ALEX RODRIGUES SILVA CAETANO

NANOPARTICLES AND NANOFIBERS INCORPORATED WITH ESSENTIAL OILS: DEVELOPMENT, CHARACTERIZATION AND EVALUATION OF ANTIFUNGAL AND INSECTICIDE ACTIVITY

LAVRAS – MG
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NANOPARTÍCULAS E NANOFÍBRAS INCORPORADAS COM ÓLEOS ESSENCIAIS: DESENVOLVIMENTO, CARACTERIZAÇÃO E AVALIAÇÃO DA ATIVIDADE ANTIFÚNGICA E INSETICIDA

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APROVADO em 9 de setembro de 2022.

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2022

A Deus, pela vitória, sabedora, inteligência e por sempre renovar a minha fé e estar ao meu lado.
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Aos meus amigos e colegas.

Dedico

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RESUMO

O Brasil é segundo maior produtor de alimentos no mundo. A cafeicultura sofre com a perda na produção devido à contaminação das folhas do cafezal pelo fungo *Hemileia vastatrix*, conhecido como ferrugem do cafeeiro, bem como a contaminação dos grãos de café pelos fungos micotoxigênicos *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae, Aspergillus flavus e Aspergillus parasiticus*. A produção de frutas vermelhas tem como principal fator que causa a perda de produção o ataque da mosca *Drosophila suzukii*. Os óleos essenciais são metabolitos secundários produzidos pelas plantas, sua composição química diversificada confere a essas substâncias diversas propriedades biológicas, como a antifúngica e a inseticida. Para tornar viável a utilização de óleos essenciais em sistemas industriais, eles têm sido incorporados em nanopartículas e nanofíbras. Esses sistemas nanoestruturados possibilitam uma volatilidade controlada dos óleos essenciais, bem como protegem seus constituintes de fatores externos que podem levar a uma mudança estrutural, bem como à sua degradação. Objetivou-se neste estudo produzir, caracterizar e avaliar a atividade antifúngica *in vivo* e *in vitro* de nanopartículas de poli(ε-caprolactona) incorporadas com os óleos essenciais de *Corymbia citriodora, Eucalyptus grandis, Eucalyptus camaldulensis* sobre o fungo *Hemileia vastatrix*; produzir, caracterizar e avaliar a atividade inseticida *in vivo* e *in vitro* de nanopartículas de poli(ε-caprolactona) incorporadas com o óleo essencial de *Rosmarinus officinalis* sobre a mosca *Drosophila suzukii*; produzir, caracterizar e avaliar a atividade antifúngica e antimicotoxigenica de nanofibras de poli(ácido lático) incorporadas com o óleo essencial de *Corymbia citriodora* e de seus constituintes isoméricos *β*-citronelol e citronelal sobre fungos micotoxigenicos. Os resultados encontrados foram promissores, verificando que os sistemas nanoestruturados foram capazes de diminuir a severidade da ferrugem do cafeeiro causada pelo fungo *Hemileia vastatrix* em até 90.75%, resultado semelhante a fungicidas sintéticos. Em relação à atividade inseticida, os sitemas nanoestruturados apresentaram um resultado de LD_{50} na concentração de 17.5 mL/L sobre a mosca *Drosophila suzukii*. Os sistemas nanofibrosos apresentaram atividade antifúngica sobre os microrganismos *Aspergillus ochraceus*, *Aspergillus carbonarius* e *Aspergillus westerdijkiae* inibindo em 99 e 100% a produção de ocratoxina A e o crescimento micelial, respectivamente. Os resultados inovadores indicam a viabilidade da aplicação de nanopartículas e nanofíbras como sistema de liberação controlada de óleos essenciais e de monoterpenos no combate a *Hemileia vastatrix* e fungos micotoxigênicos, bem como no controle à mosca *Drosophila suzukii*.

Palavras-chave: Óleos essenciais. Atividade antifúngica. Atividade inseticida. Nanopartículas. Nanofíbras. *Hemileia vastatrix*. *Drosophila suzukii*. *Aspergillus*.

ABSTRACT

Brazil is the second largest food producer in the world. Coffee production suffers from loss of production due to contamination of coffee plantation leaves by the fungus *Hemileia vastatrix*, known as coffee rust, as well as contamination of coffee beans by the mycotoxigenic fungi *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, *Aspergillus flavus* and *Aspergillus parasiticus*. The main factor that causes the loss of production of red fruits is the attack of the fly *Drosophila suzukii*. Essential oils are secondary metabolites produced by plants, their diverse chemical composition gives these substances several biological properties, such as antifungal and insecticidal. To make the use of essential oils in industrial systems viable, they have been incorporated into nanoparticles and nanofibers. These nanostructured systems enable a controlled volatility of essential oils, as well as protect their constituents from external factors that can lead to structural change as well as their degradation. The objective of this study was to produce, characterize and evaluate the *in vivo* and *in vitro* antifungal activity of poly(ε-caprolactone) nanoparticles incorporated with the essential oils of *Corymbia citriodora*, *Eucalyptus grandis*, *Eucalyptus camaldulensis* on the fungus *Hemileia vastatrix*; to produce, characterize and evaluate the insecticidal activity *in vivo* and *in vitro* of poly(ε-caprolactone) nanoparticles incorporated with the essential oil of *Rosmarinus officinalis* on the fly *Drosophila suzukii*; to produce, characterize and evaluate the antifungal and antimycotoxigenic activity of poly(lactic acid) nanofibers incorporated with the essential oil of *Corymbia citriodora* and its isomeric constituents *β*-citronellol and citronellal on mycotoxigenic fungi. The results found were promising, verifying that the nanostructured systems were able to reduce the severity of coffee rust caused by the fungus *Hemileia vastatrix* by up to 90.75%, a result similar to synthetic fungicides. Regarding insecticidal activity, the nanostructured systems showed a result of LD_{50} at a concentration of 17.5 mL/L on the fly *Drosophila suzukii*. The nanofibrous systems showed antifungal activity against the microorganisms *Aspergillus ochraceus*, *Aspergillus parasiticus* and *Aspergillus westerdijkiae*, inhibiting by 99 and 100% the production of ochratoxin A and the mycelial growth, respectively. The innovative results indicate the feasibility of applying nanoparticles and nanofibers as a controlled release system of essential oils and monoterpenes to combat *Hemileia vastatrix* and mycotoxigenic fungi, as well as to control the fly *Drosophila suzukii*.

Keywords: Essential oils. Antifungal activity. Insecticidal activity. Nanoparticles. Nanofíber. *Hemileia vastatrix*. *Drosophila suzukii*. *Aspergillus*.

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1 INTRODUÇÃO GERAL

O Brasil é um dos principais produtores de alimentos do mundo, tendo a agricultura como uma das principais atividades econômicas do país, gerando empregos diretos e indiretos. Para o ano de 2022, a estimativa é de que a produção agrícola cresça 12,5% em relação ao ano anterior, produzindo aproximadamente 284,4 milhões de toneladas de grãos. Dentre as diversas culturas cultivadas no Brasil, a cafeicultura vem ganhando destaque nesse cenário. Estima-se que a produção de café para o ano de 2022 seja de aproximadamente 55,74 milhões de sacas de 60kg, com um faturamento bruto de 71,7 bilhões de reais (BRASIL, 2022; EMPRAPA, 2020).

O cafeeiro *Coffea* ssp. é um arbusto da família Rubiaceae do gênero *Coffea* L., originário na Etiópia e abrange 103 espécies. O Brasil é o principal produtor de grãos de café do mundo. As principais cultivares no país são o *Coffea arabica* e o *Coffea canefora*, sendo o primeiro, responsável por produzir cafés finos nos mais variados sabores. O café produzido a partir da semente contida no seu fruto é uma das bebidas mais requisitadas no mundo, tornando a cafeicultura um dos pilares agrícolas que movimenta a economia global.

A cafeicultura, assim como a agricultura em geral, vem passando por transformações tecnológicas e de manejo, buscando sempre melhorias na produção. Um dos principais empecilhos na produção de grãos de café é a contaminação das lavouras pelo fungo *Hemileia vastatrix* Berk and Br, conhecido também como “ferrugem do cafeeiro”. Esse microrganismo, ao contaminar a parte abaxial das folhas do cafeeiro, é responsável por causar alterações no processo fotossintético da planta, causando necrose das folhas e eventual desfolha do cafeeiro, reduzindo a produção e os ganhos econômicos.

A produção de grãos de café, bem como a produção de cereais, cacau, especiarias, uva, frutas secas e rações, entre outros alimentos, também é comprometida pela contaminação dos fungos *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, *Aspergillus flavus* e *Aspergillus parasiticus*. Esses microrganismos, ao contaminarem os alimentos, causam transformações indesejáveis no sabor, aroma e no teor nutricional, causando danos diretos à qualidade e à venda desses alimentos. Além disso, esses microrganismos são responsáveis por produzirem micotoxinas. Essas substâncias são formadas no metabolismo secundário dos fungos e sua presença em alimentos é completamente indesejável, devido a seus malefícios causados à saúde humana, bem como seu alto potencial carcinogênico.

Além da produção de grãos, o Brasil vem ganhando visibilidade na produção de frutas vermelhas, com destaque para o morango. No ano de 2020, a produção dessa fruta foi de

aproximadamente 165 mil toneladas, com uma receita bruta de 81,7 milhões de reais (BRASIL, 2022; EMPRAPA, 2020). A produção de frutas vermelhas, como amora e morango, enfrenta como principal desafio o combate à mosca *Drosophila susukii* (Diptera: Drosophilidae). Essa mosca de origem oriental contamina as frutas vermelhas no período pré e pós-colheita, e se não for controlada, pode diminuir a produção em até 80 %. O ataque dessa mosca ocorre pela ovoposição das fêmeas. As larvas crescem e se alimentam dos frutos, causando, assim, o apodrecimento do fruto, impedindo sua comercialização.

No Brasil, a estratégia mais utilizada para combater o ataque de microrganismos e insetos na lavoura é fazendo o uso de defensivos agrícolas. O uso indiscriminado e equivocado desses produtos pode causar danos ao meio ambiente, aos seres vivos, bem como selecionar espécies resistentes a esses produtos. Com o intuito de desenvolver defensivos agrícolas fungicidas e inseticidas que possuem potencial biológico semelhante aos defensivos agrícolas disponíveis no mercado e que apresentem menos riscos à saúde dos seres vivos e do meio ambiente, pesquisas têm sido realizadas com produtos naturais, como os óleos essenciais classificados como geralmente reconhecidos como seguros (GRAS). Designação da American Food and Drug Administration (FDA) para produtos seguros.

Os óleos essenciais, conhecidos também como óleos voláteis, são metabólitos secundários produzidos pelas plantas, apresentando uma composição química complexa, rica em terpenos e fenilpropanoides. Sua composição química diversificada, constituída por substâncias com as mais variadas funções orgânicas, proporcionam aos óleos essenciais diversas atividades biológicas, como a atividade inseticida, atividade antifúngica, dentre outras.

Os óleos essenciais obtidos a partir das folhas de *Eucalipto* sp. são comumente utilizados para produção de aromatizantes e medicamentos. A composição química desses óleos essenciais é bastante complexa, sendo alguns constituintes químicos como o 1,8-cineol, conhecido também como eucaliptol, α-pineno, *β*-pineno, citronelal e citronelol, comumente encontrados.

O óleo essencial obtido das folhas de *Rosmaniruns officinalis*, planta conhecida popularmente como alecrim, também apresenta uma composição química complexa, geralmente rica em 1,8-cineol, cânfora, α-pineno e cânfeno. Esses compostos possuem atividade anticolinesterasica, que está relacionada ao mecanismo de atividade inseticida.

Para tornar possível a aplicação industrial dos óleos essenciais, controlar sua volatilidade e proteger seus constituintes químicos de fatores que podem degradar ou causar alterações em suas estruturas, eles podem ser incorporados em matrizes poliméricas de nanopartículas e nanofíbras. As nanopartículas são definidas como uma dispersão de partículas

que apresentam um tamanho de 10 - 100 nm; já as nanofibras são classificadas como filamentos ou em multifilamentos contínuos que apresentam pelo menos um diâmetro de 100 nm ou menos.

Polímeros, como a poli(ε-caprolactona) (PCL), obtida a partir da abertura do anel do éster *ε*-caprolactona e o poli(ácido lático) (PLA), que possui como monômero básico o ácido 2-hidróxi-propanoico (ácido lático), podendo ser obtido a partir de recursos naturais, como o amido, estão sendo muito estudados na produção de nanomateriais incorporados com produtos naturais, uma vez que eles possuem como características em comum a biodegradabilidade e a biocompatibilidade. Pode-se destacar que os nanomateriais biodegradáveis e biocompativeis sintetizados a partir desses polímeros apresentam vantagens únicas sobre outros sistemas coloidais de liberação controlada, devido à sua alta área superficial, baixo custo e facilidade de produção.

Objetivou-se neste estudo produzir, caracterizar e avaliar a atividade inseticida *in vivo* e *in vitro* de nanopartículas de poli(ε-caprolactona) incorporadas com o óleo essencial de *Rosmarinus officinalis* sobre a mosca *Drosophila suzukii;* produzir, caracterizar e avaliar a atividade antifúngica *in vivo* e *in vitro* de nanopartículas de poli(ε-caprolactona) incorporadas com os óleos essenciais de *Eucalyptus citriodora*, *Eucalyptus grandis*, *Eucalyptus camaldulensis* sobre o fungo *Hemileia vastatrix*; produzir, caracterizar e avaliar a atividade antifúngica e antimicotoxigenica de nanofibras de poli(ácido lático) incorporadas com o óleo essencial de *Corymbia citriodora* e de seus constituintes isoméricos *β*-citronelol e citronelal sobre os fungos micotoxigenicos *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, *Aspergillus flavus* e *Aspergillus parasiticus*.

2 REFERENCIAL TEÓRICO

2.1 Agricultura no Brasil

O Brasil é um dos principais produtores de alimentos do mundo, tendo a agricultura como uma das principais atividades econômicas do país, podendo ser comparada ao que o petróleo é para a Arábia Saudita. Para o ano de 2022, a estimativa na produção agrícola brasileira é um aumento de aproximadamente 12,5% em relação ao ano anterior, produzindo aproximadamente 284,4 milhões de toneladas de grãos. A área total cultivada no país na safra de 2022 é de aproximadamente 72,1 milhões de hectares, com um crescimento de 4,5% sobre a safra anterior. O Brasil é referência na produção e exportação de soja, algodão e café, que vem ganhando destaque a cada dia. Estima-se que a produção de café para o ano de 2022 seja de aproximadamente 55,74 milhões de sacas de 60kg, com um faturamento bruto de 71,7 bilhões de reais. Além da produção de grãos, o Brasil tem desenvolvido tecnologias e técnicas de manejo para se tornar referência na produção de frutas vermelhas, principalmente na produção de morango e amora (BRASIL, 2022).

Para que o país consiga manter a alta produtividade, é indispensável o uso de defensivos agrícolas para combater microrganismos e insetos-praga na lavoura. Estima-se que em países em desenvolvimento, os defensivos agrícolas causem anualmente 70 mil intoxicações agudas e crônicas que evoluem para óbito. No Brasil, o Ministério da Saúde estima que, por ano, mais de 400 mil pessoas são contaminadas por esses produtos, dos quais 4 mil evoluem para óbito (ARAÚJO; SANTOS; GONSALVES, 2016). Com isso, o desafio para produzir defensivos agrícolas à base de princípios ativos GRAS se torna atual e necessário para uma produção mais sustentável e com menos risco para o meio ambiente e para os seres vivos (BRANDÃO et al., 2022.

2.1.1 Cafeicultura

O cafeeiro *Coffea* ssp. é um arbusto da família Rubiaceae do gênero *Coffea* L., originário na Etiópia e abrange 103 espécies (Figura 1). Dos grãos contidos nos frutos do cafeeiro, é produzido o café, uma bebida com sabor e aroma agradável, que possui como características estimular o sistema nervoso central. O café é uma das bebidas mais consumidas em todo o mundo. Estima-se que nos anos de 2019 e 2020 houve um aumento de 2% no consumo mundial

de café, totalizando 167.592 sacas de 60 Kg consumidos (DE RESENDE et al., 2021; EMBRAPA, 2022).

O cafeeiro chegou no Brasil em 1727, vindo da Guiana Francesa, e atualmente o país é o maior produtor de café do mundo, sendo os estados de Minas Gerais, São Paulo e Espirito Santo líderes na produção. As principais cultivares no país são o *Coffea arabica* L. e o *Coffea canefora* P., sendo o primeiro responsável por produzir cafés de qualidade e finos nos mais variados sabores (BRASIL, 2018; DONG et al., 2017; GARRETT; REZENDE; IFA, 2016).

Figura 1- Aspecto geral de um cafeeiro.

Fonte: Do autor.

A padronização da bebida, bem como a sua boa qualidade, têm atraído consumidores exigentes. A composição química do café pode variar de acordo com vários fatores, como a espécie, variedade genética, condições ambientais, como altitude, temperatura, umidade e processamento pós-colheita. Entre os principais compostos bioativos presentes no café, podem-se destacar a trigonelina, cafeína e os ácidos orgânicos (ácidos clorogênicos, cítrico, málico, quínico, oxálico, tartárico, málico, succínico, lático e acético) (DE RESENDE et al., 2021; SANTIAGO et al., 2020).

A cafeicultura enfrenta diversos desafios para manter a alta na produção, sem deixar de lado a qualidade dos grãos e da bebida, principalmente no que se refere aos danos causados pelo ataque de microrganismos nas lavouras e na pós-colheita. Um dos principais fatores que tem causado diminuição na produção de grãos de café em até 30% a 50% é o ataque do fungo *Hemileia vastatrix* Berkeley & Broome, sendo esse microrganismo responsável por causar a

doença conhecida popularmente como ferrugem do cafeeiro (DE RESENDE et al., 2021). Outro microrganismo que merece destaque é a *Cercospora coffeicola*, fungo responsável por causar a cercosporiose. Quando não controlado, esse microrganismo pode causar prejuízos na produção dos grãos de café em até 30%, além de afetar a qualidade da bebida (VALE et al., 2019).

Além dos desafios enfrentados no controle de microrganismos durante a etapa de plantação e colheita, eles também causam prejuízos na pós-colheita. Os principais fungos responsáveis por contaminar grãos de café durante o transporte e armazenamento são os dos gêneros *Aspergillus* e *Penicillium*. Além de alterar os valores nutricionais dos grãos de café, algumas espécies de *Aspergillus*, como os fungos *Aspergillus westerdijkiae*, *Aspergillus ochraceus*, *Aspergillus carbonarius*, *Aspergillus flavus*, *Aspergillus parasiticus*, entre outros, são produtores de micotoxinas, substância extremamente indesejável em alimentos (BATISTA et al., 2003; TANIWAKI; PITT; MAGAN, 2018).

O Brasil, além de ser o maior produtor de café do mundo, também lidera o programa mundial de pesquisas cafeeiras, que tem como objetivo solucionar os impasses que podem diminuir a produção de café. As pesquisas são realizadas nas áreas de melhoramento genético, biotecnologia, manejo de pragas, irrigação, qualidade de produção, sustentabilidade econômica e na preservação ambiental (BRASIL, 2018).

2.1.2 Produção de frutas vermelhas

As frutas vermelhas pertencentes à família Rosaceae (morango, framboesa, amora-vermelha e cereja-doce), bem como as frutas da família Ericaceae (mirtilo e cranberry), têm sofrido um aumento no seu consumo nos últimos anos devido ao seu alto valor nutritivo e sabores característicos. Essas frutas são uma rica fonte de vitaminas (vitaminas A, C e E), minerais (cálcio, fósforo, ferro, magnésio, potássio, sódio, manganês e cobre), fibra dietética, antioxidantes, compostos fenólicos e açúcares (glicose, frutose). O consumo de frutas vermelhas pode melhorar a saúde mental e física, além de prevenir doenças neurológicas, cardiovasculares, diabetes melitus, obesidade, como também alguns tipos de câncer (COSME et al., 2022; MANGANARIS et al., 2014).

O Brasil, além de ser um dos maiores produtores de grãos do mundo, vem ganhando visibilidade na produção de frutas vermelhas, com destaque para o morango. No ano de 2020, a área total de morango plantada no país foi de 4.500 ha, com uma produção de aproximadamente 165 mil toneladas, gerando uma receita bruta de 81,7 milhões de reais. Os

principais estados produtores de morango são Minas Gerais, Paraná, São Paulo e o Rio Grande do Sul, que têm ganhado visibilidade pela qualidade do fruto, produzido de diferentes formas: orgânico, convencional, em estufa alta, no chão, hidropônico e semi-hidropônico (ABRA, 2019; EMBRPA, 2020).

Apesar do aumento da produção de frutas vermelhas no Brasil e no mundo, essas são altamente perecíveis devido ao seu amadurecimento e amolecimento excessivo, como também estão sujeitas ao ataque de insetos-praga, como o ataque da mosca *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), durante os períodos pré e pós-colheita. Essa mosca é considerada atualmente uma das principais pragas que causam a perda na produção de frutas vermelhas. Além do ataque de insetos, a produção de frutas vermelhas também sofre prejuízos causados pelo ataque de microrganismos, como a contaminação do fungo *Botrytis cinérea*, principalmente no período de pós-colheita (CAETANO et al., 2022; MANGANARIS et al., 2014).

A busca para que o Brasil possa se tornar referência mundial na produção de frutas vermelhas é constante e diversos trabalhos estão sendo realizadas no país para alcançar esse objetivo. Estudos estão sendo realizados na geração de novas cultivares; desenvolvimento de sistema de produção; condição sanitária, para que as mudas tenham condições fisiológicas para gerar maior calibre de fruta e proporcionar um sabor adocicado; bem como pesquisas voltadas no desenvolvimento de novos defensivos agrícolas seguros, que podem ser aplicados em qualquer momento da produção (EMBRAPA, 2020).

2.2 Microrganismos e insetos causadores de perda e qualidade na produção agrícola

As produções agrícolas brasileiras, com destaque para a produção de grãos de café, poderiam ser ainda maiores se não houvesse a contaminação do cafezal pelo fungo *Hemileia vastatrix*, causando perda na produção. Os grãos de cafés, como também as amêndoas, frutos secos, dentre outros alimentos, são contaminados pelos fungos *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, *Aspergillus flavus* e *Aspergillus parasiticus*, o que leva a uma perda na qualidade desses alimentos. Algumas espécies de fungos do gênero *Aspergillus*, além de contaminar os alimentos, mudando seu aspecto visual e nutricional, são capazes de produzir as micotoxinas, substâncias completamente indesejáveis em alimentos devido às suas propriedades maléficas à saúde. A produção de frutas vermelhas também vem sofrendo prejuízos devido ao ataque da mosca *Drosophila suzukii*, causando deteorização e contaminação microbiológica a esses alimentos.

