYIL, 2014). Baseado nessa premissa, os capítulos III e IV deste trabalho abordam a investigação da atividade antifúngica e interação *in vitro* de óleos voláteis obtidos de espécies do gênero *Nectandra* Rol. ex Rottb e *S. lentiscifolius* com fármacos antifúngicos utilizados na terapia convencional. De uma forma geral, ambos os óleos exibiram atividade seletiva frente a isolados dos gêneros *Microsporum* spp. e *Trichosporum* spp. No entanto, importantes diferenças resultaram das distintas combinações. A associação dos óleos de *Nectandra* spp. com terbinafina apresentou-se como indiferente em grande parte dos isolados testados, ao mesmo tempo em que interações de natureza aditiva foram observadas para ciclopirox. Efeito contrário foi verificado para *S. lentiscifolius*, cuja combinação com terbinafina resultou, em grande

parte, em efeito sinérgico. Estas divergências estão relacionadas ao diferente mecanismo de ação dos óleos investigados, bem como às características de sua composição química.

A eficiência na inibição do crescimento de microrganismos pelos óleos voláteis varia conforme o óleo testado e seu alvo no patógeno (RAUT; KARUPPAYIL, 2014). A investigação do mecanismo de ação dos óleos voláteis de *N. megapotamica* e *N. lanceolata* sugeriu efeito exercido através da interação com ergosterol presente na membrana fúngica. A terbinafina atua de forma similar, através do bloqueio da biossíntese de ergosterol, pela inibição da atividade enzimática da esqualeno epoxidase (DIAS et al., 2013), fato que pode justificar a ausência de interação sinérgica oriunda desta combinação. Além do envolvimento da membrana celular, resultados obtidos pelo ensaio de sorbitol associados à presença de alterações morfológicas das estruturas hifais, observadas por microscopia eletrônica de varredura, indicam que o efeito antifúngico do óleo volátil de *S. lentiscifolius* pode estar relacionado, ainda, à interação com a parede fúngica. Neste caso, o dano à parede celular causado pelo tratamento com o óleo pode estar envolvido no aumento do conteúdo intracelular do fármaco disponível para exercer seu efeito, caracterizando as interações sinérgicas e aditivas provenientes desta combinação. Segundo Carrillo-Muñoz e colaboradores (2014), um efeito sinérgico é resultante, principalmente, da ação em múltiplos alvos. Dessa forma, duas substâncias atuando concomitantemente em diferentes alvos da célula fúngica podem resultar em aumento da eficácia (BADDLEY; PAPPAS, 2005). O efeito antifúngico do óleo de *Schinus* apresentou ainda características fungicidas, o que denota vantagem no tratamento de infecções dermatofíticas, caracterizadas por fraca adesão ao tratamento e importante número de quadros de recorrência (PERES et al., 2010).

O principal mecanismo de ação antifúngico dos óleos voláteis está relacionado à sua capacidade de interação com a membrana fúngica, causando alterações estruturais e de permeabilidade, resultando em extravasamento de componentes vitais e morte celular (ASDADI et al., 2015). Esta hipótese corrobora os resultados obtidos nesse estudo. No entanto, pressupõe-se ainda, que estes metabólitos exerçam efeito antifúngico em virtude de sua propriedade de hidrofobicidade (VIUDA-MARTOS et

al., 2011; RAUT; KARUPPAYIL, 2014), bem como da presença de compostos oxigenados, como álcoois e substâncias fenólicas (LANG; BUCHBAUER, 2012). No caso do óleo de *Schinus*, a fração de sesquiterpenos oxigenados apresentou-se como predominante (50%), além da presença de elemol, γ-eudesmol, β-eudesmol e α-eudesmol entre os compostos majoritários. Já para *N. lanceolata* e *N. megapotamica* foram identificados, predominantemente, sesquiterpenos hidrocarbonetos, representando 69% e 79% do conteúdo total do óleo, respectivamente. Neste caso, entre os compostos oxigenados, apenas espatulenol pode ser considerado como majoritário (11%) para *N. lanceolata*.