2.2.1 *Hemileia vastatrix* (ferrugem do cafeeiro)

A *Hemileia vastatrix* Berkeley & Broome é um fungo biotrófico, basidiomiceto, da família dos Pucciniaceae. Esse microrganismo é responsável por causar a doença conhecida como ferrugem do cafeeiro, devido ao seu aspecto alaranjado que se assemelha a uma oxidação do ferro (Figura 2). O nome ferrugem do cafeeiro também é utilizado popularmente para se referir a esse microrganismo. Os sintomas dessa doença incluem grande massa de esporos alaranjados na superfície abaxial da folha do cafeeiro. Sua contaminação diminui a área fotossintética da folha, ocasionando a sua queda prematura, em casos mais agravantes, pode levar à morte dos ramos e, consequentemente, da planta (DE RESENDE et al., 2021; ZAMBOLIM, 2016).

Figura 2 - Aspecto geral da ferrugem do cafeeiro.

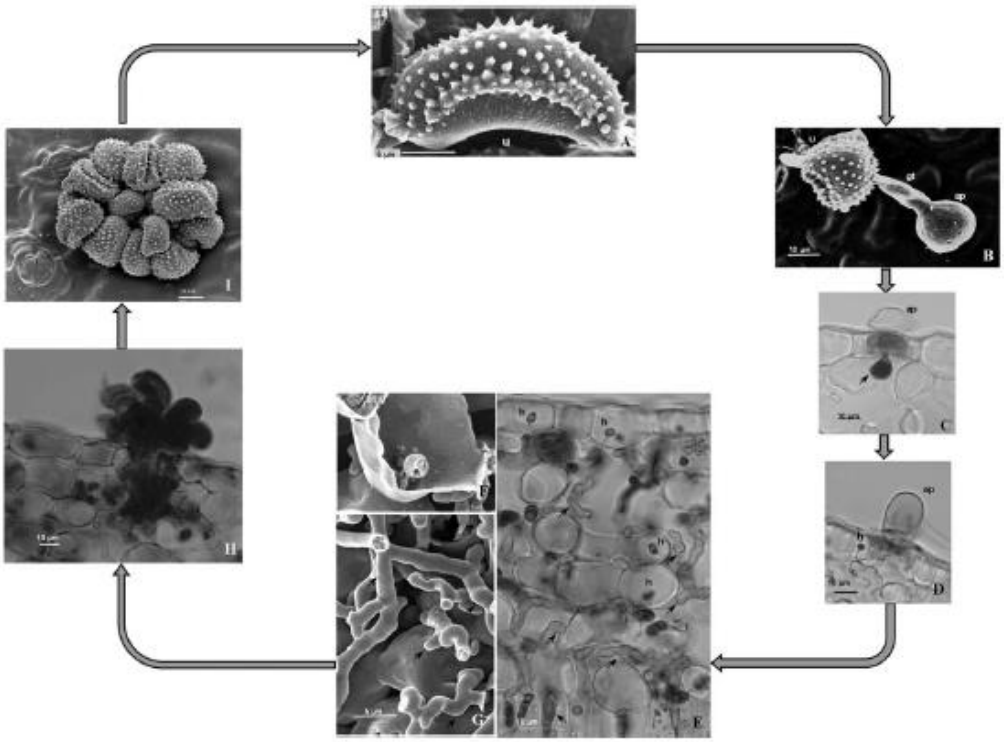
Fonte: Do autor.

A *H. vastatrix*, identificada pela primeira vez por Berkely no Sri Lanka em 1869, foi detectada pela primeira vez no Brasil no sul da Bahia em 1970. A partir de então, esse microrganismo se disseminou por todos os cafezais do país, sendo considerada atualmente por muitos produtores como a principal doença do cafezal. Em plantações de café do gênero *C. arabica* e *C. canefora*, que são susceptíveis a essa doença e onde o clima é mais quente e úmido, as perdas na produção podem chegar a 30 %, caso a doença não seja controlada (CAPUCHO et al., 2013; ROZO et al., 2012; ZAMBOLIM, 2016).

O fungo *H. vastatrix* é hemicíclico, o qual produz uredinosporos, teliosporos e basidiósporos. Os urediniosporos são dicarióticos e representam o ciclo assexual, reinfectando as folhas sempre que as condições ambientais forem favoráveis. Os telósporos ocorrem raramente e germinam *in situ*, produzindo um promicélio a partir do qual são formados quatro basidiósporos. Já os basidiósporos não podem infectar o cafeeiro. O ciclo de vida da *H. vastatrix*

e o processo de infecção no cafeeiro podem ser observados na Figura 3 (TALHINHAS et al., 2017).

Figura 3 - Ciclo de vida da *Hemileia vastatrix*/infecção no cafeeiro.



Legenda: A: uredionosporos; B: uredionosporos germinado (tubo germinativo) 17 h após inoculação; C: hifas de penetração; D: hifas intracelulares com haustório dentro de uma célula subsidiária; E: hifas intracelulares e haustoria nas células epidérmicas e mesófilas 20 dias após inoculação; F: haustório dentro de uma célula parênquima; G: hifas intercelulares na célula de parênquima; H: urediniosporos após 21 dias de incubação; I: uredionosporos analisados da parte de cima.

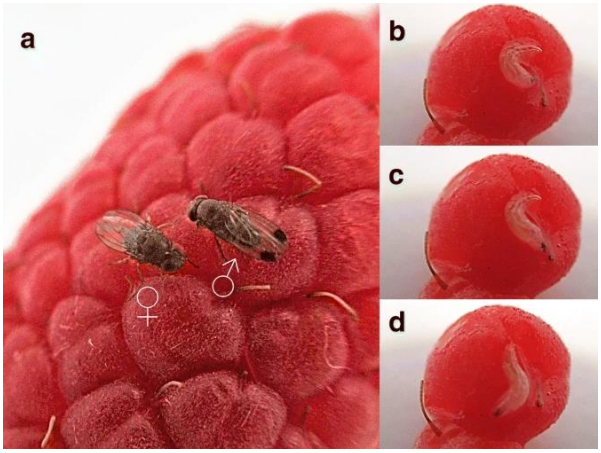
Fonte: Talhinhas et al. (2017).

Para amenizar os danos causados por esse microrganismo, o método mais utilizado é fazer o uso de fungicidas sintéticos à base de Piraclostrobina e Epoxiconazol, como o Opera®. Porém, esses fungicidas, quando utilizados de forma errônea e equivocada, podem causar danos aos seres vivos e ao meio ambiente, além de possibilitar a seleção de espécies resistentes. Alternativas mais seguras, como a plantação de cultivares resistentes a essa doença, como a Araponga MG 1, Catiguá MG 2 e Pau-Brasil MG 1, estão sendo utilizadas por produtores. O controle biológico da ferrugem do cafeeiro é atualmente alvo de pesquisas, e alguns microrganismos, como *Bacillus subtilis* Y1336 e *Lecanicillium lecanii*, se mostraram promissores no controle da *H. vastatrix* (DE RESENDE et al., 2021). Produtos naturais como o óleo essencial de *Eucalyptus* e extrato etanólico de tomilho 2% também se mostraram eficazes no controle da ferrugem do cafeeiro (CAETANO et al., 2020).

2.2.2 *Drosophila suzukii*

A *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) é uma mosca de origem oriental que apresenta dimorfismo sexual: os machos apresentam uma mancha escura nas asas, enquanto as fêmeas, não (Figura 4). Atualmente essa mosca é uma das principais pragas de insetos que causam perdas na produção de frutas vermelhas em todo o mundo. A infestação dessa mosca no campo, se não for controlada, pode levar a uma perda na produção em até 80%. O ataque de *D. suzukii* ocorre pela oviposição das fêmeas no período de pré e pós-colheita. Posteriormente, as larvas crescem e se alimentam dos frutos, causam apodrecimento e impedem a comercialização (CAETANO et al., 2022; DAM, MOLITOR AND BEYER, 2019; ENRIQUEZ et al. 2020).

Figura 4 - Aspecto geral de um macho e fêmea de *Drosophila suzukii*.



Legenda: (a) Adultos fêmeas (esquerda) e machos (direita, com asas manchadas) de *Drosophila suzukii* em um fruto de framboesa. (b – d) Série temporal (b 0 s, c 45 s, d 100 s) de uma larva de *D. suzukii* alimentando-se de um fruto de framboesa.

Fonte: Dam, Molitor e Beyer (2012)

Para amenizar e evitar os danos causados por essa mosca, a prática de manejo mais utilizada se baseia na aplicação de inseticidas sintéticos pertencentes às famílias dos piretroides ou organofosforados. Porém, esses inseticidas, quando aplicados demasiadamente e de forma errônea, podem causar danos similares aos fungicidas sintéticos. Em alguns países, onde o registro de inseticidas é restrito e a aplicação é limitada, como na Alemanha, juntamente com baixa eficácia dos inseticidas, esse método não está sendo muito preciso para eliminar essa praga. Sendo assim, outras medidas de manejo, como a remoção de folhas na zona de cachos, remoção seletiva de frutos maduros, o cobrimento das culturas com redes e a redução dos intervalos de colheita, têm sido utilizadas (DAM; MOLITOR; BEYER, 2019; WINKLER et

al., 2020). O uso de óleos essenciais, como o de *Rosmarinus officinalis*, também se mostrou eficaz no controle dessa mosca, sendo uma alternativa aos inseticidas sintéticos (CAETANO et al., 2022).

A *D. suzukii* possui preferência por frutas maduras, sendo assim, dependendo do estágio do fruto e em frutos já colhidos que estão prontos para o consumo, a aplicação de inseticidas sintéticos é descartada. Com isso, estudos que visam ao desenvolvimento de inseticidas seguros que podem ser aplicados em toda a cadeia produtiva estão em andamento, sendo que o controle biológico de *D. suzukii* ainda está sob fase inicial de investigação em todo o mundo. Estudos com produtos naturais, como os óleos essenciais, também estão em andamento, os mesmos se mostram promissores, uma vez que, os compostos químicos presentes nesses óleos, apresentam atividade inibitória da enzima acetilcolinesterase, sendo esse um mecanismo da ação inseticida (CAETANO et al., 2022; DAM; MOLITOR; BEYER, 2019).

2.2.3 Gênero *Aspergillus*

Os fungos do gênero *Aspergillus* pertencentes à família Trichocomaceae, ao Reino Fungi, divisão Ascomycota, ordem Eurotiales, são um dos principais fungos utilizados na indústria de alimento, porém, alguns são causadores de doença e produtores de micotoxinas. Os *Aspergillus* são os fungos que mais se adaptaram ao crescimento nos trópicos, apresentando temperatura de crescimento acima de 10 ºC e crescem vigorosamente a 37 ºC ou a temperaturas acima. Esses fungos são capazes de se desenvolverem com uma atividade de água (aw) em alimentos de até 0.80. Os valores ótimos de temperatura e aw para o crescimento dos fungos do gênero *Aspergillus*, bem como para a produção de Ocratoxina A (OTA), pode variar entre os isolados da mesma espécie. Durante a fase de crescimento, as colônias apresentam coloração branca e posteriormente a cor vai se transformando para preto, amarelo, verde, castanho, de acordo com cada espécie (TANIWAKI; PITT; MAGAN, 2018).

2.2.3.1 *Aspergillus carbonarius*

Os fungos da espécie *Aspergillus carbonarius* são pertencentes ao gênero *Aspergillus*, seção Nigri, sendo esse considerado um dos principais produtores de OTA. O crescimento ótimo para esse microrganismo ocorre na temperatura de 30 °C e com uma aw de 0.97. Já as condições ótimas de temperatura e aw para a produção de OTA ocorrem em aproximadamente 20 ºC, com aw de 0.99. Esse microrganismo pode ser encontrado naturalmente em café, cacau,

frutos secos e atualmente é considerado um dos principais contaminantes de OTA em uvas e seus derivados (BRANDÃO et al., 2022; MONDANI et al., 2020; MUTLU-İNGÖK et al., 2020).

2.2.3.2 *Aspergillus ochraceus*

Os fungos da espécie *Aspergillus ochraceus* são pertencentes ao gênero *Aspergillus*, seção Circumdati. A temperatura ótima de crescimento para esse fungo e sua atividade de água são de 30 ºC e 0.99 aw, respectivamente. Esse microrganismo também produz em seu metabolismo secundário a micotoxina OTA. A condições ótimas de temperatura e aw para produção de OTA são iguais a de seu crescimento. A presença de OTA em café foi relatada pela primeira vez em 1970, e sabe-se hoje que o *A. ocrhaceus*, juntamente com *A. carbonarius* e *A. westerdijkiae*, são os principais produtores dessa substância nos grãos. Além de grãos de café, esse microrganismo contamina cereais, arroz, trigo, aveia, cevada e vinho (OLIVEIRA et al., 2019; TANIWAKI; PITT; MAGAN, 2018).

2.2.3.3 *Aspergillus westerdijkiae*

Os fungos da espécie *Aspergillus westerdijkiae* são pertencentes ao gênero *Aspergillus*, seção Circumdati. Esses microrganismos apresentam temperatura de crescimento e aw ótima de 30 °C e 0.97 aw, respectivamente. Embora o fungo *Aspergillus ochraceus* seja considerado como potencial produtor de OTA, particularmente em climas quentes, o fungo *Aspergillus westerdijkiae* consegue produzir em até 100 vezes maior quantidade de OTA que o *Aspergillus ochraceus*, sendo considerado, dentro de sua seção, o principal produtor de OTA. As condições ótimas de temperatura e aw para produção de OTA são de 25 °C e 0.97 aw, respectivamente. Esse microrganismo pode ser encontrado naturalmente em alimentos de origem vegetal, como cereais, frutos secos e bebida; além disso, esse fungo é atualmente reconhecido como a principal fonte de OTA em café *arabica*. (TANIWAKI; PITT; MAGAN, 2018; VIPOTNIK; RODRIGUEZ; RODRIGUES, 2017).

2.2.3.4 *Aspergillus flavus*

Os fungos da espécie *Aspergillus flavus* são pertencentes ao gênero *Aspergillus*, seção Flavi. As condições ótimas de temperatura e aw para seu desenvolvimento são de 33 ºC e 0,99

aw, respectivamente. Esse fungo também é um potencial produtor de micotoxinas, sendo elas conhecidas como aflatoxina B1 e B2. As condições ótimas de temperatura e aw para a produção de alfatoxina são de 30 ºC e 0,95 aw, respectivamente. Esses microrganismos são encontrados em amêndoas, rações, nozes e castanha-do-Pará, dentre outros alimentos. (SCHMIDT-HEYDT et al., 2009; TANIWAKI; PITT; MAGAN, 2018).

2.2.3.5 *Aspergillus parasiticus*

A espécie *Aspergillus parasiticus* pertencente ao gênero *Aspergillus*, seção Flavi, é um fungo micotoxigenico produtor de aflatoxinas B_1, B_2, G_1 e G_2. As condições ótimas de temperatura e aw, para seu crescimento e para a produção de aflatoxinas B1, são de 35 °C; 0,99 aw e de 37 ºC; 0,99, respectivamente. Em comparação com o *Aspergillus flavus*, mais de 90% dos isolados de habitats naturais desse microrganismo são capazes de produzir altas concentrações de aflatoxina, sendo que, os isolados de *Aspergillus flavus*, apenas 50% sintetizam essas substâncias. Espécies de *Aspergillus parasiticus* podem ser encontradas naturalmente em amêndoas, rações, milho, nozes e castanha-do-Pará, dentre outros alimentos. Em relação ao *Aspergillus flavus*, esse microrganismo pode ser encontrado com mais frequência em solos, contaminando assim amendoins, entre outros alimentos do solo (SCHMIDT-HEYDT et al., 2010).

2.2.4 Micotoxinas

As micotoxinas são classificadas como substâncias de baixa massa molar produzidas pelo metabolismo secundário de fungos filamentosos, sendo os principais produtores os fungos do gênero *Aspergillus*, *Penicilium* e *Fusarium*. A produção de micotoxinas pode ser influenciada por fatores, como: disponibilidade de nutrientes, fatores ambientais, atividade de água, temperatura, fungicidas, fertilizantes e as interações entre espécies fúngicas toxigênicas, dentre outros. A presença dessas substâncias em alimentos, mesmo que em baixas concentrações, é completamente indesejável, uma vez que as micotoxinas podem causar danos à saúde humana, além de apresentarem alto potencial carcinogênico (BENNETT; KLICH, 2003; GREEFF-LAUBSCHER et al., 2020; SWEENEY, 1998).

Dados obtidos da Food and Agriculture Organization mostram que as micotoxinas são responsáveis pela contaminação de aproximadamente 25% das culturas alimentares do mundo, incluindo milho, uva, café, amendoim, coco, nozes, oleaginosas, entre outros. A contaminação

de alimentos por micotoxinas se estende a todo o processo de produção, até chegar ao produto final, podendo gerar grandes perdas econômicas. Uma vez que seus limites ultrapassem o estabelecido pelos órgãos regulatórios nacionais e transnacionais, esses alimentos tendem a ser descartados ou relocados para alimentação animal, porém, mesmo quando eles são destinados ao trato animal, resíduos dessas substâncias podem aparecer em produtos derivados desse animal. Vacas, ao consumirem alimentos contaminados com aflotoxinas B1, podem biotransformar metabolicamente essa substância em uma forma hidroxilada conhecida como aflatoxina M1, sendo essa uma forma indireta de contaminação de micotoxinas em seres humanos (ABD-ELSALAM; RAI, 2020; BENNETT; KLICH, 2003).

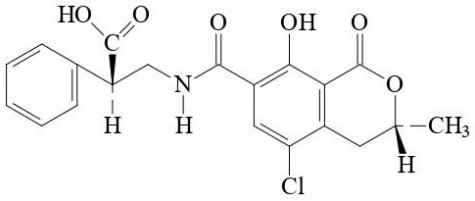
A forma mais segura para evitar a contaminação de alimentos por micotoxinas é impedir o desenvolvimento de fungos micotoxigênicos durante o processo de cultivo, armazenamento e transporte desses alimentos, uma vez que o alimento já esteja contaminado, a remoção dessas substâncias se torna um desafio difícil de ser superado. As estruturas químicas complexas das micotoxinas proporcionam a essas substâncias possíveis ligações de hidrogênio e ligações dipolo-dipolo intermoleculares, promovendo uma relativa estabilidade térmica e dificultando sua remoção por processos térmicos (ABD-ELSALAM et al., 2017; BOONME et al., 2020).

No Brasil, a Agência Nacional de Vigilância Sanitária (Anvisa) é o órgão responsável por estabelecer limites máximos dessas micotoxinas em alimentos. Entre as micotoxinas, podem-se destacar como as principais a OTA e a aflatoxina B1.

2.2.4.1 Ocratoxina A

A OTA é uma micotoxina produzida por fungos do gênero *Aspergillus* (Figura 5). Essa micotoxina foi descoberta em 1965 a partir de isolados *Aspergillus ochraceus*, e pouco tempo depois, foi identificada em isolados de fungos do mesmo gênero, como *Aspergillus alliaceus*, *Aspergillus carbonarius* e *Aspergillus niger* (BENNETT e KLICH, 2003).

Figura 5 – Estrutura química da ocratoxina A.



Fonte: Do autor.

A produção de ocratoxina A, bem como das outras micotoxinas, pode ser influenciada pelo substrato no qual os fungos crescem, bem como o nível de umidade, temperatura e presença de microflora competitiva. A quantidade de OTA produzida pelos diversos gêneros de *Aspergillus* pode variar, sendo que, entre os gêneros *A. carbonarius, A. westerdijkae* e *Aspergillus ochraceus*, os maiores produtores são os dois primeiros (BRANDÃO et al., 2020; PASSAMANI, et al., 2014).

A presença de OTA em alimentos é comumente encontrada em aveia, centeio, trigo, grãos de café, vinhos, nos quais as uvas foram contaminadas por *A. carbonarius* e em cevada, sendo o último com um alto grau de contaminação. O consumo de alimentos contaminados por essa micotoxina pode levar a casos de micotoxicose, gerando danos adversos à saúde humana. A OTA é classificada pela Agência Internacional para Pesquisa em Câncer (International Agency for Research on Câncer – IARC) como possível carcinógeno humano do Grupo 2B, além de apresentar efeitos nefrotóxicos, imunossupressores e teratogênicos. Além disso, entre as micotoxinas, a OTA é a que apresenta o tempo de meia-vida mais longo; sendo assim, é a micotoxina que demora mais tempo para ser eliminada do organismo (BENNETT e KLICH, 2003; BOONME et al., 2020; IARC, 1993).

Os limites estabelecidos para OTA pela Resolução RDC n° 7, de 18 de fevereiro de 2011, modificada pela Resolução RDC n° 138, de 8 de fevereiro de 2017, estão descritos no Quadro 1 (BRASIL, 2011 e 2017).

Quadro 1 - Limites máximos (μg Kg^{-1}) de OTA em alguns alimentos estabelecidos no Brasil.

Micotoxinas	Alimentos	LMT (μg/kg)
Ocratoxina A	Cereais e produtos de cereais, incluindo cevada malteada	10
	Feijão	10
	Café torrado (moído ou em grão) e café solúvel	10
	Vinho e seus derivados	2
	Suco de uva e polpa de uva	2
	Especiarias: *Capsicum* spp. (o fruto seco, inteiro ou triturado, incluindo pimentas, pimenta em pó, pimenta decaiena e pimentão-doce) *Piper* spp. (o fruto, incluindo a pimenta-branca e a pimenta-preta) *Myristica fragrans*(noz-moscada) *Zingiber officinale* (gengibre) *Curcuma longa* (curcuma) Misturas de especiarias que contenhamuma ou mais das especiarias acima indicadas	30
	Alimentos à base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	2
	Produtos de cacau e chocolate	5
	Amêndoa de cacau	10
	Frutas secas e desidratadas	10
	Cereais para posterior processamento, incluindo grão de cevada	20

Legenda: LMT – limites máximos tolerantes. Fonte: Brasil (2011 e 2017).

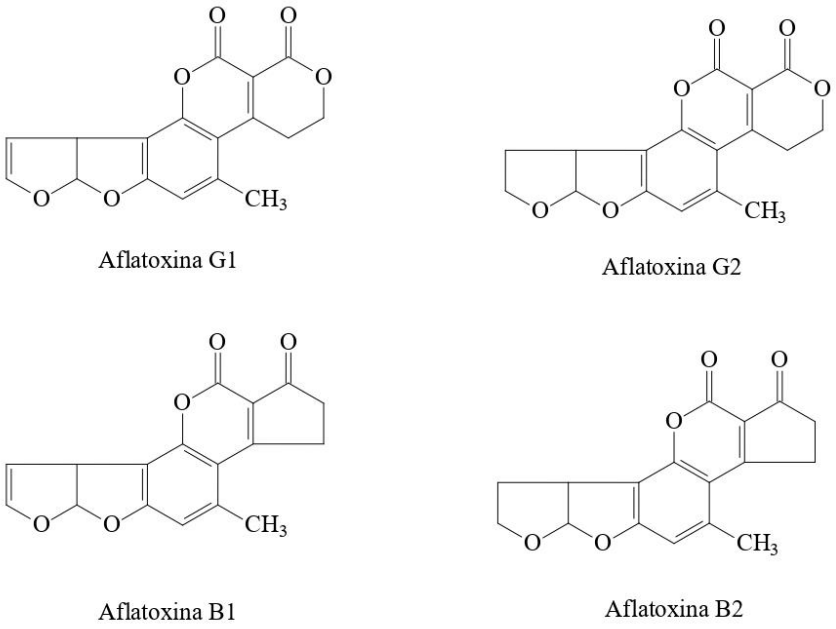
2.2.4.2 Aflatoxinas

As aflatoxinas, como a OTA, também são produzidas por fungos do gênero *Aspergillus*. As principais Aflatoxinas são a B1, B2, G1 e G2, sendo a Aflatoxina B1 a que apresenta o maior potencial carcinogênico entre as micotoxinas (Figura 6). A contaminação de alimentos por essas micotoxinas tem causado grandes prejuízos econômicos mundiais. Nos Estados Unidos, a safra do ano de 2020 teve um prejuízo de aproximadamente US$ 932 milhões, devido à contaminação por aflatoxinas e pelas micotoxinas fumonisinas e desoxinivalenol (ABD-ELSALAM; RAI, 2020; BENNETT; KLICH, 2003).

Essas micotoxinas são derivadas da difuranocumarina, produzida por uma via policetídica por cepas como *Aspergillus flavus* e *Aspergillus parasiticus*, sendo o primeiro um dos principais contaminantes na agricultura. A faixa de temperatura ótima de crescimento de fungos Aflatoxigenicos, bem como a temperatura ótima de produção dessas substancias, são quase idênticas, variando de 10 - 43°C e 12 - 40°C, respectivamente. As aflatoxinas são encontradas naturalmente em toda cadeia produtiva de alimentos, como figo, milho, amendoim,

oleaginosas, nozes, entre vários outros produtos (BENNETT; KLICH, 2003; GREEFF-LAUBSCHER, et al., 2020).

Figura 6 - Estrutura química das aflatoxinas B1, B2, G1 e G2.



Fonte: Do autor.

Os limites estabelecidos para aflatoxinas B1, B2, G1 e G2 pela Resolução RDC n° 7, de 18 de fevereiro de 2011, modificada pela Resolução RDC n° 138, de 8 de fevereiro de 2017, estão descritos no Quadro 2 (BRASIL, 2011 e 2017).

Quadro 2 - Limites máximos (μg Kg^{-1}) de Aflatoxinas B1, B2, G1 e G2 em alguns alimentos estabelecidos no Brasil.

Micotoxinas	**Alimentos**	**LMT (μg/kg)**
Aflatoxinas B1, B2, G1 e G2	Cereais e produtos de cereais, exceto milho e derivados, incluindo cevada malteada	5
	Feijão	5
	Castanhas exceto castanha-do-brasil, incluindo nozes, pistachios, avelãs e amêndoas	10
	Frutas desidratadas e secas	10
	Castanha-do-brasil com casca para consumo direto	20
	Castanha-do-brasil sem casca para consumo direto	10
	Castanha-do-brasil sem casca para processamento posterior	15
	Alimentos à base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	1
	Fórmulas infantis para lactentes e fórmulas infantis de seguimento para lactentes e crianças de primeira infância	1
	Amêndoas de cacau	10
	Produtos de cacau e chocolate	5
	Especiarias: *Capsicum* spp. (o fruto seco, inteiro ou triturado, incluindo pimentas, pimenta em pó, pimenta decaiena e pimentão-doce); *Piper* spp. (o fruto, incluindo a pimenta-branca e a pimenta-preta) *Myristica fragrans*(noz-moscada) *Zingiber officinale* (gengibre) *Curcuma longa* (curcuma). Misturas de especiarias que contenhamuma ou mais das especiarias acima indicadas	20
	Amendoim (com casca), (descascado, cru ou tostado), pasta de amendoim ou manteiga de amendoim	20
	Milho, milho em grão (inteiro, partido, amassado, moído), farinhas ou sêmolas de milho	20
	Castanha-do-brasil sem casca para processamento posterior	15
	Alimentos à base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	1
	Fórmulas infantis para lactentes e fórmulas infantis de seguimento para lactentes e crianças de primeira infância	1

Legenda: LMT – limites máximos tolerantes. Fonte: Brasil (2011 e 2017).

2.3 Óleos essenciais

Os óleos essenciais são usados pelos seres humanos para diversos fins, antes do século IX, sendo descritos como o processo de destilação que os árabes usaram para extrair esses óleos e levá-los para a Europa. São denominados também por óleos voláteis, óleos etéreos ou essências, e definidos pela ISO (International Standard Organization) como produtos obtidos de diferentes partes das plantas mediante destilação por arraste com vapor d'água, bem como produtos obtidos por expressão dos pericarpos de frutos cítricos. Esses óleos apresentam-se geralmente líquidos a temperatura ambiente e exalam um aroma agradável. Suas principais características que os diferem dos óleos fixos são sua alta volatilidade e sua composição química predominantemente de terpenos e fenilpromanoides. Os óleos essenciais possuem alta solubilidade em solventes apolares, como éteres e acetona, e são praticamente insolúveis em água; porém, sua solubilidade limitada em água é suficiente para aromatizar soluções aquosas, denominadas hidrolatos (HANIF et al., 2019; SIMÕES et al., 2017).

Quando recém-extraídos geralmente apresentam-se incolores ou ligeiramente amarelados com exceção para os óleos que apresentam como constituinte químico o azuleno. Em relação a sua estabilidade, eles são pouco estáveis na presença de ar, luz, calor, umidade e metais. A maioria dos óleos essenciais apresenta atividade ópticas, ou seja, conseguem desviar o plano de luz polarizada devido à presença de C* (carbono quiral) na estrutura química de seus constituintes (SIMÕES et al., 2017).

A composição química de um óleo essencial é bastante complexa, em que a classe de seus constituintes químicos terpenos e fenilpropanoides podem se apresentar com as mais variadas funções orgânicas (álcoois, aldeídos, ésteres, éteres, cetonas, compostos de enxofre e fenóis). Os constituintes químicos dos óleos essenciais que se apresentam em maior proporção em relação aos demais são denominados constituintes químicos majoritários, sendo o restante denominados constituintes minoritários. Além disso, em sua composição, também é possível encontrar constituintes traços, que são substâncias que se apresentam em baixíssima quantidade, comumente difíceis de serem identificados e quantificados (SIMÕES et al., 2017).

Os óleos essenciais, por serem metabólitos secundários, podem variar sua composição química dentro da mesma espécie de planta, devido a fatores, como a sazonalidade, temperatura, radiação ultravioleta, nutrientes do solo, ataque de patógenos, altitude, poluição atmosférica, disponibilidade hídrica, estímulos mecânicos, idade da planta e época de colheita (DUARTE et al., 2018; GOOBO-NETO; LOPES, 2007; SIMÕES et al., 2017). A composição química diversificada dos óleos essenciais proporciona a essas substâncias diversas atividades

biológicas, como a atividade antifúngica, inseticida, carrapaticida, bactericida e antioxidante. A diversidade de atividades biológicas, atrelada a sua segurança de aplicação, uma vez que esses óleos são considerados substâncias GRAS pelo FDA (American Food and Drug Administration), fazem com que eles sejam alvo de pesquisas e desenvolvimento de produtos na indústria agrícola, de alimentos e farmacêutica (BRANDÃO et al., 2022; CAETANO et al., 2022; DA SILVA LUNGUINHO et al., 2021; NOGUEIRA et al., 2021).

Nas plantas, os óleos essenciais exercem a função de atuar na proteção contra herbívoros e microrganismos, atraem agentes polinizadores e dispersores de semente, além de apresentarem atividades alelopáticas (GOOBO-NETO; LOPES, 2007).

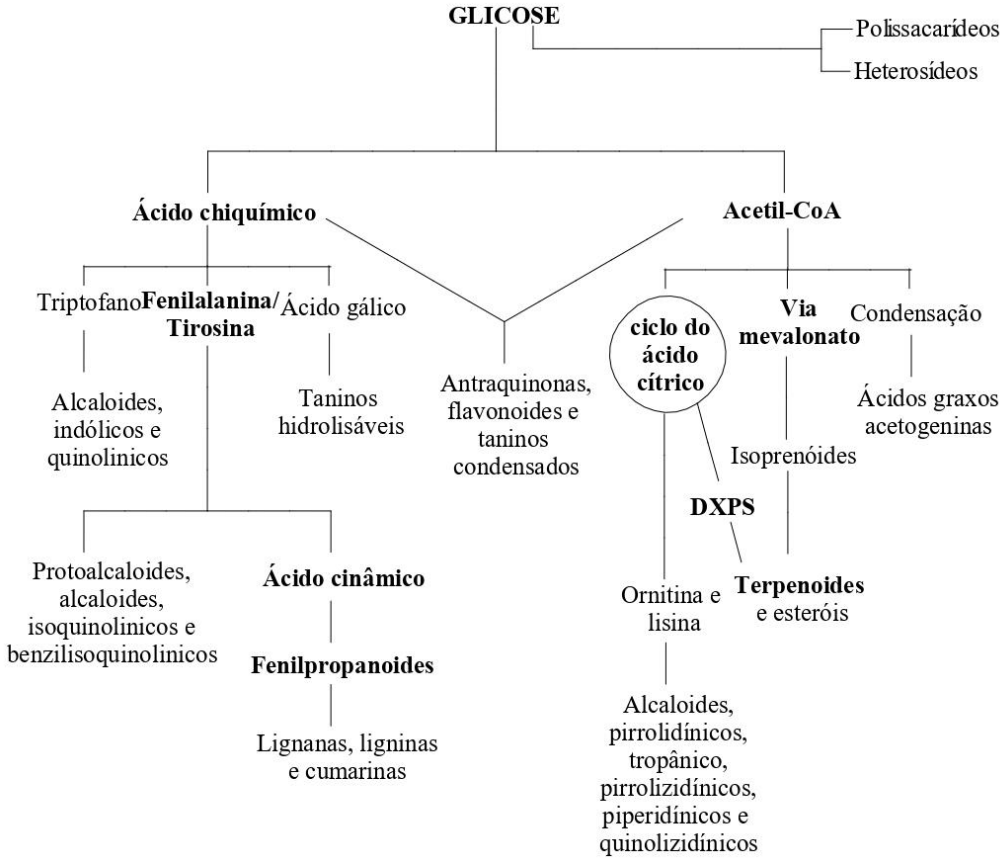
Os constituintes químicos dos óleos essenciais são encontrados em todos os órgãos das plantas, como nas flores (rosa, lavanda e no botão floral do cravo-da-índia), nas folhas (eucalipto, alecrim, segurelha, hortelã e tomilho), rizomas (gengibre), raízes (vetiver), sementes (coentro e carvi), frutas (anis, funcho e epicarpos cítricos), madeira e casca (em sândalo, canela e pau-rosa), entre várias outras espécies de plantas (HANIF et al., 2019; SIMÕES et al., 2017).

Os óleos essenciais podem ser extraídos por técnicas, como a prensagem (método comumente empregado para extrair óleos essenciais de pericarpos de frutos cítricos), arraste por vapor d'água e hidrodestilação, sendo os dois últimos os mais empregados industrialmente (ROOHINEJAD et al., 2017; SIMÕES et al., 2017).

2.3.1 Biossíntese dos compostos dos óleos essenciais

A formação de todos os metabolitos secundários produzidos pelas plantas pode ser resumida a partir do metabolismo da glicose, via dois intermediários principais: o ácido chiquímico, que dará origem aos compostos aromáticos e o Acetil-CoA (Figura 7), que dará origem a compostos pertencentes às classes dos terpenos e fenilpropanoides, sendo os principais constituintes dos óleos essenciais (SIMOES et al., 2017).

Figura 7 - Ciclo biossintético dos metabólitos secundários.

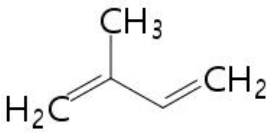


Fonte: Simões et al. (2017).

2.3.1.1 Terpenos

Os terpenos são os constituintes predominantes na composição química dos óleos essenciais e podem ser produzidos por duas vias sintéticas distintas, a via do mevalonato, que ocorre nos citosol e no retículo endoplasmático, conhecida também como via clássica para a formação dos terpenos e pela via 1-deoxi-D-xilulose-5-fosfato (DXPS) de ocorrência nos plastídios. Entre as classes dos terpenos, os monoterpenos e sesquiterpenos que apresentam 10 e 15 átomos de carbono, respectivamente, são os compostos mais encontrados formados por unidades básicas de 2-metil-1,3-butadieno (isopreno) (Figura 8). (BUCHANAN; GRUISSEM; JONES, 2000; DEWICK, 2009; SIMÕES et al., 2017).

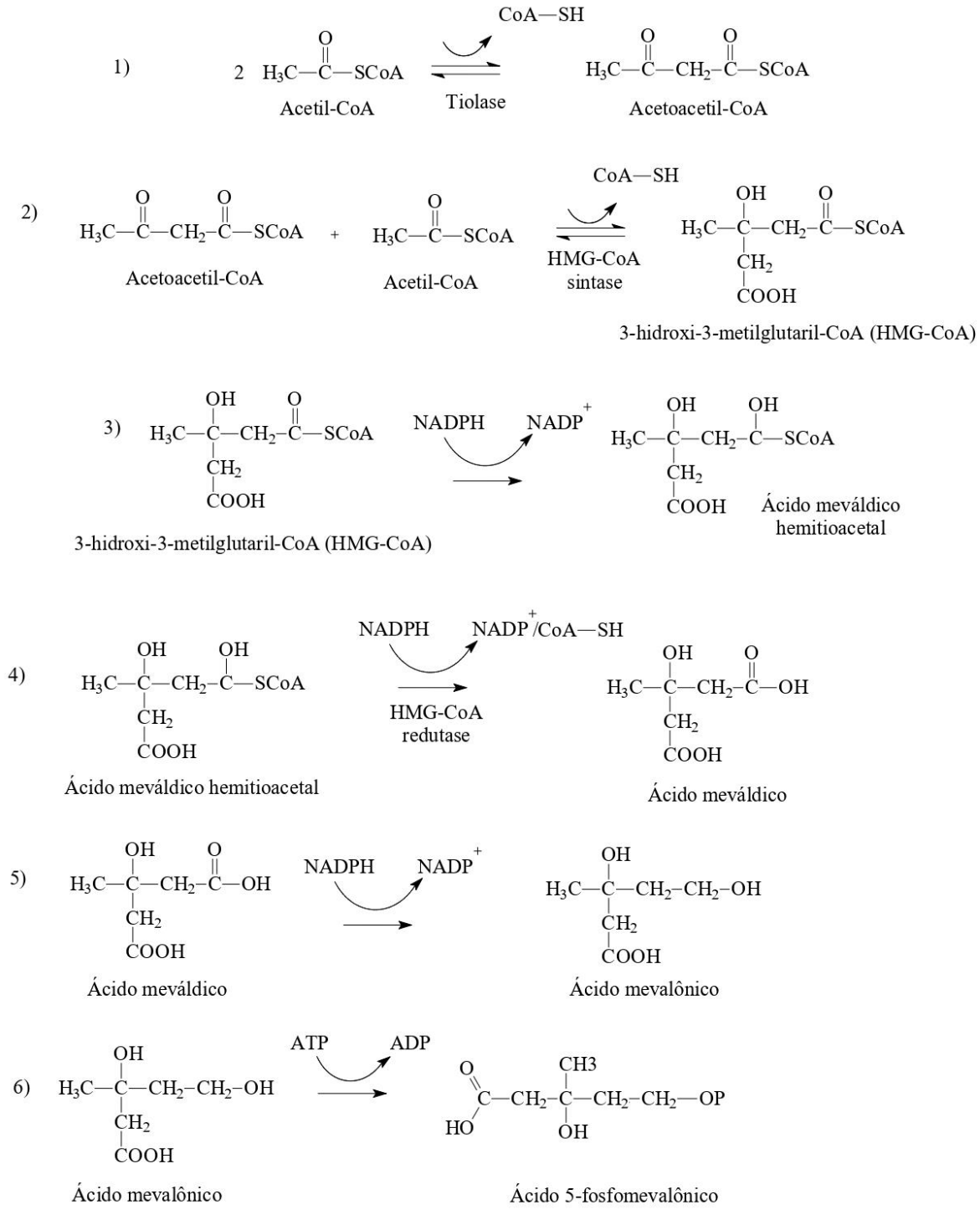
Figura 8 - Estrutura química do isopreno.

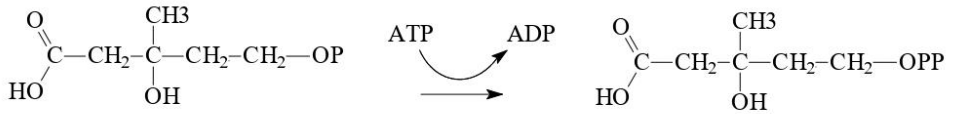


Fonte: Do autor.

O mecanismo detalhado para a formação dos terpenos pela via do mevalonato está apresentado na Figura 9. Na primeira etapa da síntese, ocorre a condensação de três moléculas de Acetil-CoA catalisada pelas enzimas Tiolase e Hidroximetilglutaril-CoA, formando como produto o 3-hidroxi-3-metilglutaril-CoA (HMG-CoA). Posteriormente, o HMG-CoA é reduzido a ácido mevalônico pela enzima HMG-CoA redutase utilizando 3 moléculas de NADPH. Em seguida, o ácido mevalônico sofre 3 fosforilações com 3 moléculas de ATP para formar o ácido 3-fosfo-5-pirofosfatomevalônico. Subsequentemente, o ácido 3-fosfo-5-pirofosfatomevalônico sofre uma descarboxilação catalisado pela coenzima biotina, formando o Isopentenil PP (IPP). O produto formado catalizado por uma enzima isomerase forma o Dimetilalil PP (DMAPP), que sofre uma desfosforilação por ação de um ADP seguido de uma adição eletrofílica do IPP, formando o Geranil PP (GPP), que dará origem a todos os monoterpenos e sesquiterpenos (ADAM 1998; BUCHANAN; GRUISSEM; JONES, 2015; DEWICK, 2009).

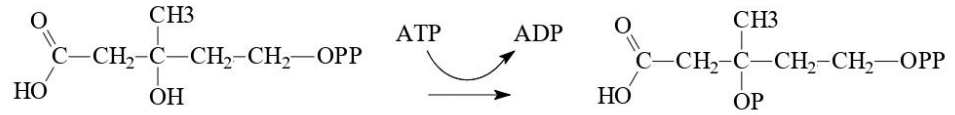
Figura 9 - Rota metabólica para a formação de monoterpenos a partir do ácido mevalônico.



7) 

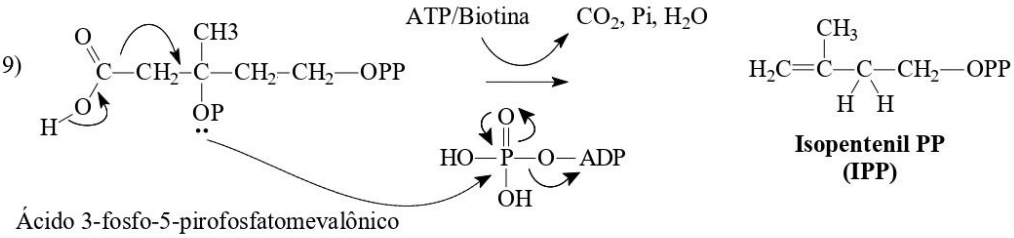
Ácido 5-fosfomevalônico

Ácido 5-pirofosfatomevalônico

8) 

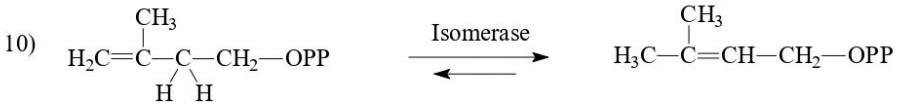
Ácido 5-pirofosfatomevalônico

Ácido 3-fosfo-5-pirofosfatomevalônico

9) 

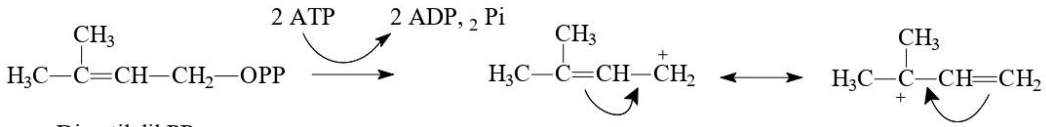
Ácido 3-fosfo-5-pirofosfatomevalônico

Isopentenil PP (IPP)

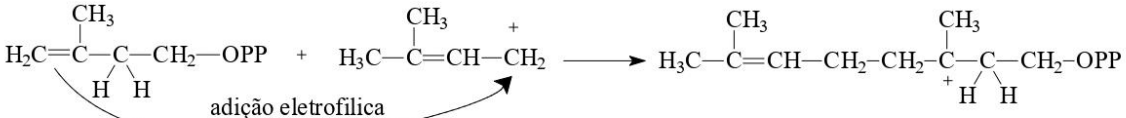
10) 

Isopentenil PP (IPP)

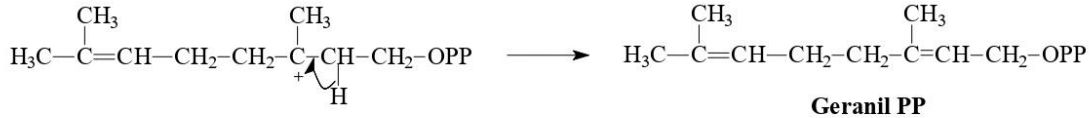
Dimetilalil PP (DMAPP)

11) 

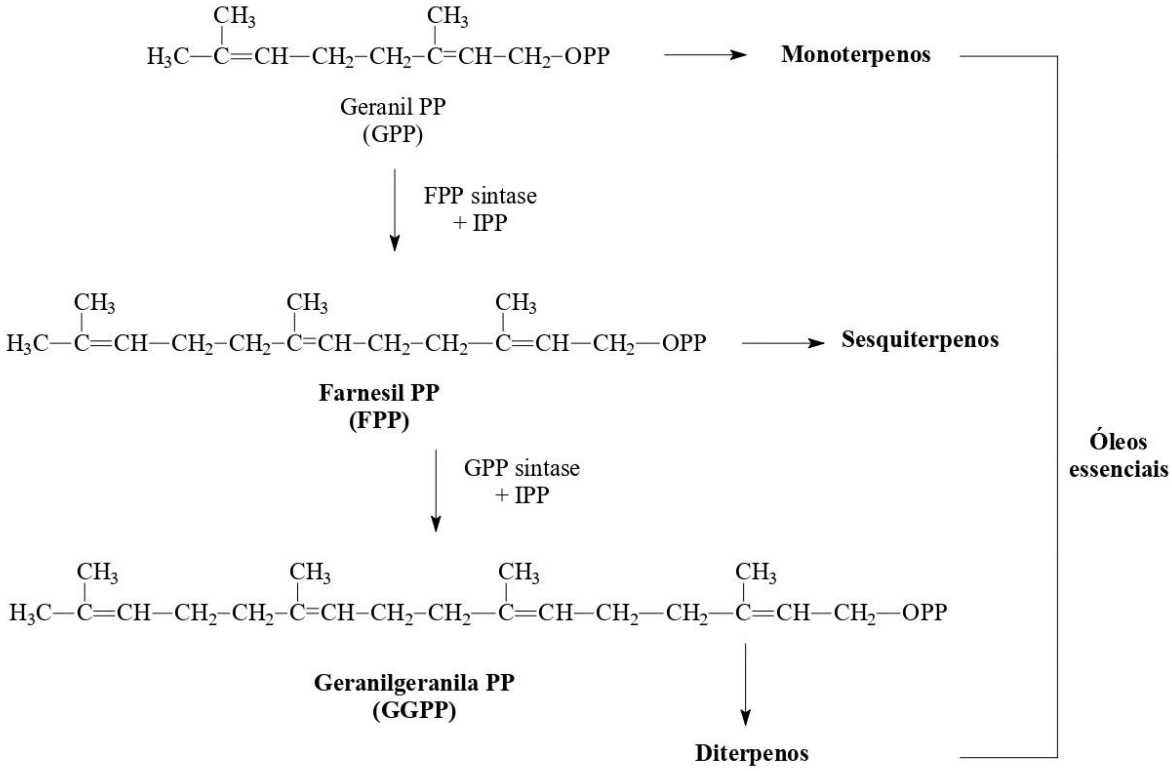
Dimetilalil PP (DMAPP)