Associado ao efeito antifúngico, os óleos voláteis apresentaram importante efeito antiquimiotáxico, indicando ação na fase aguda do processo inflamatório (MEDZHITOV, 2008). Essa propriedade, em conjunto com mecanismos antioxidantes, auxilia tanto no processo de cura de uma infecção fúngica, como no alívio dos sintomas. Ao acessar o tecido do hospedeiro, os dermatófitos induzem uma resposta imune pelos queratinócitos, responsável pelo desencadeamento do processo inflamatório do organismo em face ao agressor (PERES et al., 2010). No entanto, quando a resposta inflamatória é exacerbada e prolongada, acarreta em prejuízo ao sistema de defesa e à evolução terapêutica (ROMANI, 2011; KIM et al., 2015). No mesmo sentido, a produção excessiva de radicais livres pelas células de defesa durante a fagocitose do patógeno, pode intensificar os danos no tecido injuriado (BOUDIAF et al., 2016; ORHAN et al., 2016). Muito embora de uma forma não tão significativa, os óleos avaliados apresentaram efeito de sequestro de radical livre mediado pelo mecanismo de doação de hidrogênio (SCHAICH et al., 2015). Desta forma, além do efeito antifúngico, as ações anti-inflamatória e antioxidante conjuntas podem contribuir na rápida redução dos sintomas, promoção da cura do processo infeccioso e prevenção do desenvolvimento de cronicidade (HUBE et al., 2015).

Além da atividade inibitória no crescimento de dermatófitos e efeito sinérgico e aditivo aos fármacos comerciais, o óleo volátil de *S. lentiscifolius* apresentou inibição na formação de biofilme em um isolado de *Microsporum canis*, principal agente etiológico de tinea capitis. De fato, verificou-se, pela primeira vez, a capacidade de formação de biofilme por fungos filamentosos deste gênero. Biofilmes

são considerados uma das principais causas de falha na terapia antimicrobiana (ACKER et al., 2014). Devido a sua estrutura complexa que denota aumento de resistência aos agentes terapêuticos e à resposta imune do hospedeiro, a formação de biofilme é considerada um importante fator de virulência (DESAI et al., 2014; GONZÁLEZ-RAMÍREZ et al., 2016). Até o momento, a capacidade de formação desta estrutura por dermatófitos havia sido descrita apenas para o gênero *Trichophyton* spp. (COSTA-ORLANDI et al., 2014). Através de microscopia eletrônica de varredura verificou-se a presença de expansão miceliar, incluindo uma rede de hifas altamente organizada e envolta em matriz extracelular polissacarídica, compatível com a estrutura de um biofilme maduro. Considerando-se a formação de biofilme como fator de virulência a importância deste achado se dá na determinação de uma abordagem terapêutica efetiva, evitando episódios de recorrência.

A atividade do óleo volátil de *S. lentiscifolius* frente ao biofilme de *M. canis*, foi caracterizada pela importante redução da matriz extracelular e completa inibição da formação do biofilme em concentrações sub-inibitória e inibitória, respectivamente. Além de prevenir a aderência da célula fúngica à superfície, fator imprescindível na formação de tais estruturas, os óleos voláteis apresentam a vantagem de atuar tanto em células séssies quanto planctônicas (KHAN; AHMAD, 2012; MANGANYI et al., 2015). No caso de infecções causadas por *M. canis*, com tinea capitis, a formação do biofilme pode ser considerada uma barreira ao tratamento, mantendo as hifas protegidas da ação do fármaco e facilitando sua aderência ao pelo.