12) 

Isopentenil PP (IPP)

13) 

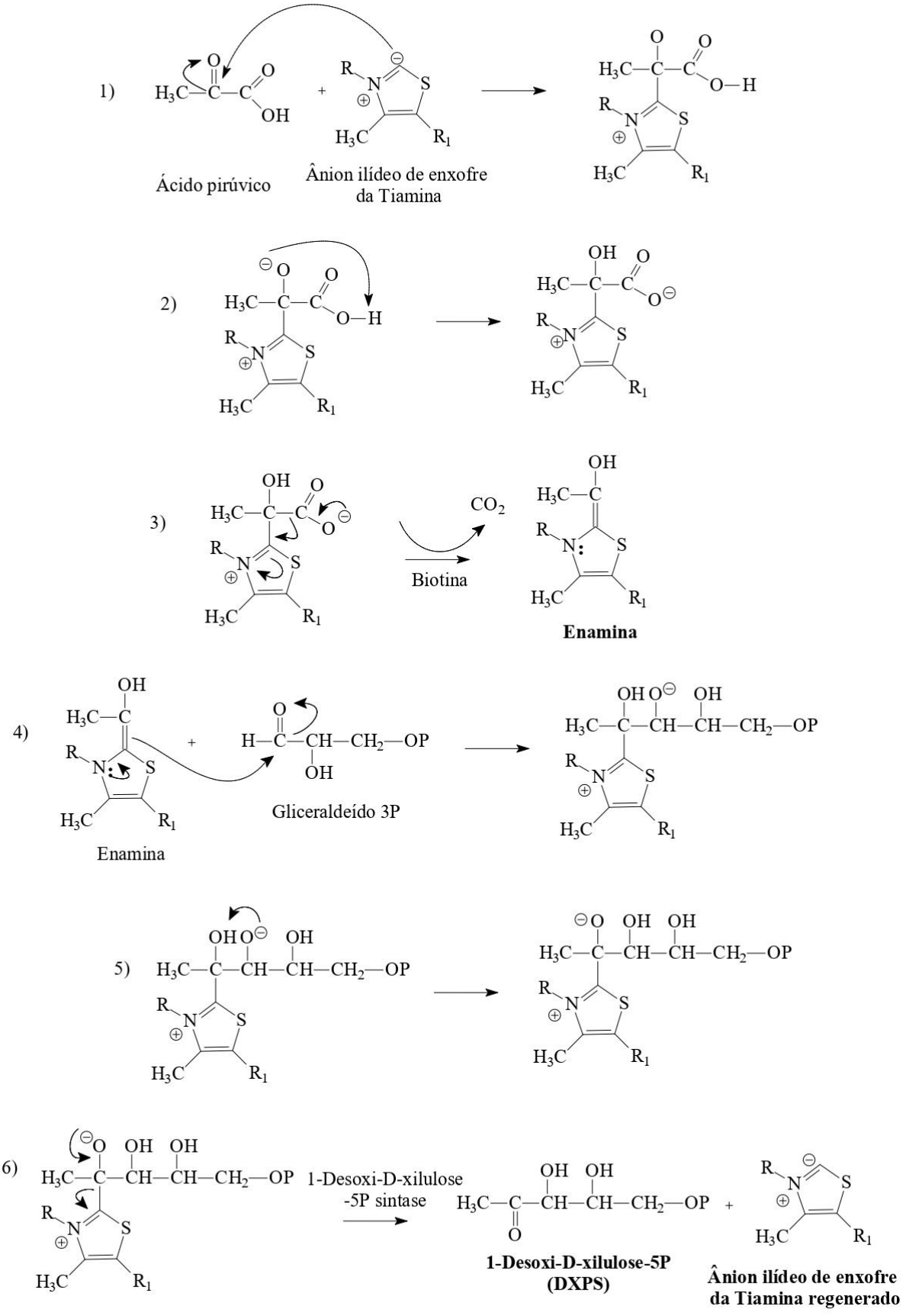
Geranil PP (GPP)

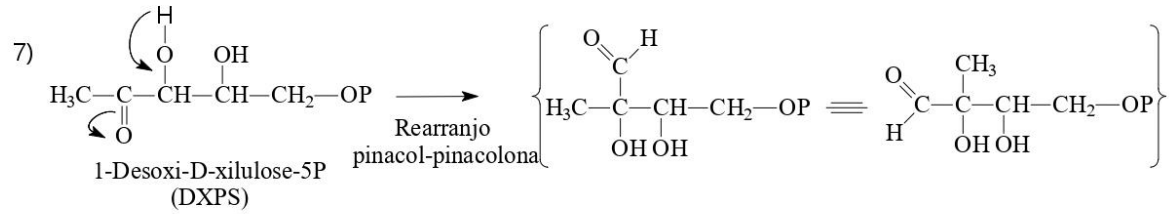


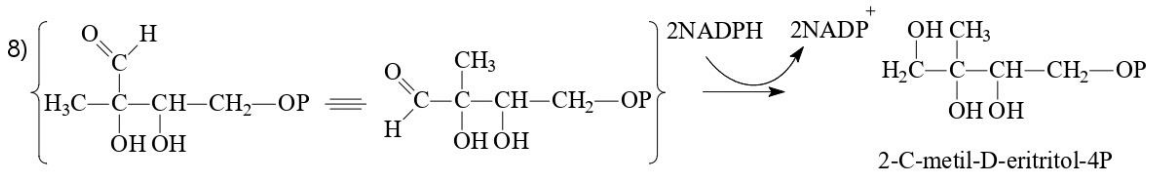
Fonte: Fonte: Adaptada de Buchanan; Gruissem e Jones (2015); Dewick (2009).

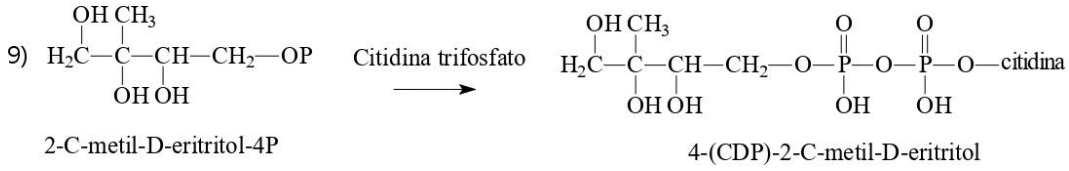
A formação do IPP pela rota alternativa DXPS ocorre inicialmente pela adição ao grupo carbonílico do piruvato pelo ânion ilideo de enxofre da tiamina (Figura 10). Em seguida, o produto formado é catalisado pela coenzima biotina, formando como produto a enamina. Posteriormente, a enamina se rearranja e é catalisada pela enzima 1-Desoxi-D-xilulose-5P-sintase, formando como produto a 1-Desoxi-D-xilulose-5P (DXPS), regenerando também ânion ilideo de enxofre da tiamina. Em seguida, a DXPS sofre um rearranjo pinacol-pinacolona e, em seguida, é reduzida a 2-C-metil-D-eritritol-4P, usando 2 moléculas de NADPH. O produto formado sofre, então, a adição da cistidina e posterior fosforilação, formando a 2-fosfo-4-(CDP)-2-C-metil-D-eritritol-4P. Em seguida, essa molécula se rearranja liberando cistidina fosfato e formando como produto 2-C-metil-D-eritritol-2,4-ciclofosfato. Posteriormente, esse composto cíclico sofre um tautomerismo ceto-enólico, seguido por uma redução (NADPH), remoção de H_2O, redução (NADPH) e remoção de H_2O para formar o isopentenil PP (IPP). A partir desse estágio, a reação segue o mesmo mecanismo da via do mevalonato para formação dos monoterpenos (ADAM 1998; BUCHANAN; GRUISSEM; JONES, 2015; DEWICK, 2009).

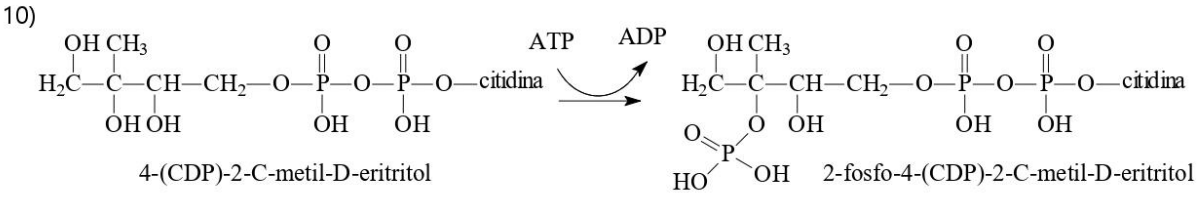
Figura 10 - Rota metabólica para a formação de monoterpenos a partir da via DXPS.

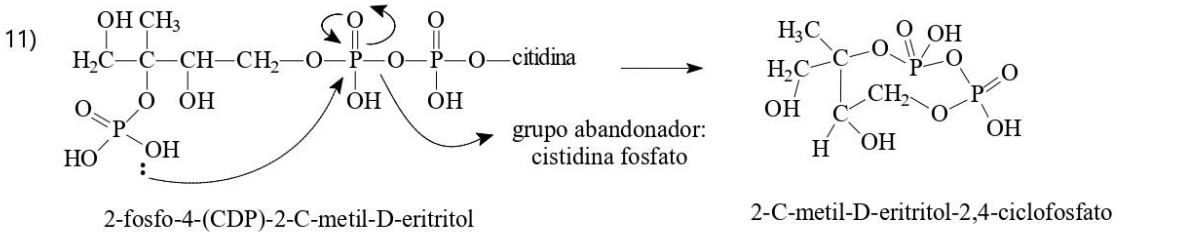


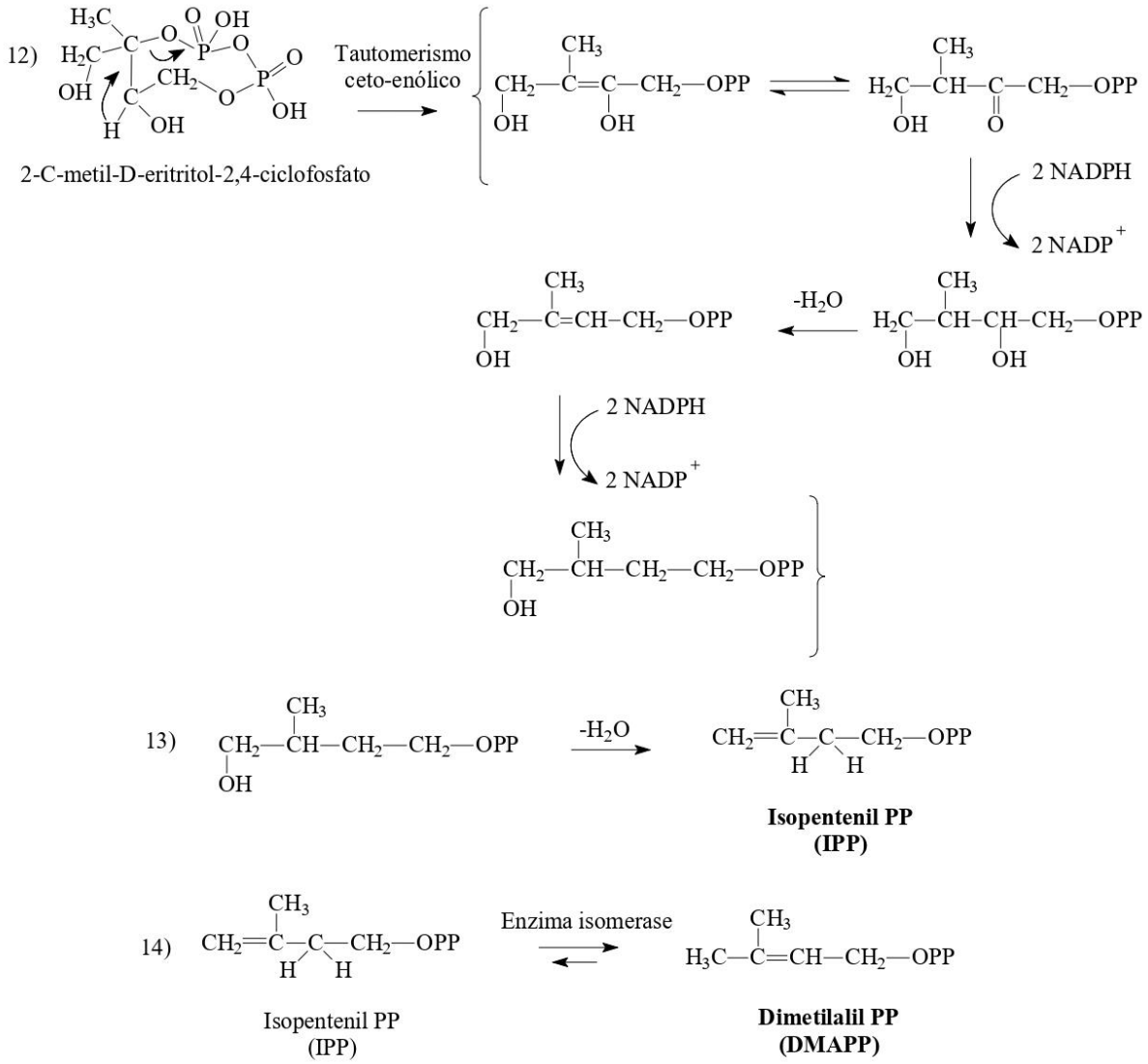

7)
1-Desoxi-D-xilulose-5P
(DXPS)
Rearranjo
pinacol-pinacolona


8)
2NADPH
2NADP+
2-C-metil-D-eritritol-4P


9)
Citidina trifosfato
2-C-metil-D-eritritol-4P
4-(CDP)-2-C-metil-D-eritritol


10)
ATP
ADP
4-(CDP)-2-C-metil-D-eritritol
2-fosfo-4-(CDP)-2-C-metil-D-eritritol


11)
grupo abandonador:
cistidina fosfato
2-fosfo-4-(CDP)-2-C-metil-D-eritritol
2-C-metil-D-eritritol-2,4-ciclofosfato



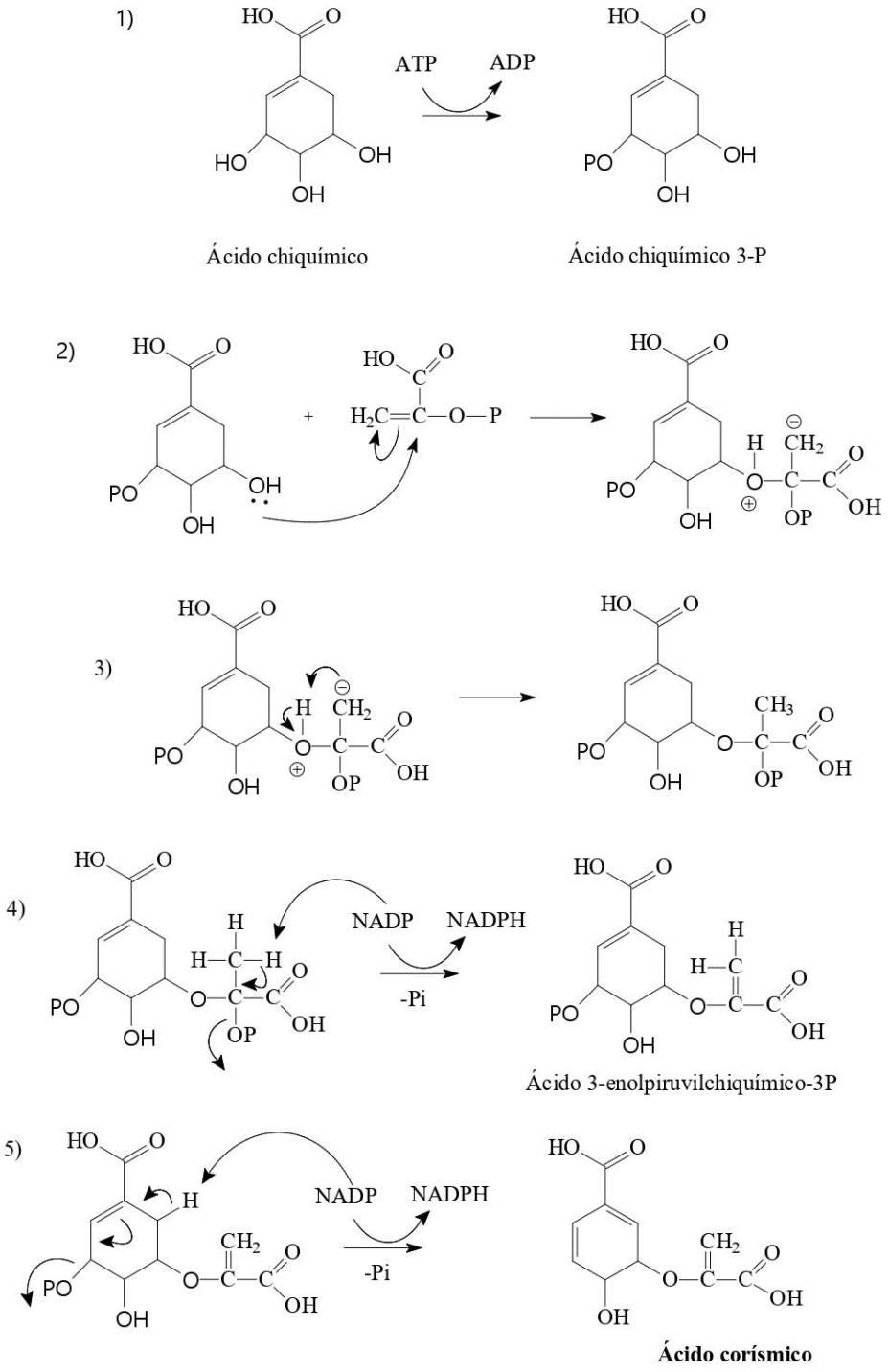
Fonte: Adaptada de Buchanan; Gruissem e Jones (2015); Dewick (2009).

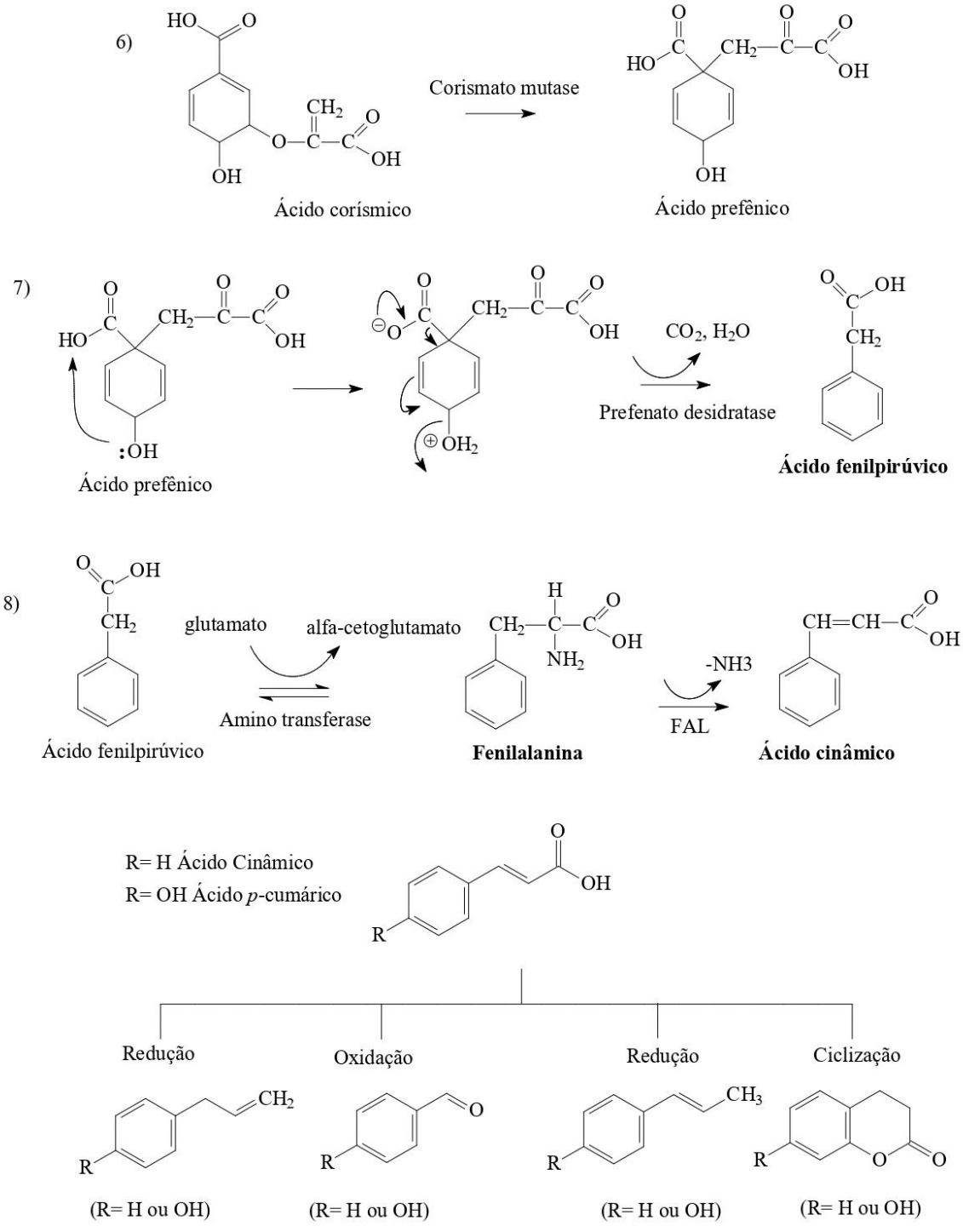
2.3.1.2 Fenilpropanoides

Os fenilpropanoides são formados a partir do ácido chiquímico, inicialmente fosforilado, formando o ácido chiquímico-3P, que posteriormente reage com o fosfoenolpiruvato seguido de uma oxidação e liberação de Pi para formar o ácido 3-enolpiruvilchiquimico-3P. Em seguida, esse ácido sofre uma redução, formando o ácido corísmico. Pela ação da enzima corismato mutase, o ácido corísmico é transformado em ácido prefênico, que, por sua vez, é catalizado pela enzima prefenato desidratase para formar ácido fenilpirúvico. Esse ácido formado reage com o glutamato, em que a reação é catalisada pela

enzima amino transferase, para formar como produto a fenilalanina. A enzima fosfatase alcalina (FAL), então, atua sobre a Fenilalanina, gerando como produto o ácido cinâmico. O ácido formado pode sofrer redução enzimática, produzindo propenilbenzeno e/ou alilbenzenos e, por meio de uma oxidação e redução das cadeias laterais, geram os diferentes fenilpropanoides (Figura 11).

Figura 11 - Biossíntese de fenilpropanoides.





Fonte: Adaptada de Dewick (2009) e Simões et al. (2017).

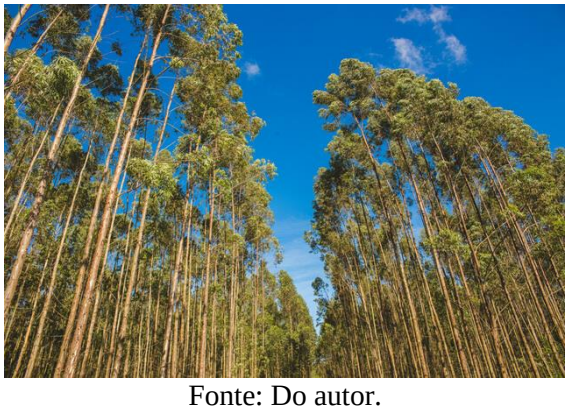
2.3.2 Óleos essenciais de *Eucalyptus* e *Rosmarinus officinalis*

Entre os principais óleos essenciais utilizados, podem-se destacar os obtidos a partir das folhas das plantas das espécies de *Eucalyptus* e *Rosmarinus officinalis*.

2.3.2.1 *Eucalyptus*

Os eucaliptos (Figura 12) são uma das plantas mais utilizadas para produção de celulose. O gênero *Eucalyptus* foi descrito pela primeira vez em 1788 pelo botânico francês L'Heritier e abrange mais de 800 espécies. O gênero é originário da Austrália e Tasmânia. Atualmente, essa planta pode ser encontrada em todo o mundo, devido à sua fácil adaptabilidade e rápido crescimento. No Brasil, há relatos do plantio das primeiras mudas em meados de 1824 no Jardim Botânico do Rio de Janeiro. O cultivo do eucalipto é prioritário para a obtenção de celulose, produção de madeira e carvão. Das suas folhas podem-se extrair os óleos essenciais; porém, essa prática industrial é baixa, mas tem aumentado nos últimos anos. Aproximadamente 300 espécies de *Eucalyptus* apresentram óleos essenciais em suas folhas, entre elas, 20 espécies apresentam alta concentração de 1,8-cineol (mais de 70%), fazendo com que esses monoterpeno oxigenado ficasse conhecido também por eucaliptol (CASTRO et al., 2016; DHAKAD et al., 2018; SABO; KNEZEVIC, 2019; SILVA; BRITO; SILVA JUNIOR, 2006).

Figura 12 - Aspecto geral de uma espécie de *Eucalyptus* sp.

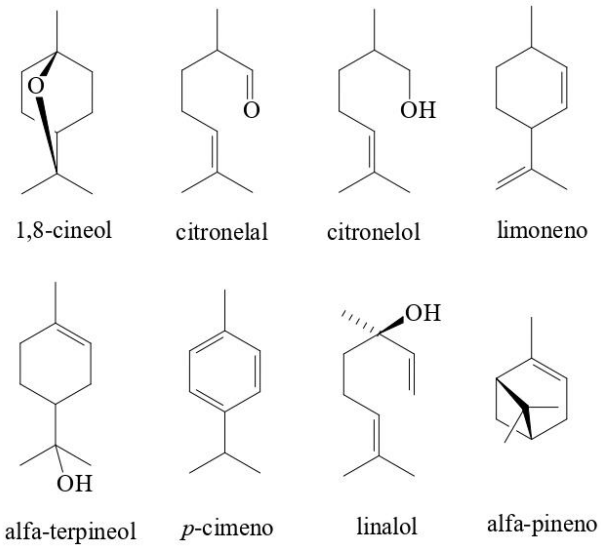


Fonte: Do autor.

A composição química do óleo essencial obtido das folhas de espécies de *Eucalyptus* é bastante diversificada e pode variar de acordo com a espécie. Fatores como teor de umidade do solo e a temperatura do ar podem influenciar na produção do óleo essencial de *Eucalyptus*. No verão, quando as temperaturas são mais elevadas e as chuvas são frequentes, há maior produção

de óleo essencial em relação à primavera. Na Figura 13, estão representados alguns dos principais constituintes químicos comumente encontrados nos óleos essenciais obtidos das folhas de *Eucalyptus* spp. (ESTANISLAU et al., 2001; SALGADO et al., 2003; SILVA; BRITO; SILVA JUNIOR, 2006).

Figura 13 - Constituintes químicos comumente encontrados nos óleos essenciais de *Eucalyptus*.



Fonte: Do autor.

As principais espécies de eucaliptos cultivadas para extração de óleos essencias são *C. citriodora* e *E. globolus*, nos quais são encontradas altas concentrações de citronelal e 1,8-cineol em aproximadamente 90% e 50%, respectivamente. Esses óleos essenciais são destinados às indústrias farmacêuticas e perfumaria, e utilizados como controladores microbiológicos. Os óleos essenciais de *Eucalyptus* possuem como propriedades: atividades antimicrobianas, antioxidantes, inseticidas, acaricida, nematicida e alelopática (CAETANO et al., 2020; DHAKAD et al., 2018; BARBOSA; FILOMENO; TEIXEIRA, 2016).

A atividade antifúngica dos óleos essenciais das espécies de *Eucalyptus* já foi relatada na literatura, e eles se mostraram promissores diante de diversos microrganismos. Pesquisas de Caetano et al. (2020) sobre a caracterização e atividade antifúngica dos óleos essenciais de *Eucalyptus citriodora*, *Eucalyptus grandis*, *Eucalyptus camaldulensis* e *Eucalyptus microcorys* sobre o fungo *Hemileia vastatrix* os mesmos mostraram eficazes no controle desse microrganismo. A composição química desses óleos essenciais apresentou como constituinte químico majoritário o 1,8-cineol, na proporção de 37,43%; 41,61% e 39,08%, respectivamente,

exceto para o óleo essencial de *Eucalyptus citriodora*, em que o constituinte majoritário encontrado foi o citronelal com 88,83%.

Gakuubi et al. (2017) estudando a caracterização e a atividade antifúngica do óleo essencial de *Eucalyptus camaldulensis* sobre os fungos *Fusarium oxysporum, Fusarium solani, Fusarium verticillioides, Fusarium proliferatum* e *Fusarium subglutinans*, encontraram resultados positivos no controle desses microrganismos. Os autores encontraram o 1,8-cineol (16,2%), *α*-pineno (15,6%), *α*-felandreno (10,0%) e *p*-cimeno (8,1%) como constituintes majoritários.

Outros estudos fitoquímicos relatam a atividade antifúngica dos óleos essenciais de *Eucalyptus* sobre vários fungos de importância econômica, incluindo *Aspergillus flavus, Aspergillus parasiticus, Colletotrichum gloeosporioides, Botrytis cinérea, Bipolaris sorokiniana, Penicillium digitatum, Pythium ultimum, Rhizoctonia solani, Fusarium oxysporum, Fusarium solani* e *Fusarium subglutinans* (KATOOLI; MAGHSODLO; RAZAVI, 2011; SALGADO et al., 2003; VILELA et al., 2009).

2.3.2.2 *Rosmarinus officinalis*

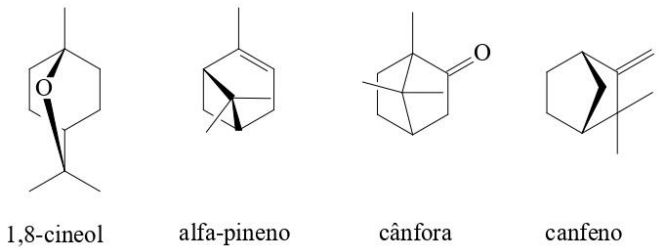
O alecrim (*Rosmarinus officinalis* L.), conhecido popularmente como alecrim-da-horta, alecrim-de jardim, alecrim-de-cheiro ou alecrim-rosmarinho, é uma planta pertencente à família Lamiaceae, originária da Europa (Figura 14). No Brasil, essa planta é cultivada em todos os estados. O alecrim é um subarbusto muito ramificado, sempre verde, com hastes lenhosas, folhas pequenas, sésseis, finas, opostas e lanceoladas, de sabor picante. A parte inferior das folhas é de cor verde-acinzentada, enquanto a superior é quase prateada. Essa planta é utilizada na medicina popular como antibiótico, anti-inflamatório, digestivo (hepatoprotetor, colerético), antiespasmódico, antioxidante, estimulante, reduzindo a permeabilidade capilar, diurético, mucolítico e antiparasitário (MAY et al., 2010; TAKAYAMA et al., 2016).

Figura 14 - Aspecto geral de uma espécie de *Rosmarinus officinalis*.

Fonte: Do autor.

O óleo essencial de alecrim destaca-se entre os óleos essenciais utilizados pelas indústrias agrícolas, farmacêuticas, de alimentos, de cosmética e de higiene. O rendimento do seu óleo essencial é de aproximadamente 1,0 por cento em relação ao peso do material fresco. O rendimento desse óleo não é bruscamente afetado durante as 4 estações do ano; porém, a proporção entre os seus constituintes pode variar. Em sua composição química, são encontrados como constituintes químicos majoritários o 1,8-cineol, cânfora, alfa-pineno e canfeno (Figura 15). Essas substâncias proporcionam a esse óleo essencial propriedades biológicas, como inseticida, antifúngica, anticarcinogênica e anticolinesterásica, que estão diretamente relacionadas ao mecanismo de atividade inseticida. (CAETANO et al., 2022; MAY et al., 2010; RIBEIRO et al., 2012; TAKAYAMA et al., 2016).

Figura 15 - Principais constituintes químicos encontrados nos óleos essenciais de alecrim.



Fonte: Do autor.

A atividade inseticida do óleo essencial de *Rosmarinus officinalis* atrelada à inibição da enzima acetilcolinesterase foi relatada por Caetano et al., (2022). Os autores caracterizaram o

óleo essencial de *Rosmarinus officinalis*, e incorporaram-no em nanopartículas de PCL para avaliar a atividade inseticida e a inibição da enzima acetilcolinesterase, encontrando resultados promissores. O óleo essencial apresentou como constituintes majoritários a cânfora (35,38%), 1,8-cineol (17,05%) e α-pineno (12,90%), os quais causaram a mortalidade de moscas da espécie *D. suzukii* e inibiram a atividade enzimática da acetilcolinesterase em 75%.

Diversos estudos fitoquímicos relatam a atividade inseticida do óleo essencial de *Rosmarinus officinalis* sobre vários insetos de importância econômica, incluindo *Callosobruchus maculatus* (gorgulho do feijão-caupi); *Trichoplusia ni* (lagarta-do-repolho); *Tetranychus urticae* (ácaro-rajado); *Tribolium castaneum* (besouro-vermelho-da-farinha); *Ulomoides dermestoides* (besouro-de-amendoim), entre outros (CABALLERO-GALLARDO et al., 2021; KHOOBDEL; AHSAEI; FARZANEH, 2017; KRZYŻOWSKI et al., 2020; MIRESMAILLI; BRADBURY; ISMAN, 2006; TAK; JOVEL; ISMAN, 2016).

2.3.3 Mecanismo da atividade inseticida dos óleos essenciais

A utilização de inseticidas comerciais é a principal forma de manejo para se controlar o ataque de insetos em plantações e alimentos; porém, o uso desses produtos de forma errônea ao longo do tempo tem selecionado espécies de insetos resistentes aos inseticidas tradicionais. Nos Estados Unidos, em 2001, estudos apontaram que ácaros-aranha desenvolveram resistência a mais de 80 acaricidas, sendo essa resistência relatada em mais de 60 países. Sendo assim, estudos que visa a elucidar e compreender o mecanismo de ação inseticida de produtos naturais, como os óleos essenciais, são facilitadores para o desenvolvimento de novos inseticidas eficazes e seguros (MIRESMAILLI; BRADBURY; ISMAN, 2006).

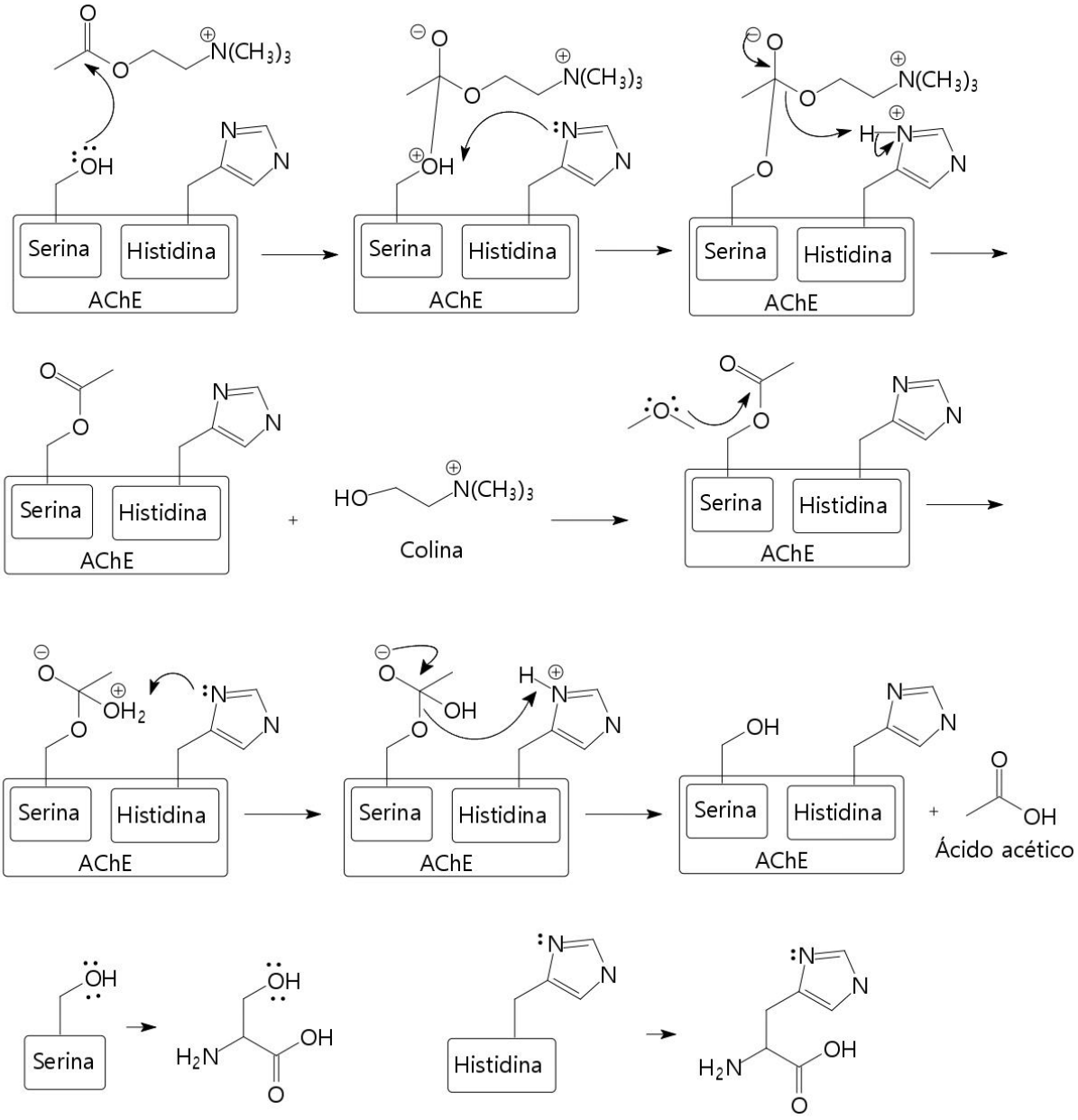
O mecanismo da atividade inseticida de metabolitos secundários, como os óleos essenciais, pode ocorre por meio de interações dos seus constituintes químicos com o sistema de sinalização celular, modificando o funcionamento de enzimas vitais, alterando a sinalização do sistema nervoso, interferindo na síntese, armazenamento, liberação, ligação e receptação dos neurotransmissores, ativação e função dos receptores e bloqueio de enzimas envolvidas na transdução de sinais (WINK, 2000).

O mecanismo de ação neurotóxicos é um dos mais estudados. Os sintomas mais proeminentes são a hiperatividade, seguida da hiperexitação, que pode levar à queda do inseto e, posteriormente, sua imobilização. Outro suposto mecanismo de ação são os danos causados nos processos bioquímicos que afetam especificamente o equilíbrio endocrinológico dos

insetos, podendo causar danos aos reguladores de crescimento dos insetos, interrompendo o processo normal de morfogênese (RATTAN, 2010).

No mecanismo de ação neurotóxico, o alvo específico dos constituintes dos óleos essenciais é a inibição da enzima Acetilcolinesterase (AChE). Essa enzima é conhecida também como colinesterase de glóbulo vermelho, colinesterase verdadeira ou acetil-colina. A AChE é encontrada nas hemácias e desempenha um papel-chave nas sinapses colinérgicas, essenciais para insetos e animais superiores. Sua principal função é hidrolisar o neurotransmissor acetilcolina em ácido acético e colina (Figura 16). A acetilcolina atua desempenhando uma função de transmitir a mensagem de um neurônio para o outro. A sua inibição causa o acúmulo de acetilcolina nas sinapses, de modo que a membrana pós-sináptica está em estado de estimulação permanente, resultando em alterações no funcionamento do sistema nervoso central e periférico, levando a diversos perturbações no sistema fisiológico e causando ataxia (ARAÚJO; SANTOS; GONSALVES, 2016; RATTAN, 2010).

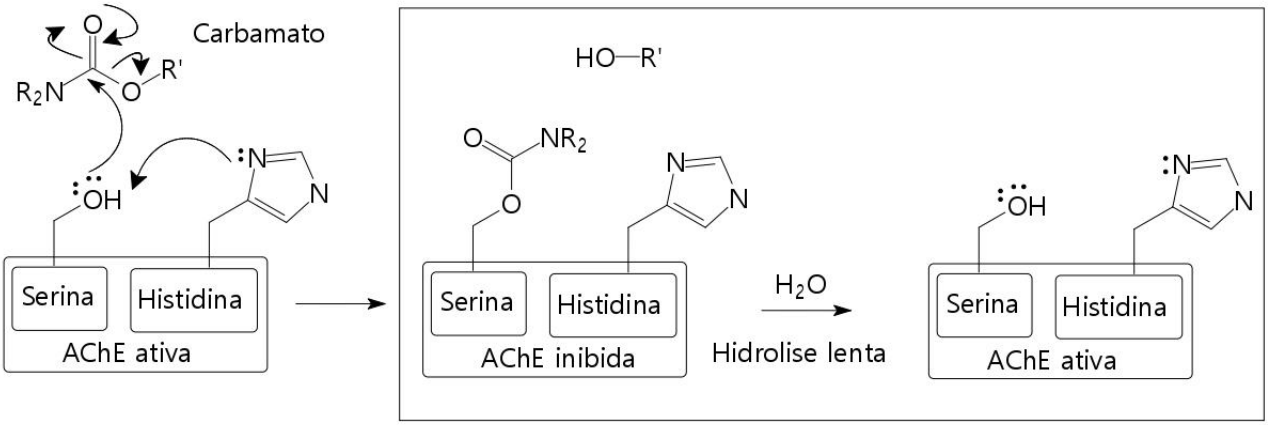
Figura 16 - Hidrólise da acetilcolina em ácido acético e colina.



Fonte: Adaptada de Araújo et al. (2016).

Produtos comerciais à base de organanofosforados (R1OPOOR2OR3) e carbamatos (R1NCOOR2) têm sido a classe de inseticidas mais utilizados, apresentando como mecanismo de ação a inibição da enzima AChE. Alguns fatores têm diminuído a eficiência desses inseticidas comerciais, como a alteração estrutural dessa enzima em insetos-praga, além de que a inibição por carbamatos é uma reação reversível (Figura 17). O mecanismo de inibição da enzima AChE por carbamato pode ser similar ao mecanismo de inibição pelos constituintes químicos dos óleos essenciais (ARAÚJO; SANTOS; GONSALVES, 2016; HEMINGWAY et al.,1986).

Figura 17- Mecanismo de inibição da AChE por carbamato.



Fonte: Adaptada de Araújo et al. (2016).

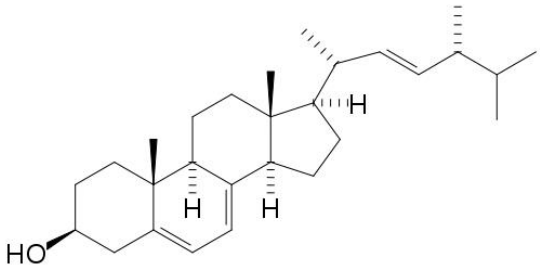
Trabalhos encontrados na literatura relatam a atividade inibitória da AChE por óleos essenciais de *Rosmarinus officinalis*, *Satureja montana*, *Myristica fragrans*, *Cymbopogon flexuosus*, *Backhousia citriodora*, *Callistemon viminalis*, *Cinnamodendron dinisii*, *Lippia thymoides*, *Thymus vulgaris*, O*riganum ehrenbergii*, *Anisophyllea disticha*, *Azadirachtina indica*, *Mentha* spp., *Lavendula* spp., entre outros. Padrões de constituintes químicos presentes nos óleos essenciais também já foram testados e apresentaram atividade inibitória da enzima AChE, incluindo o 1,8-cineol, carvona, cuminaldeído, limoneno, linalol e fenchona (ABDELGALEIL et al., 2009; ARAÚJO; SANTOS; GONSALVES, 2016; DA SILVA LUNGUINHO et al., 2021; KRZYŻOWSKI et al., 2020; REZENDE et al., 2021; SALLEH et al., 2021; SILVA et al., 2019).

2.3.4 Mecanismo da atividade antifúngica dos óleos essenciais

A partir do momento em que a resistência antimicrobiana está se tornando um problema mundial, a busca por formas alternativas para combater esses microrganismos vem aumentando. Os óleos essenciais são considerados como promissores nessa busca, sendo alvo de novas pesquisas. A atividade antifúngica dos óleos essenciais está relacionada com a capacidade de seus constituintes químicos interagirem com a membrana lipofílica dos fungos. A atividade de um óleo pode estar relacionada a um constituinte majoritário ou ao sinergismo de vários constituintes químicos presentes nos óleos essenciais (KISOVÁ et al., 2020; SIMÕES et al., 2017; TANG et al., 2018).