De uma forma geral, os óleos voláteis apresentam um amplo espectro de atividades biológicas (RAUT; KARUPPAYIL, 2014). Esta característica pode ser atribuída à variabilidade e complexidade de sua composição química, a qual é diretamente influenciada por fatores internos e externos ao vegetal. Localização geográfica da planta, variação genética, aplicação de fertilizantes, estresse durante o crescimento e a maturidade do indivíduo, variações sazonais e climáticas, condições de estocagem e secagem, afetam a distribuição química dos constituintes de um óleo (RAUT; KARUPPAYIL, 2014; GRULOVA et al., 2015). Pesquisas relacionadas à influência da variação sazonal, fase do desenvolvimento do vegetal e condições agronômicas nas atividades biológicas do óleo são foco de estudos farmacológicos e

de engenharia metabólica (OLMEDO et al., 2015; ALMEIDA et al., 2016b; KARIMI et al., 2016). Através da aplicação de ferramentas quimiométricas observou-se a formação de grupamentos entre as amostras de óleo volátil de *N. megapotamica* obtidas de diferentes fases do desenvolvimento do vegetal, resultante do perfil de similaridade de efeitos biológicos. Além da influência da presença de monoterpenos, principalmente limoneno, α- e β-pineno, nas atividades biológicas do óleo, observou-se correlação entre a amostra obtida na fase de pré-floração e todas as atividades avaliadas, a saber, antifúngica, antiquimiotáxica e antioxidante. As análises quimiométricas auxiliam no estabelecimento de uma relação entre um grande número de amostras de óleo, considerando sua complexidade química, a fim de estabelecer tendências, definir marcadores e qimiotipos (PINHEIRO et al., 2016; SORO et al., 2016; YANG et al., 2016).

Em suma, a atividade antifúngica dos óleos voláteis avaliados foi caracterizada pelo efeito quimiosensibilizante da célula fúngica à ação de agentes terapêuticos convencionais, tendo a membrana e parede celular como alvos para esta atividade, de forma a corroborar com a principal hipótese de mecanismo antifúngico de óleos voláteis. Desta forma, o emprego destas substâncias de forma combinada pode ser potencial na complementação à terapia convencional de uso tópico em infecções dermatofíticas. Além disso, a utilização de análises multivariadas no estudo da variabilidade química de óleos voláteis demonstrou-se uma ferramenta de grande utilidade na correlação de bioatividades e características químicas, bem como na determinação da fase de desenvolvimento do vegetal potencialmente ativa.

CONCLUSÕES

- A composição química dos óleos voláteis de *N. megapotamica*, *N. lanceolata* e *S. lentiscifolius* foi determinada, destacando-se a presença da fração sesquiterpênica como majoritária para ambas as espécies;

- Ambos os óleos em estudo apresentaram efeito antidermatofítico e afinidade pelo ergosterol, indicando possível ação ao nível de membrana fúngica, no entanto, para *S. lentiscifolius* verificou-se ainda, a presença de hifas com dano estrutural e resultado positivo para o ensaio de sorbitol, sugerindo que a parede celular também seja um dos alvos para esta amostra;

- A combinação dos óleos obtidos das espécies de *Nectandra* com terbinafina apresentou predominantemente interações classificadas como indiferentes, enquanto que a associação com ciclopirox demonstrou aditividade e sinergismo para o óleo de *N. lanceolata*. O efeito oposto foi observado para o óleo de *S. lentiscifolius*, cujas interações sinérgicas foram obtidas principalmente com terbinafina;

- Os óleos apresentaram significativo efeito antiquimiotáxico, inibindo a migração leucocitária em direção ao fator quimioatrativo;

- *Microsporum canis* apresentou capacidade de formar biofilme caracterizado pela presença de matriz extracelular polissacarídica;

- O óleo volátil de *S. lentiscifolius* inibiu a formação de biofilme por *M. canis* de modo concentração-dependente;

- Análises multivariadas revelaram a formação de grupamentos para as amostras do óleo volátil de *N. megapotamica* obtidas de diferentes fases do desenvolvimento do vegetal, devido ao perfil de similaridade de seus efeitos nas atividades biológicas avaliadas;

- A variação da composição da fração monoterpênica para o óleo de *N. megapotamica*, principalmente no conteúdo de limoneno, α-pineno e β-pineno, afetou diretamente na bioatividade do óleo.

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