Nos fungos, a membrana plasmática desempenha um papel-chave na manutenção de um ambiente homeostático, troca de materiais, transferência de energia e informações para manter as células saudáveis. Os óleos essenciais, por apresentaram como característica a lipofilicidade, são capazes de interagir com a membrana plasmática dos fungos, constituída por quitina, *β*-glucanos e principalmente por ergosterol (Figura 18), que é o principal esterol responsável por manter a vitalidade celular dos fungos. As interações dos constituintes dos óleos essenciais com a membrana plasmática, seguido do rompimento dessa membrana e inibição na biossíntese do ergosterol, é um dos principais mecanismos da ação antifúngica dessas substâncias (HU et al., 2017; TANG et al., 2018).

Figura 18- Estrutura química molecular do ergosterol.



Fonte: Do autor.

Após a interação com a membrana plasmática dos fungos, os constituintes dos óleos essências, além de inibirem a atividade do ergosterol, podem afetar diretamente as membranas mitocondriais que controlam a entrada e saída de diferentes componentes, como a ciclagem de Ca^{2+} e outros canais iônicos. Os constituintes dos óleos essenciais também podem afetar a expansão da membrana e alterar sua fluidez, desregulando, assim, a bomba de prótons e de ATP, que levam à desordem de substâncias macromoleculares intracelulares, como os ácidos nucleicos e proteínas, alternando também as atividades enzimáticas e o metabolismo energético. Sendo assim, os óleos essenciais promovem a inibição na taxa respiratória intracelular, fosforilação e fosforilação oxidativa, o que causa a morte celular por apoptose e necrose (HU et al., 2017; RICHTER; SCHLEGEL, 1993; YOON et al., 2000).

Os constituintes químicos dos óleos essenciais também podem inibir o desenvolvimento de esporos e interagir com as hifas, causando deformações nelas, como também podem causar alterações nos conidióforos e conídeos. As distorções nas estruturas morfológicas desses microrganismos podem estar relacionadas ao vazamento de conteúdos de íons e materiais celulares (KEDIA et al., 2015; RASOOLI; REZAEI; ALLAMEH, 2006).

Estudos de Kisová et al., (2020) mostraram de maneira específicas onde os óleos essenciais podem atuar no controle desses microrganismos. Entre os principais alvos, pode-se citar alteração nas vias metabólicas, como: transporte e metabolismo de nucleotídeos; processos de tradução e biossíntese de proteínas; metabolismo de aminoácidos; metabolismo de cofatores e vitaminas; processos celulares básicos; via de metabolismo básico e produção de energia. Os autores também encontraram como mecanismo de ação dos óleos essenciais alteração no gêne de Pc22g00420, que é anotado como acetil-CoA-acetiltransferase, também conhecido como acetoacetil-CoA tiolase. Esse gene tem um papel fundamental na regulação da síntese de ergosterol. Baixos níveis de expressão desse gene foram encontrados após o tratamento com óleos essenciais. Alterações também foram observadas no nível da enzima aminoacil-tRNA sintetase, sendo essa, fundamental para síntese proteica, fator limitante para a sobrevivência celular.

Na literatura, é possível encontrar diversos trabalhos nos quais os óleos essenciais se mostraram eficazes no controle de fungos, como *Hemielia vastatrix*, *Aspergillus carbonarius*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus westerdjikiae*, *Aspergillus parasiticus*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium digitatum*, *Penicillium italicum*, *Penicillium aurantiogriseum*, *Penicillium expansum*, *Penicillium chrysogenum*; *Botrytis cinérea*, *Candida krusei*, *Candida albicans*, entre vários outros microrganismos (BIDGOLI, 2021; BRANDÃO et al., 2021; BRANDÃO et al., 2022; CAETANO et al., 2020; RAHMATI-JONEIDABAD et al., 2021; REZENDE et al., 2021; VALKOVÁ et al., 2022; WARDANA et al., 2022).

2.4 Nanotecnologia na agricultura

A agricultura é um dos principais fatores da economia mundial. A produção de alimentos desempenha um papel-chave no crescimento da Produção Interna Bruta (PIB) de um país. Alguns fatores como o uso de fertilizantes, pesticidas, mudanças climáticas, saúde do solo, entre outros, afetam diretamente a taxa de produção de alimentos e o crescimento da agricultura. Tecnologias que possibilitam regular condições adversas da agricultura podem potencializar a produção de alimentos, além de reduzir custos e proporcionar uma agricultura de maior precisão (CHHIPA, 2019).

A nanotecnologia é a ciência de materiais que têm seu tamanho na faixa de nanômetros. Nessa escala de tamanho, os materiais apresentam propriedades químicas e óticas específicas e únicas, que em comparação com o material a granel, apresentam como principal característica

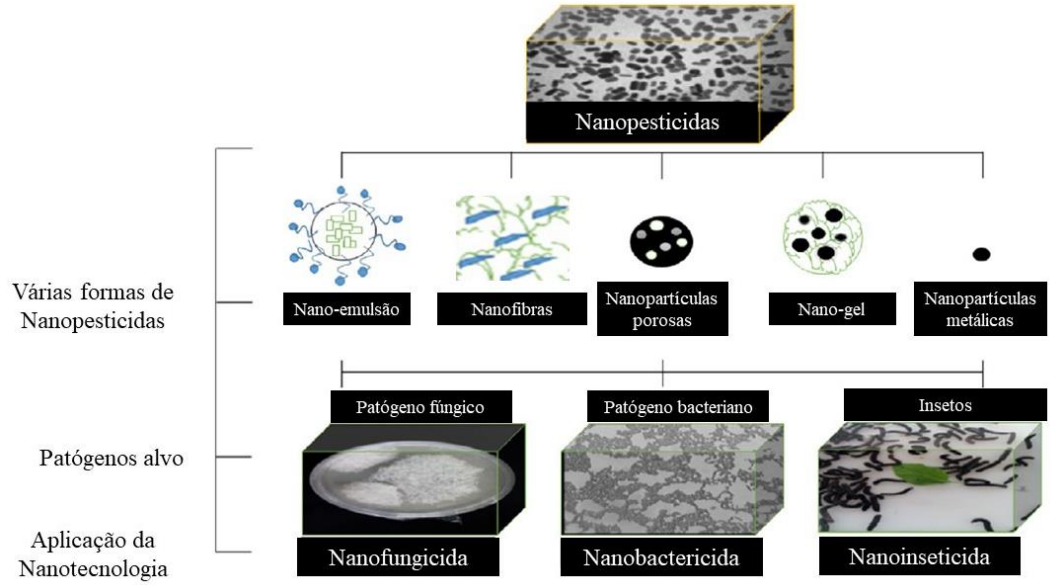
alta área superficial. A aplicação da nanotecnologia na agricultura pode proporcionar o desenvolvimento de nanofertilizantes, nanopesticidas e nanosensores, entre outros produtos, podendo, assim, contribuir para que a agricultura consiga alcançar os desafios emergentes, como a segurança alimentar para o aumento da população, a produção reduzida em terras cultiváveis, a baixa eficiência de insumos agrícolas, a baixa vida útil dos produtos alimentícios, as perdas pós-colheita de produtos alimentícios e o aumento de ataques de pragas e doenças de plantas (CHHIPA, 2019; KHAN; RIZVI, 2014; YOGESH, et al., 2015).

A aplicação da nanotecnologia na agricultura, além de diminuir a poluição química de efluentes e de organismos não visados, pode minimizar o custo de fertilizantes e pesticidas. Uma vez que a aplicação nanotecnologia melhora as características inteligentes dos agroinsumos, ela proporciona a esses produtos a entrega direcionada e liberação controlada dos princípios ativos, aumentando a solubilidade e a vida útil deles (CHHIPA, 2019).

O desenvolvimento de nanopesticidas pode ajudar a solucionar o que é atualmente um dos principais desafios da agricultura. A contaminação das lavouras por pragas em todo o mundo pode causar perdas de safras anuais de aproximadamente US$ 220 bilhões. Os patógenos responsáveis por causar as doenças em plantas são as bactérias, fungos, vírus, nematoides, parasitas, insetos e protozoários. A forma mais utilizada para controlar essas pragas é fazendo uso de pesticidas químicos e biológicos. Porém, o uso equivocado desses produtos tem causado desequilíbrio ambiental, bem como o aumento à resistência das pragas. Para amenizar esses danos e proporcionar uma agricultura de maior precisão, diversos nanopesticidas estão sendo desenvolvidos e se apresentam promissores (CHHIPA, 2019; SHARMA; KOONER; ARORA, 2017).

Entre os principais nanopesticidas produzidos (Figura 19), podem-se destacar as nano-emulsões, naofibras, nanopartículas porosas, nano-gel e nanoparticulas-metálicas. Cada nanopesticida apresenta uma característica e vantagens específicas. Sua produção deve ser priorizada de acordo com o método de aplicação. As nanopartículas porosas geralmente são utilizadas para aplicação no campo na forma de aspersão. As nanofibras também podem ser aplicadas no campo, como também podem ser utilizadas em galpões e embalagens.

Figura 19 - Aplicação de nanopesticidas na agricultura.



Legenda: Aplicação de nanopesticidas na agricultura: os nanopesticidas têm sido usados para controlar vários patógenos fúngicos, bacterianos e de insetos na forma de nanofungicida, nanobactericida e nanoinseticida.

Fonte: Chhipa (2019).

Diversos nanoinseticidas já foram formulados e se mostraram promissores no controle de insetos-praga. Nanoinseticidas produzidos à base de óleo essencial de *Artemisia arborescens* e *Lippia sidoides* já foram estudados, e a nanoformulação proporcionou aos óleos essenciais um aumento da solubilidade e vida útil, como também proporcionou a atividade inseticida sem afetar organismos não visados. Nanoinseticida formulado a partir de óleo essencial de *R. oficinallis* incorporado em nanoparticula de poli(ε-caprolactona) também foram estudados e se mostraram promissores no controle da mosca *Drosophila suzikii*. Nanoinseticidas formulados à base de silica estão em fase avançada de estudos, apresentando atividades sobre a mosca-branca, ácaro-do-coco, gorgulho-da-mostarda e gorgulho-do-arroz. Além disso, seu mecanismo de ação já foi elucidado, verificando-se que sua atividade se deve à fisissorção de lipídios da cutícula de insetos, o que estimula a sua morte (BARIK, KAMARAJU e GOWSWAMI, 2012; CAETANO, et al., 2022; CHHIPA, 2019).

A contaminação por fungos nas lavouras é responsável por mais de 70% das doenças das plantações, e caso esse patógeno não seja controlado, as perdas na produção podem chegar em até 100%. Diversos nanofungicidas já foram formulados e apresentaram resultados promissores no controle desses patógenos. Nanofungicidas produzidos a partir de nanopartículas de prata se mostraram eficazes no controle de *Magnaporthe grisea*, um patógeno fúngico que causa a doença da brusone do arroz, *Phoma glomerata*, responsável por causar

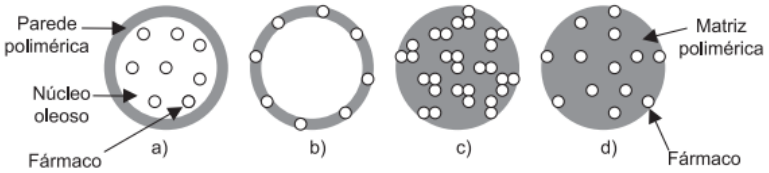
danos a lã e *Fusarium semitectum*, inibidor no desenvolvimento de sementes. (BAKER et al., 2017; ELAMAWI; EL-SHAFEY, 2013; GAJBHIYE et al., 2009).

A utilização em escala de nanopesticidas seguros em futuro próximo trará diversos benefícios para a agricultura, meio ambiente e para a sociedade em geral, proporcionando aplicações sem resíduos químicos, eficácia em baixas doses, redução na contaminação de microrganismos em alimentos e rações, sustentando, assim, a demanda global por alimento e uma agricultura de qualidade (ABD-ELSALAM et al., 2017).

2.4.1 Nanopartículas

As nanopartículas são definidas como uma dispersão de partículas que apresentam um tamanho de 10 - 100 nm. O termo nanopartícula inclui as nanocápsulas e as nanoesferas, as quais diferem entre si segundo a composição e organização estrutural. As nanocápsulas são sistemas em que o princípio ativo é confinado em um núcleo cercado por uma membrana polimérica única ou adsorvido nas paredes poliméricas. Já as nanoesferas apresentam um arranjo de distribuição uniforme no qual o princípio ativo está disperso ou adsorvido na matriz (Figura 20). As nanopartículas podem ser dividias em várias classes, como nanopartículas poliméricas, nanopartículas metálicas, nanopartículas lipídicas sólidas, entre outros. (ESPINOZA et al., 2020; MOHANRAJ; CHEN, 2006; SCHAFFAZICK, et al., 2003).

Figura 20 - Representação esquemática de nanocapsulas a) e b), e nanoesferas c) e d).



Fonte: Schaffazick et al. (2003).

Na produção de nanopartículas, o princípio ativo é dissolvido em uma matriz polimérica, sendo aprisionado, encapsulado ou ligado a uma matriz de nanopartículas. Dependendo do método de preparação, polímero utilizado e das condições do sistema, podem ser formadas nanocapsulas e nanoesferas. Durante a produção das nanopartículas, são utilizados surfactantes para facilitar a formação do estado nanomérico e assegurar sua estabilidade cinética durante o armazenamento, uma vez que ele diminui a energia interfacial entre as fases orgânica e aquosa, no qual a parte polar (cabeça) do surfactante interage com o núcleo hidrofílico e a

parte apolar (cauda) interage com o núcleo hidrofóbico (SINGH et al., 2017; MCCLEMENTS 2012; MOHANRAJ; CHEN, 2006).

As nanopartículas têm sido amplamente estudadas e aplicadas na indústria de alimentos, de cosméticos, na medicina, na agricultura, entre outras aplicações. Essas partículas também são empregadas para solubilizar e proteger os princípios ativos contra fatores ambientais adversos (oxidação, pH, hidrólise), para atingir alvos específicos, explorando efeito de permeabilidade e retenção do princípio ativo, que podem ser aumentados ou diminuídos. Apresentam como principal característica o aumento da área superficial, promovendo propriedades físico-químicas únicas e uma maior distribuição do princípio ativo nanoencapsulado (SINGH et al. 2017; ANTON; VANDAMME, 2011).

Na agricultura, nanopartículas poliméricas de poli(ε-caprolactona) estão sendo utilizadas para incorporar princípios ativos já utilizados pelos produtores rurais, como a atrazina, para potencializar sua eficácia, além de direcionar sua atividade em alvos específicos, não causando danos, assim, a culturas de interesse (PESTOVSK; MARTINEZ-ANTONIO, 2017).

2.4.1.1 Produção de nanopartículas pelo método de emulsão/evaporação de solvente

As nanopartículas poliméricas podem ser sintetizadas por diversos métodos, os quais incluem os métodos físicos, químicos e biológicos. O método deve ser selecionado de acordo com as propriedades físico-químicas do polímero e do princípio ativo, salientando que, durante o processo de síntese, a técnica escolhida não possa ser capaz destruir ou inativar o princípio ativo, uma vez que, alguns métodos de síntese empregam diversos solventes orgânicos, ultra-sonicação, temperatura e agitação (BADRI et al., 2017; SAJID; PŁOTKA-WASYLKA, 2020.).

Entre os diversos métodos possíveis para a produção de nanopartículas poliméricas, o método de nanoprecipitação (Figura 21), conhecido também como emulsão/evaporação do solvente ou método de deslocamento do solvente, foi desenvolvido pela primeira vez por Fessi et al., (1989). Na síntese de nanopartículas poliméricas utilizando esse método, são necessários o preparo de duas fazes. A fase orgânica composta pelo substrato estudado e pelo polímero, e a fase aquosa, composta por água e agente estabilizante, como o Tween 80. Em seguida, a fase orgânica é adicionada à fase aquosa, podendo ser formadas então partículas coloidais após a evaporação do solvente orgânico. Nesse método, alguns parâmetros podem influenciar nas propriedades físico-químicas das nanopartículas (tamanho de partícula, potencial zeta e morfologia), como a técnica de evaporação do solvente, taxa de injeção da fase orgânica na fase

aquosa, natureza e concentração do agente estabilizante, concentração do polímero, velocidade de agitação, volume da fase aquosa (BADRI et al., 2017).

Figura 21 - Método de emulsão/evaporação do solvente.

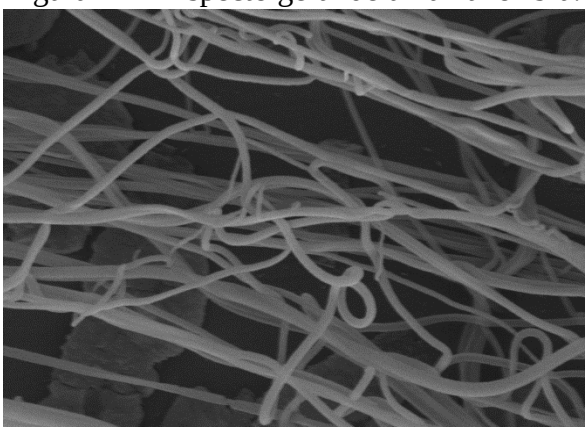
Fonte: Bradri et al. (2017).

Entre as principais vantagens na preparação de nanopartículas poliméricas pelo método de emulsão/evaporação do solvente, podem-se destacar a alta reprodutibilidade em escala manométrica, a possibilidade de produzir nanopartículas, fazendo o uso de polímeros biodegradáveis e biocompativeis, como o poli(ε-caprolactona) (PCL), poli(ácido lático) PLA e Quitosana, além da possibilidade de se utilizarem princípios ativos e estabilizantes considerados de baixa toxicidade, como os óleos essenciais e o Tween 80. Além disso, essa técnica faz o uso de baixo volume de água, energia, tempo, como também apresenta uma simplicidade no processo experimental (BADRI et al., 2017; CHORNY et al., 2002).

2.4.2 Nanofibras

As nanofibras (Figura 22) são polímeros em filamentos ou em multifilamentos contínuos que apresentam pelo menos uma dimensão de 100 nm ou menos. Elas podem ser produzidas por técnicas, como eletrofiação, fiação por sopro de solução (SBS), entre outras. As nanofibras possuem como características principais a biocompatibilidade, biodegradabilidade, alta área superficial, porosidade e padrão de liberação controlada de princípios ativos. As nanofibras podem ser preparadas a partir de polímeros naturais, polímeros sintéticos, nanomaterias à base de carbono, nanomaterias semicondutores, entre outros (BRANDÃO et al., 2022; HUANG et al., 2003; LIM et al., 2017; NEPOMUCENO et al., 2018).

Figura 22 - Aspecto geral de uma nanofibra.



Fonte: Do autor.

Devido às suas características e propriedades, as nanofibras estão sendo largamente estudadas para encapsular e fornecer substâncias com atividades biológicas, como os óleos essenciais e seus constituintes isolados, com o objetivo de proteger e possibilitar uma liberação controlada deles, tornando essa prática de grande interesse para as indústrias agrícolas, farmacêuticas e de alimentos. As nanofíbras também estão sendo aplicadas nas áreas de geração e armazenamento de energia, tratamento de água e remediação ambiental e engenharia biomédica e de saúde (LIM et al., 2017; MIRANDA et al., 2019).

Na agricultura, as nanofíbras poliméricas estão sendo utilizadas para solucionar diversos desafios. Nanosensores produzidos a partir de membranas fibrosas de solução de poli(ácido lático)/polianilina estão sendo utilizados na avaliação da maturação de frutas. As nanofibras poliméricas de poli(butileno adipato-co-tereftalato) (PBAT) carregadas com Zn também têm sido utilizadas como nanofertilizantes e se mostraram promissoras na liberação controlada desse metal em culturas de milho (*Zea mays*). Na pecuária, nanofibras de zeína incorporadas com aminoácidos essenciais têm sido desenvolvidas para fornecer quantidades ideais de triptofano para a dieta de peixes, e as mesmelas se mostraram promissoras (BRANDÃO et al., 2022; NATARELLI et al., 2021; SILVA et al., 2020; SILVA et al., 2022).

Nanofibras poliméricas de PLA produzidas pela técnica de (*Solution Blow Spinning*) SBS incorporadas com os óleos essenciais de *Alpinia speciosa* e *Cymbopogon flexuosus* se mostraram eficazes no controle dos fungos micotoxigenicos, como o *Aspergillus ochraceus* e o *Aspergillus westerdijkiae*, sendo uma alternativa no desenvolvimento de novos produtos com atividade antifúngica e antimicotoxigênica (BRANDÃO et al., 2022).

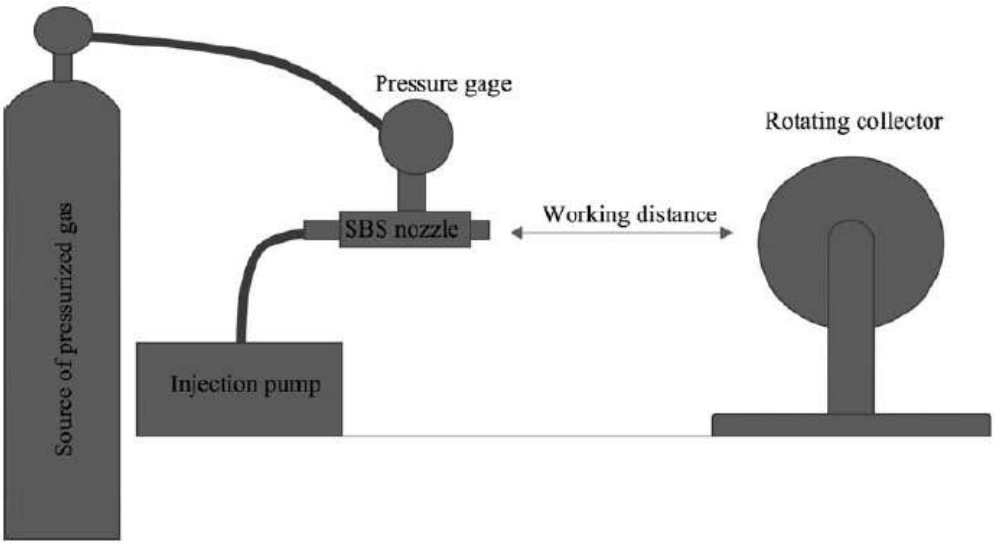
A ampla gama de aplicações e benefícios da utilização de nanofibras poliméricas biodegradáveis nas diferentes áreas, em especial na agricultura, faz com que esse nanomaterial seja promissor no desenvolvimento de soluções inovadoras. Sabendo-se do seu potencial no

controle de fungos micotoxigenicos, a eficácia de liberação controlada de princípios ativos voláteis, como os óleos essenciais e a possibilidade da entrega eficaz em concentrações ideais de fertilizantes, faz das nanofibras substâncias de interesse agroindustrial.

2.4.2.1 Fiação por sopro de solução

A técnica fiação por sopro de solução (*Solution Blow Spinning*) (SBS) (Figura 23) foi desenvolvida com o intuito de superar algumas restrições que a técnica comumente utilizada de eletrofiação para síntese de nanofíbras possui, como a dificuldade de síntese *in situ* de nanofíbras e os requisitos de alto potencial elétrico e alvos condutores. A técnica de SBS é consideravelmente simples, segura, versátil e de baixo custo, possibilitando a produção de micro e nanofibras, podendo utilizar-se de diversos polímeros para esse fim. Em comparação com técnicas tradicionais, ela produz uma maior quantidade de fibras (BEHRENS et al., 2014; OLIVEIRA et al., 2011).

Figura 23 - Esquema de sopro de solução para produção de nanofibras.



Fonte: Nepomuceno et al. (2018).

A técnica de SBS consiste no uso de uma bomba e uma seringa para alimentar bicos concêntricos no qual a solução polimérica contendo o princípio ativo e um gás pressurizado são simultaneamente injetados; a diferença de pressão e o cisalhamento na interface gás/solução produz filamentos de solução de polímero em direção ao coletor, sendo que, durante o voo até o coletor, os solventes são volatilizados formando, então as nano e microfibras. Alguns parâmetros, como a taxa de injeção, a pressão do fluxo de gás, a concentração do polímero,

viscosidade da solução, a distância de trabalho e a geometria do bico podem influenciar diretamente na morfologia e na taxa de produção das fibras (BRANDÃO et al., 2022; MEDEIROS et al., 2009; NEPOMUCENO et al., 2018).

Alterações morfológicas de nanofibras podem ser causadas pela variação na concentração do polímero. Concentrações mais baixas de polímero tendem a aumentar a formação de estruturas contra-fio, ao contrário do que, em concentrações mais altas, são formadas fibras lisas. Em relação ao diâmetro das nanofibras, esse parâmetro tende a aumentar com o aumento da concentração do polímero e diminuir com a taxa de alimentação (OLIVEIRA et al., 2011).

A viscosidade da solução também influencia diretamente nas propriedades das nanofibras. À medida que a viscosidade aumenta, o estiramento da fibra se torna mais difícil, formando, então, fibras mais grossas e com uma distribuição mais ampla de diâmetro. Outro fator que afeta a distribuição do diâmetro das nanofibras é o fluxo de ar. A força de cisalhamento gerada pelo arrasto aerodinâmico da solução polimérica é responsável pelo alongamento das fibras e pela distribuição no diâmetro das fibras. Sendo assim, para soluções poliméricas de alta viscosidade, onde o alongamento das fibras é mais difícil, menos eficiente e instável, o aumento no fluxo de ar necessário para sintetizar as nanofibras provoca uma distribuição mais ampla do diâmentro (OLIVEIRA et al., 2013).

Parâmetros ambientais como umidade também influenciam na produção e morfologia das nanofibras. Investigações recentes mostram que a umidade pode alterar diretamente o diâmetro das nanofibras. Porém, o efeito causado pela umidade no diâmetro médio das nanofibras é relativo em relação à matriz polimérica. Para fibras produzidas a partir de acetato de celulose, o aumento de umidade está diretamente relacionado com o aumento do diâmetro médio, enquanto na matriz polimérica de poli(vinilpirrolidona), o diâmetro médio diminui. A umidade também influencia no tamanho e na produção de nanoporos. Para nanofibras produzidas a partir de poliestireno, policarbonato e poli(metil metacrilato) utilizando solventes voláteis, o aumento da umidade proporcionou o aumento e maior desenvolvimento de nanoporos na superfície das nanofibras (HUANG et al., 2011).

2.5 Polímeros

No desenvolvimento de nanomateriais, diversos polímeros podem ser utilizados, incluindo aqueles naturais, e aqueles sintéticos, materiais à base de carbono, materiais semicondutores, entre outros. A escolha do polímero é uma etapa primordial, no

desenvolvimento de nanomaterias, uma vez que o material sintetizado apresentará características especificas referente a cada matriz polimérica. Essa escolha é guiada principalmente pelas propriedades mecânicas, térmicas, ópitcas, magnéticas, equilíbrio hidrofóbico/hidrofílico, estabilidade química, biodegradabilidade e biocompatibilidade. Os polímeros comumente utilizados na produção de nanomaterias são a poli (ε-caprolactona) (PCL); poli (ácido lático) (PLA); polivinilpirrolidona (PVP), álcool polivinílico (PVA); polietilenoglicol (PEG); poliacrilonitrila (PAN), entre outros (JEON; BAEK, 2010; LIM et al., 2017; VILCHEZ et al., 2020).

Na síntese de nanopartículas e nanofíbras, polímeros biodegradáveis e biocompatíveis, especialmente os poliésteres biodegradáveis como o PLA, PCL e PGA, têm sido destaque. Esses polímeros são eficazes atuando como carreadores, proporcionando a liberação controlada de princípios ativos, bem como protegendo-os de degradação por fatores externos (pH, oxidação, hidrolise). Esses polímeros também estão sendo estudados na biomedicina para síntese de dispositivos médicos absorvíveis, como sutura, placas e parafusos de fixação óssea, como também no desenvolvimento de plásticos ambientalmente degradáveis na produção de produtos de consumo descartáveis (KOBAYASHI; MÜLLEN, 2015; SINGH et al. 2017).

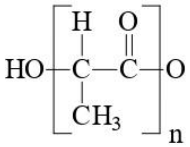
2.5.1 Poli (ácido lático)

O poli (ácido lático) (PLA) (Figura 24) é um poliéster alifático, termoplástico, semicristalino ou amorfo. Esse polímero pode ser sintetizado a partir de monômeros do ácido 2-hidróxi-propanoico (ácido lático) ou obtido de recursos naturais, como o milho e a cana-de-açúcar. O ácido lático é uma molécula quíral (C*) que apresenta propriedades opticas, sendo encontrado nas suas duas formas enantioméricas, L(+) ácido lático e D(-) ácido lático. Esse monômero pode ser obtido a partir de fermentação ou por via sintética, sendo o primeiro método o mais empregado. Na sua obtenção por fermentação, pode-se utilizar como fonte de carboidratos o amido de batata, a partir do qual são formados a glicose, maltose e dextrose, entre outros carboidratos. No processo de fermentação, são utilizadas bactérias do ácido lático, sendo a mais empregada as do gênero *Lactobacillus*. (BRITO et al. 2011; GARLOTTA, 2001; GHAFFAR et al., 2014).

O PLA apresenta-se como principais características a biodegradabilidade, a biocompatibilidade e boa processabilidade, sendo uma alternativa para a substituição dos polímeros tradicionais obtidos a partir do petróleo. Os produtos obtidos a partir de sua degradação são; água (H_2O) e dióxido de carbono (CO_2), não proporcionando, assim, riscos à

saúde humana e ao meio ambiente (BRITO et al. 2011; JONOOBI et al., 2010; REIS et al., 2021; XIAO et al., 2012).

Figura 24 – Estrutura química do ácido polilático.



Fonte: Brito et al. (2011).

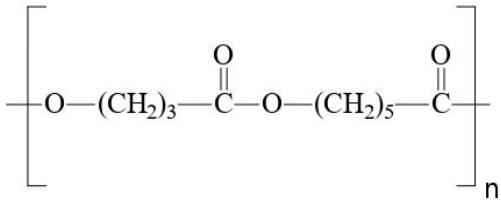
O PLA apresenta como características físicas uma temperatura de transição vítrea (Tg) de 55 ºC, temperatura de fusão (Tm) de 165 °C, temperatura de cristalização (Tc) de 90 ºC e temperatura de processamento entre 185 a 210 ºC; porém, os valores dessas temperaturas podem variar dependendo dos tipos de "grades". Devido às suas características físicas, o que lhe proporciona razoável rigidez e resistência mecânica, ele é utilizado na fabricação de embalagens de alimentos, garrafas de água e leite, sacolas plásticas degradáveis, bem como aplicações automotivas (BRANDÃO et al., 2022; BRITO et al., 2011; JONOOBI et al., 2010; REIS et al., 2021).

Devido à sua alta biocompatibilidade, biodegradabilidade, transparência e razoável resistência, o PLA tem sido um candidato para a produção de filmes utilizados na fabricação de embalagens inteligentes, na produção de fibras utilizadas na indústria têxtil, na agricultura, e também na área da saúde, como material de implante cirúrgico, sistema de administração de medicamentos e fibras para suturas (BRITO et al., 2011).

2.5.2 Poli (ε-caprolactona)

A poli (ε-caprolactona**)** (Figura 25) é um polímero biodegradável e biocompatível sintetizado pela primeira vez no início de 1930, que se tornou comercialmente viável décadas depois, devido à busca por polímeros sintéticos biodegradáveis. Esse polímero é caracterizado como sendo semicristalino e hidrofóbico, possuindo temperatura de transição vítrea (Tg) de − 60 °C e temperatura de fusão (Tm) variando de 59 − 64°C, dependendo da sua natureza cristalina. Esse polímero é solúvel à temperatura ambiente em clorofórmio, diclorometano, tetracloreto de carbono, benzeno, tolueno, ciclohexanona e 2-nitropropano e outros solventes orgânicos, como a acetona, sendo insolúvel em água e etanol (FRANK et al., 2015; SINHA et al., 2004).

Figura 25 - Estrutura química da poli (ε-caprolactona).

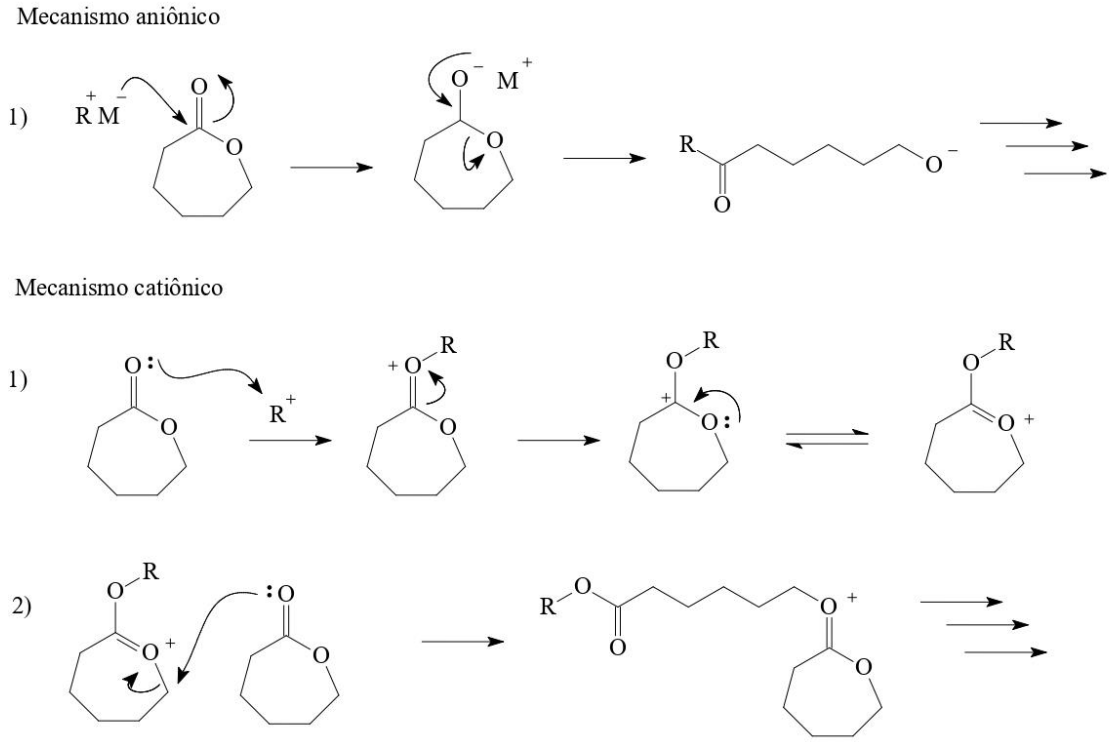


Fonte: Espinoza et al. (2020).

A biodegradação do PCL, em comparação com alguns polímeros, como o ácido poliglicólico, é lenta, sendo assim, esse polímero pode ser muito bem utilizado para entrega de princípios ativos, que se estende por um período de mais de um ano. O PCL vem sendo empregado nas diversas áreas do conhecimento, principalmente no encapsulamento de produtos naturais, pois possui alta permeabilidade a pequenas moléculas, possibilitando a liberação controlada de substâncias ao longo do tempo, protegendo o princípio ativo da degradação por variação no pH do meio e impedindo a degradação enzimática e a fotodegradação (FRANK et al., 2015; SINHA et al., 2004).

A síntese de PCL pode ser realizada por meio da polimerização por abertura do anel éster da *ε*-caprolactona ou por policondensação, sendo o primeiro considerado o método mais apropriado, pois possibilita a obtenção de polímeros com maiores pesos moleculares e menores valores de polidispersidade. O produto polimérico final conterá grupos terminais com função álcool, amina, entre outros, dependendo do iniciador aplicado. Vários mecanismos podem ser utilizados para realizar a síntese de PCL por polimerização, por abertura do anel éster da *ε*-caprolactona, sendo os mais utilizados os mecanismos catiônicos e os aniônicos (Figura 26) (ESPINOZA et al., 2020).

Figura 26 – Ataque nucleofílico para síntese de poli (ε-caprolactona).



Fonte: Espinoza et al. (2020).

No mecanismo aniônico, um iniciador carregado negativamente, como, por exemplo, o reagente de Grignard, ataca o átomo de carbono carbonílico da *ε*-caprolactona monomérica, sendo esse o que apresenta o menor LUMO, ocorrendo, assim, a abertura do anel pela quebra da ligação acil-oxigênio. Após a quebra da ligação, há formação de alcoxidos, que podem propagar a reação como iniciadores. Possiveis reações intramoleculares podem ocorrer, resultando então em polímeros cíclios. Essas reações podem sem evitadas, parando a reação em estágios iniciais (ESPINOZA et al., 2020).

O mecanismo catiônico se inicia com o anel da *ε*-caprolactona, adquirindo uma carga positiva após a reação do oxigênio hibridizado sp^2, com uma espécie positiva, podendo ser um carbocátion. Posteriormente, ocorre uma reação de substituição nucleofílica bimolecular (SN_2) nessa molécula carregada positivamente pelo oxigênio da carbonila de outro monômero de *ε*-caprolactona, induzindo a abertura do anel. Em seguida, o polímero resultante carregado positivamente reage novamente com outro monômero de *ε*-caprolactona, propagando, assim, o crescimento da cadeia polimérica (ESPINOZA et al., 2020).

Após a produção da poli(*ε*-caprolactona), diversos métodos podem ser utilizados na preparação das suas nanopartículas, como o métodos de polimerização por dispersão,

polimerização por suspensão e emulsificação/evaporação de solventes, entre outros (BADRI et al., 2017).

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SEGUNDA PARTE – ARTIGOS

Este trabalho gerou 3 artigos, 2 já foram publicados e 1 encontra-se aceito para publicação. Todos eles estão apresentados de acordo com as normas das revistas Austral Entomology e Letters in Applied Microbiology.

ARTIGO 1 - *Rosmarinus officinalis* essential oil incorporated into nanoparticles as an efficient insecticide against *Drosophila suzukii* (Diptera: Drosophilidae). Volume 61, páginas 265 – 272, data de publicação: 22/03/2022.

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Rosmarinus officinalis essential oil incorporated into nanoparticles as an efficient bioinsecticide against the *Drosophila suzukii* (Diptera: Drosophilidae) fly

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Running Title

Essential oil against the fly *Drosophila suzukii*

Abstract

The effective control of an insect pest such as the *Drosophila suzukii* (Diptera: Drosophilidae) fly has been challenging because its attack on red fruits can cause losses in production of up to 80% worldwide. The objective of this work was to extract and characterize the essential oil from rosemary (*Rosmarinus officinalis*), produce and characterize nanoparticles incorporated with this essential oil, evaluate the *in vivo* insecticide activity of free and nanoencapsulated essential oil against *D. suzukii*, and evaluate the effect of that oil on the enzyme acetylcholinesterase. The nanoparticles produced with the essential oil from rosemary had a particle size and surface charge of 236.6 nm and -11.8 mV, respectively. According to the FTIR

data, the essential oil was incorporated into the polymeric matrix of poly(ε-caprolactone). Through *in vivo* toxicity analysis, the free and nanoencapsulated essential oils were found to possess insecticidal activity, with an LD50 close to 9.1 $g.L^{-1}$. The maximum anticholinesterase activity for the essential oil was 75% at a concentration of 0.10 $mg.mL^{-1}$. The incorporation of the essential oil from *R. officinalis* into biodegradable nanoparticles of poly(ε-caprolactone) potentiated and prolonged its *in vivo* insecticidal activity against *D. suzukii*.

Keywords: Phytochemistry, Essential oil, Berries, *Drosophila suzukii*, Polymeric nanoparticles.

Introduction

Red fruits or berries, as they are also known, are a group that includes fruits such as blackberry, strawberry, raspberry and blueberry. The main characteristics of these fruits are their high concentrations of vitamin C and phenolic compounds, characteristics that contribute color, flavor and antioxidant properties to these fruits (Manganaris *et al*., 2014; Pavinatto *et al.*, 2019). Despite the increase in production in Brazil and in the world, red fruits are highly perishable, and they are subject to attack by insect pests, such as *Drosophila suzukii* (Diptera: Drosophilidae), during the pre- and post-harvest periods.

The *D. suzukii*, a fly of oriental origin, is currently one of the main insect pests that cause losses in the production of red fruits throughout the world (Andreazza *et al*., 2016; *Mendonca et al*., 2019). Its infestation in the field can cause up to 80% loss of the fruit if it is not controlled (Santos, 2014). The attack of *D. suzukii* occurs via the oviposition by the females. The larvae grow and feed on the fruits, cause rot and prevent commercialization (Enriquez *et al*., 2020).

The most widely used control strategy for fighting this insect is by means of synthetic insecticides. However, the improper and excessive use of these products can lead to the resistance of insects, in addition to the environmental effects and the operational costs that they generate. Thus, the development of new products for insect control is considered to represent a challenge (Gonzáles *et al*., 2017; Lourenço *et al*., 2018).

Essential oils stand out as possible substitutes for synthetic chemical pesticides. These oils are natural products extracted from plants, and they are composed of a complex mixture of terpenes and phenylpropanoids that provide biological activities such as insecticidal actions (Rezende *et al*., 2017; Haddi *et al*., 2020; Santos *et al*., 2021).

The essential oil from rosemary (*Rosmarinus officinalis*) stands out among the essential oils used by the agricultural and food industries. The principal components in the oil are 1,8-cineol, camphor, alpha-pinene, and camphene. These compounds have anticholinesterase activity, which is related to the mechanism of insecticidal activity (Ribeiro *et al*., 2012; Takayama *et al*., 2016). Several phytochemical studies have reported the insecticidal potential of rosemary essential oil against a wide range of economically important insects, including *Tribolium confusum, Orgyia trigotephras, Bemisia tabaci, Anopheles stephensi, Aedes aegypti,* and *Culex quinquefasciatus* (Prajapati *et al*., 2005; Sertkaya *et al*., 2010; Badreddine *et al*., 2015; Ahsaei *et al*., 2020).

To prolong the biological activity of essential oils and protect their chemical constituents from degradation by external factors, these oils are currently being incorporated into different nanomaterials, including biodegradable nanopolymeric matrices such as poly(ε-caprolactone). In addition, the incorporation of the active ingredient into the nanopolymeric matrix increases its surface area and leads to a greater distribution of the active ingredient (Natta *et al*., 1934; Singh *et al*., 2017; Ferreira *et al*., 2019). The aim of the present study was to extract and chemically characterize the essential oil from *R. officinalis*; encapsulate this essential oil in biodegradable polymeric nanoparticles and evaluate its insecticidal activity against the *D. suzukii* fly.

Materials and Methods

Collection of plant material and extraction of the essential oil

The material chosen for extraction of the essential oil was the leaves of the *R. officinalis* shrubs. The leaves were collected in February 2020 in the city of Carmo da Mata, MG, Brazil (-20.548018 S; -44.8762125.19 W), in the morning, on a hot and sunny day, at a temperature of approximately 25 °C. Subsequently, the leaves were sent to the Department of Biology of the Federal University of Lavras (DBI-UFLA) for identification and confirmation of the species. The leaves were were cleaned, chopped, weighed and added to a 5-L round bottom flask in the Organic Chemistry Laboratory - Essential Oils (DQI-UFLA). A 2.5-L volume of water was added to this flask, and the flask was subjected to the hydrodistillation process using a modified Clevenger device for a period of 2 hours (Brasil, 2010). The essential oil was separated from the hydrolate by centrifugation using a horizontal cross-piece bench centrifuge (Fanem Baby®I Model 206 BL) at 965*g* for 5 minutes. The essential oil was removed with the aid of a Pasteur pipette and stored in an amber container under refrigeration (8°C ±2).

Chemical characterization of the essential oil from *Rosmarinus officinalis*

The chemical characterization of the essential oil was performed at the UFLA Chemical Analysis and Prospecting Center (CAPQ) using a gas chromatograph equipped with a mass spectrometer detector (GC-MS) and a gas chromatograph equipped with a flame ionization detector (GC-FID). The constituents of the essential oil were identified using a Shimadzu GC-17A instrument with a model QP 5050A mass detector under the following experimental conditions; capillary column of fused silica (30 m x 0.25 mm) with a DB5 bound phase (film thickness, 0.25 μm). The carrier gas was helium at a flow rate of 1.18 $mL.min^{-1}$ at 210 °C. The column temperature was programmed from 60 °C, increasing to 240 °C at 3 °C min^{-1}; the temperature then increased at 10 °C min^{-1} to 300 °C, where this temperature was maintained for 7 minutes. The injector temperature was 220 °C, and the detector (or interface) temperature was 240 °C. To perform the analysis, an aliquot of 0.1 μL of the sample diluted 1:100 in hexane was injected; a mixture of hydrocarbons (C_9H_{20}, $C_{10}H_{22}$, ..., $C_{24}H_{50}$, $C_{25}H_{52}$, $C_{26}H_{54}$) was also injected. The impact energy was 70 eV.

The quantification of the constituents was performed using a gas chromatograph (Shimadzu CG-17A) equipped with a FID. The experimental parameters for the analyses were the same as those used in the identification of chemical constituents by GC-MS; however, the carrier gas used was compressed air, and the temperature of the detector was 300 °C.

The constituents were identified by comparing the retention indices for the homologous series of alkanes (nC8-nC18), calculated according to the method of Van & Kratz (1963) with extrapolation to C19 and C20, using the literature retention indices from Adams (2014). The data were also compared with the NIST107 and NIST2 libraries available in the instrument. It was also possible to compare the spectra of the samples with those already in the literature (Nist, 2010).

Production of nanoparticles containing the essential oil from *Rosmarinus officinalis*

The poly(ε-caprolactone) polymer, PCL, (MW = 50,000 $g.mol^{-1}$) was obtained from Perstorp (Warrington, United Kingdom). Acetone (CAS number 67-64-1) and Tween 80 (CAS number 9005-65-6) were purchased from Synth (SP, Brazil) and used to prepare the biodegradable polymeric nanoparticles used in this study.

The biodegradable polymeric nanoparticles were obtained by the solvent emulsification-evaporation technique, with some modifications (Reis *et al*., 2006; Rao & Geckeler 2011). For this purpose, a PCL-acetone solution was prepared in which 130 mg of PCL polymer was added to 27 ml of acetone. The mixture was subjected to magnetic stirring at

37 °C until complete dissolution of the polymer. Four mL of Tween 80 surfactant was added. This solution was stirred at room temperature for 15 minutes until complete homogenization. With the aid of an injection pump, the suspension containing PCL/acetone/Tween 80 was added at a speed of 300 µL/min to 53 mL of water with magnetic stirring. After addition, the solution composed of PCL/acetone/Tween 80/distilled water was stirred at room temperature until the solvent (acetone) completely evaporated. The volume of acetone lost by evaporation was replaced by the addition of distilled water to the system.

The procedure for encapsulating the essential oil from *R. officinalis* in the PCL nanoparticles was accomplished in a manner similar to that of the particle synthesis described above. However, it differed in the fact that, after the dissolution of the polymer (PCL) in the solvent (acetone), 4 ml of rosemary oil were added for the preparation of the respective encapsulated systems: PCL nanoparticles (PCL) and PCL nanoparticles containing *R. officinalis* oil (NP-EO).

Characterization of nanoparticles

Measurements of particle size and zeta potential

The zeta potential, average nanoparticle diameter and polydispersity index were determined by dynamic light scattering in a Zetasizer Nano ZS instrument (Malvern Instruments Inc., Worcestershire, UK). Distilled water was used as a dispersant to avoid effects of multiple dispersions, dispersion and interactions between nanoparticles. The accumulated mean diameter (mean z) and the polydispersity index (PdI) were used to describe the size and distribution of the nanoparticles, respectively (Fraj *et al*., 2019).

Infrared Analysis

FTIR data were recorded on an IRAffinity-1 FTIR spectrophotometer (Shimadzu, Kyoto, Japan). The FTIR spectrophotometer was continuously purged with nitrogen. A total of 64 scans (400-4000 cm^{-1}) were collected with a resolution of 2 cm^{-1}. Infrared spectra were recorded in the transmission mode using 5 µL of nanoparticle colloids that were deposited on potassium bromide (Fraj *et al*., 2019).

Creation and maintenance of *Drosophila suzukii.*

The *D. suzukii* (Matsumura) (Diptera, Drosophilidae) flies used in the bioassays were obtained from the collection maintained in the Molecular Entomology and Ecotoxicology laboratory of the Entomology Department (Federal University of Lavras). They were raised in cages kept in

an air-conditioned room at 23 ± 2 °C and 50% relative humidity with a 12:12 h photoperiod (light: dark). *D. suzukii* flies were fed with an artificial diet as previously described (Emiljanowicz *et al*., 2014; Andreazza *et al*., 2018; Mendonca *et al*., 2019).

Toxicity assessment of free and nanoencapsulated essential oil against *Drosophila suzukii*

The toxicity test was performed for nanoparticles incorporated with *R. officinalis* essential oil and also for poly(ε-caprolactone) nanoparticles to assess whether this polymer exerts insecticidal activity against *D. suzukii*. The synthetic insecticide Decis® was used as a positive control.

The mortality rate of the *D. suzukii* flies was evaluated in quadruplicate according to the method described by IRAC (2020), with modifications. Briefly, a dental cotton (2 cm) treated with 2 mL of the solution composed of DMSO, 20% aqueous sugar solution and the essential oil in concentrations ranging from 5 to 100 mL was deposited in a glass vial (200 mL). Twenty-five non-sexed, same-age insects were introduced into the glass bottles, and the bottles were closed with foam plugs and kept in a biological oxygen demand incubator (BOD) at 23 ± 2 °C and 50% RH, with a 12 L:12 D photoperiod, to be evaluated after 24 hours.

In the evaluation, the flies were classified as (a) unaffected, which were those that, when gently stimulated by shaking the containers, responded normally (making a coordinated movement); or (b) dead or affected, which were those that, when stimulated, did not respond or showed an abnormal movement. The results were expressed as a percentage of mortality using the dose-response curve.

Evaluation of the insecticidal activity of free and nanoencapsulated essential oil against *D. suzukii* over time

To assess the durability of the insecticidal action of free and nanoencapsulated essential oil, an insecticidal activity test was performed over a 72-hour period. The method used to perform this analysis was a modification of the method used to assess the toxicity of the active principles against *D. suzukii*. The concentration of active ingredients used was 20 $mL.L^{-1}$ on the basis of the promising results observed previously. The evaluation was performed every hour during the first 6 hours, and then the evaluation was performed every 24 hours until no more effect of the active ingredients could be observed. Every 24 hours, all the insects were removed from the flasks, and new insects were added.

Anticholinesterastic activity

The anticholinesterase inhibitory activity of the free and nanoencapsulated essential oil of *R. officinalis* was assessed following the methodology of Ellman *et al*., (1961).

To a test tube, 2970 µL (50 mmol.L^{-1}) of the Tris-HCl buffer, pH 8, and 254 µL of the 1000 U.mL^{-1} acetylthiocholine solution were added. The mixture was incubated at 37 °C for 5 minutes, and 25 µL of the essential oil diluted in ethanol at concentrations of 0.25; 0.50; 1.00; 5.00; 10.0; 50.0 and 100 µg.mL^{-1} was added; 100 µL of 10 mmol.L^{-1} Ellman's reagent and 80 µL of the 0.02 mol.L^{-1} substrate solution were also added. The mixture was incubated again at 37 °C for 15 minutes, and the absorbance was measured on a spectrophotometer at 412 nm. For the blank, 3.2 ml Tris-HCl buffer was used. The evaluation of the anticholinesterase activity of the nanoparticles was accomplished by the same method used for the essential oil.

Considering that acetylthiocoline undergoes spontaneous hydrolysis, non-enzymatic controls were performed for each concentration of essential oil and nanoparticles, replacing the enzyme with Tris-HCl buffer. A solution for the negative control was prepared containing all the reagents except the essential oil and the nanoparticles, which were replaced by ethanol. As a positive control the carvacrol was used, since it has anticholinesterase activity.

The tests were performed with five repetitions. The results were expressed as a percentage of inhibition by applying the following equation:

Equation 1:

$$I(\%) = 100 - \left(\frac{A_T - A_C}{A_O}\right).100$$

Where: A_T: Sample absorbency; *Ac*: Control absorbency; A_O: Blank absorbency.

Statistical analysis

The toxicity bioassay data were subjected to Probit analysis to estimate lethal doses (LD_{50} and LD_{95}) and chi-square values (X^2) with a 95% confidence limit using the statistical software package SAS (SAS Institute, Cary, NC, USA). The results obtained for the different treatments were analyzed by multiple comparisons by the Scott-Knott test ($p < 0.05$ using the statistical software "R", version 4.0.2.

Results

The result of the analysis of the essential oil from rosemary is shown in Table 1. Nine constituents were identified in the essential oil, and the principal components were camphor (35.38%), 1.8-cineole (17.05%) and α-pinene (12.90%).

Table1.

The incorporation of the essential oil from rosemary in the PCL nanoparticles changed the surface charge and particle size, in addition to influencing the polydispersity index. The size of the particles containing the rosemary essential oil (237 nm – 35%) was larger than that of the PCL particles (187 nm – 100%) without the oil.

The variation in particle size is confirmed by the values of the polydispersity index, which was 0.59 for particles containing the oil, whereas the nanoparticles synthesized with only PCL were more homogeneous, having a polydispersion index of 0.08. The zeta potentials obtained for the nanoparticles were - 8.0 mV and -12.0 mV for the PCL and for the nanoparticles containing the essential oil, respectively.

Among the analyses that are necessary for the characterization of nanoparticles incorporated with organic compounds, that of FTIR is fundamental because the spectra can prove whether or not the active principle was incorporated into the polymeric matrix. The spectra of the samples are shown in Fig 1.

Fig 1.

The toxicity of free and nanoencapsulated essential oil after 24 h of application on the *D. suzukii* flies varied according to the dose applied and the essential oil disposition (Fig 2).

Fig 2.

Toxicity was observed for the essential oil in its free and nanoencapsulated form after 24 h of exposure in a dose-dependent correlation. The LD_{50} values were 6.7 and 17.5 $mL.L^{-1}$, and the LD_{95} were 20.23 and 64.20 $mL.L^{-1}$, respectively. No insecticidal activity against the *D. suzukii* flies was observed in the treatment performed with poly(ε-caprolactone) nanoparticles without the inclusion of the essential oil from *R. officinalis*. LD_{50} values of 0.09 $mL.L^{-1}$ and LD_{95} of 3.31 $mL.L^{-1}$ were observed in the treatment performed with the synthetic insecticide Decis®. Although the results presented in the Figure 2 indicate that the treatment with free essential oil presents a slightly greater insecticidal action, promising results were obtained with the nanoparticles incorporated with this essential oil during a longer period (Fig 3).

Fig 3.

Over the 72-h assessement period, the highest mortality (82%) of the flies was observed with the treatment containing the free essential oil from *R. officinalis* during the first 24 h of evaluation (Fig 3). In the same period of analysis, a mortality of 60% of the flies was obtained with the nanoencapsulated essential oil. However, the nanoencapsulated essential oil stood out in relation to the free essential oil by maintaining the insecticidal activity for 48 hours at 42% mortality compared to 7% mortality for the free essential oil. In the 72-h evaluation period, no results were observed for free essential oil, only for the nanoencapsulated essential oil, for which a mortality rate of 22% was observed.

The results for the anticholinesterase activity of the free and nanoencapsulated essential oil from *R. officinalis* are shown in Fig 4.

Fig 4.

The maximum inhibitory activities for free and nanoencapsulated essential oil were 75% and 25%, respectively, at a concentration of 0.10 $mg.mL^{-1}$. The positive control, carvacrol, inhibited enzymatic activity by 90% at the same concentration, and no enzymatic activity was observed for the poly(ε-caprolactone) polymer.

Discussion

The essential oils are composed of secondary metabolites, and they are produced as a form of plant defense. Their chemical compositions can vary within the same species of plant, providing different chemical entities (Simões *et al*., 2007; Do *et al*., 2015). Other factors that may explain the differences in chemical constituents and their concentrations for the essential oil of rosemary from this study and the data found in the literature are the edaphoclimatic effects. Variations in temperature, rainfall, incidence of ultraviolet radiation, and attack by pathogens and insects can influence the production of essential oil constituents (Gobbo-Neto & Lopes 2007).

Ahsaei et al. (2020) determined that the principal components of the essential oil from rosemary were 1.8-cineole (26.1%), camphor (15.8%), 3-carene (13.8%) and camphene (10.24%). Furthermore, Neves et al. (2019) studied the composition of rosemary essential oil and found α-pinene (8%), 1,8-cineole (34%) and camphor (52%) as the principal constituents. These results are in agreement with those found in this work, however, with different percentages for the principal constituents.

The increase in the particle size might be due to the encapsulation effect. This characteristic is related to the stability. The more negative the value, the greater the tendency for repulsion between the particles and, consequently, the greater their stability (Souza *et al*., 2012). It can be inferred that the incorporation of rosemary essential oil in the PCL nanoparticles increased the stability of these particles in relation to the PCL nanoparticles without essential oil.

The increase in zeta potential in PCL nanoparticles after the incorporation of the essential oil from rosemary can be also related to the adsorption of the surfactant Tween 80 on the surface of the particle as this compound has hydroxyl groups that can dissociate. In fact, the zeta potential of a particle is related to the surface properties, such as the number of ionized groups and the presence of charged or uncharged polymers on the surface of the particles (Abriata *et al*., 2019).

Additionally, the nature of incorporated oil and the techniques used for nanoparticle synthesis can influence the physical parameters of nanoparticles incorporated with oil. For example, Jummes et al. (2020) characterized PCL nanoparticles incorporated with the essential oil from *Cymbopogon martinii* and found a particle size of 289.3 ± 1.5 nm, a polydispersity index of 0.140 and the zeta potential equal to -30 mV, whereas a study by Khoobdel *et al*. (2017) on the characterization of PCL nanocapsules incorporated with rosemary essential oil prepared by using the nanoprecipation technique resulted in a particle size of 145 nm, a polydispersion index of 0.3 and a zeta potential of -11.0 mV.

The spectrum of the poly(ε-caprolactone) nanoparticles is in accordance with data found in the literature. Strong bands appear at 1720 cm^{-1} that are characteristic of the stretching vibrational mode of the carbonyl group. The 1294 cm^{-1} band is characteristic of the elongation of the main chain (C-C) and (C-O). The intense, wide band that appears at approximately 1100 cm^{-1} is characteristic of the vibrations of the ester bonds of Tween 80, which is used as an emulsifier in the production of nanoparticles (Zanetti *et al*., 2019).

The fact that the rosemary essential oil was incorporated into the nanoparticles is demonstrated by the presence of the oil bands at 1720, 1500, 1400 and 1100 cm^{-1} in the spectrum of nanoparticles containing the oil. Some bands characteristic of the functional groups present in the PCL spectrum suffered distortion after the addition of the essential oil to the polymer. This distortion might have occurred as a result of intermolecular interactions between the functional groups of the polymer and the functional groups of the constituents of the essential oil.

An intense band at 1720 cm^{-1} in the FTIR spectrum of the essential oil refers to the stretching vibration of a carbonyl bond (C=O), and it is probably due to the carbonyl of the principal camphor constituent present in this oil. After incorporating the oil into the poly(ε-caprolactone) nanoparticles, which also possess the carbonyl funtional group, an increase in the intensity of the band at 1720 cm^{-1} was observed.

The bands at 1400-1500 cm^{-1} refer to the unsaturation (C=C) present in the constituents of the essential oil, such as limonene, camphene, α-penene, β-pinene and mircene. The intense, wide band that appears at 1100 cm^{-1} in the spectrum obtained for the oil-containing nanoparticles refers to the vibrations of the ester bonds (COOC) of PCl and Tween 80, and also to the ether bond (COC) of the 1,8-cineol present in the OAS. The absorptions corresponding to the vibration of both functional groups occur at the same wavelength.

Peaks in the spectrum at 1112 cm^{-1} and 1669.56 cm^{-1} were observed by Gharenaghadeh *et al*., (2017) in the characterization of a nanoemulsion containing the essential oil from *Salvia multicaulis*. These peaks refer to 1,8-cineol and camphor, which possesses ether and carbonyl bonds, respectively. These results were also found in this work.

The insecticidal activities can be related to the controlled release of the constituents of the essential oil from *R. officinalis* contained in the nanoparticles. Thus, the essential oil from *R. officinalis* is being gradually released from the biodegradable nanoparticles of poly(ε-caprolactone), and this slow release prolongs its insecticidal activity. These results prove that the encapsulation of the essential oil in biodegradable nanoparticles is efficient for the controlled release of its constituents, and it can be related to the controlled release of the constituents of the essential oil from *R. officinalis* contained in the nanoparticles. Thus, the essential oil from *R. officinalis* is being gradually released from the biodegradable nanoparticles of poly(ε-caprolactone), and this slow release extends its insecticidal activity for a longer period of time.

The insecticidal activity of the essential oil from *R. officinalis* has already been reported and attributed to its main constituents, 1,8-cineol, camphor and α-pinene (Krzyzowski *et al*., 2020). The lipophilic character of the constituents of essential oils, as well as the low molecular weight of these substances, collaborates their toxicity against insects by altering the functionality of membranes and inhibiting respiration and ion transport (Souza *et al*., 2020). Essential oils can also cause neurotoxicity through mechanisms that involve blockage of octopamine receptors, inhibition of gamma-aminobutyric acid (GABA) and inhibit the enzyme acetylcholinesterase (Chaubey, 2012; Mossa, 2016). In addition, EOs can cause disregulation

of pheromones, hormones, and biochemical and physiological dysfunctions. EOs can also act by inhibiting insect growth and inhibiting food intake (Mohafrash *et al*., 2020).

According to work performed by Park *et al*. (2016) regarding the insecticidal activity of the essential oil from *Mentha piperita* and *Perilla frutescens* against *D. suzukii* females, the authors determined that the LD_{50}'s were 4.10 and 3.31 $mg.L^{-1}$, respectively. These results differ from those found in this study because the LD_{50} value found for the free essential oil from *R. officilais* in non-sexed flies was approximately 6.7 $mL.L^{-1}$. The difference in insecticidal activity of the essential oils mentioned in relation to the essential oil under study can be related to the difference in the chemical composition. Although these oils have chemical constituents in common, their concentrations in each oil are different.

Work performed by Renkema *et al*. (2016) on the insecticidal activity of the essential oil from *R. officilais* against *D. suzukii* at a concentration of 15 g L^{-1} found that the mortality rates were 38.3% and 68.6% for male and female flies, respectively, during the first six hours, and the mortality was 1% and 7.3% for male and female flies, respectively, after 24 hours. These data corroborate those found in this study, in which the oil under study, at a concentration of 20 $mL.L^{-1}$, caused a mortality of approximately 77% of the flies without sexing during the first 6 hours of evaluation and a mortality of approximately 5% during the remaining 18 hours in a 24-hour evaluation trial. Better results in relation to the treatments with free oils was obtained with the nanoencapsulated essential oil from *R. officilai*s at a concentration of 20 $mL.L^{-1}$. The mortality rates for this treatment were approximately 60, 59, 42 and 22% in the evaluation periods of 6, 24, 48 and 72 hours, respectively.

Acetylcholinesterase (AChE) inhibitory activity can be observed for both samples, and there was a correlation between the increase in the concentration of these active principles and the increase in inhibitory activity. The inhibition of this enzyme is the main mechanism of action of insecticides based on organophosphates and carbamates (Wang et al., 2004).

Studies performed by De Souza et al. (2012) mention the presence of 1,8-cineol and α-pinene in essential oils as potent inhibitors of the enzyme acetylcholinesterase. Furthermore, Miyazawa & Yamafuji (2005) showed that monoterpenoids exert a high degree of inhibition of this enzyme. The non-oxygenated monoterpenes camphene, limonene, β-pinene and mircene found in the essential oil from *R. officinalis* might have contributed individually or synergistically to the anticholinesterase activity of this oil.

The greater enzymatic inhibition observed for the free oil than for the nanoencapsulated oil can be related to the fact that the chemical constituents of the oil in the nanoparticles are mostly contained in the particle nucleus. The encapsulation efficiency for the

essential oil of R*osmarinus officinalis* in PCL can reach a value of 78.20%, as reported by Khoobdel et al., (2017). Thus, only a part of the essential oil is adsorbed on the surface of the particle, so direct contact with the enzyme acetylcholinesterase is difficult.

Finally, insecticidal activity against the fly *D. suzukii* was observed for the free and nanoencapsulated essential oil of *R. officinalis*. The incorporation of this essential oil into PCL polymeric nanoparticles slightly reduces its insecticidal potential. However, the volatility of the essential oil was lower for the encapsulated oil, and, consequently, its insecticidal effect was prolonged, so that industrial applications of this essential oil are possible.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Chemical composition of the essential oil from *Rosmarinus officinalis*.

	Compound[a]	RI[b]	RIcal[c]	%[d]
1	α-pinene	932	933	12.90
2	camphene	946	949	3.85
4	β-pinene	974	978	2.24
5	myrcene	988	989	5.24
7	limonene	1024	1030	1.27
8	1,8-cineol	1026	1032	17.05
9	camphor	1141	1147	35.38
Percent of the total área				78.38

[a] The components are listed in order of elution on the apolar capillary column of fused silica with DBS bound stationary phase. [b] Retention index. [c] Calculated retention index, [d] (%): mean percentage of each compound.

Figure Legends

Figure 1. Fourier transform infrared analysis of free and nanoencapsulated essential oil from *Rosmarinus officinalis*.

Figure 2. Toxicity of free and nanoencapsulated *Rosmarinus officinalis* essential oil to the *Drosophila suzukii* fly.

Figure 3. Insecticidal activity of free and nanoencapsulated *Rosmarinus officinalis* essential oil during an evaluation period of 72 hours.

Figure 4. Anticholinesterase activity of free and nanoencapsulated *Rosmarinus officinalis* essential oil.

Figures

Fig. 1

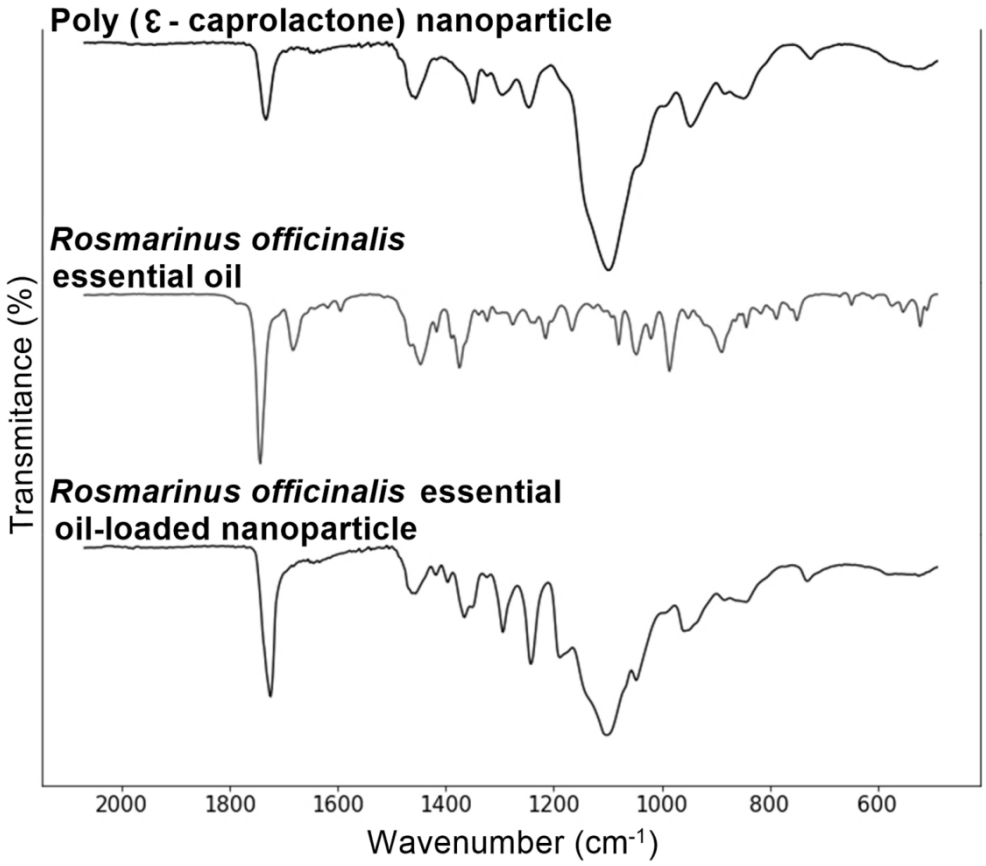

Figure 1. Fourier transform infrared analysis of free and nanoencapsulated essential oil from *Rosmarinus officinalis*.

Fig. 2

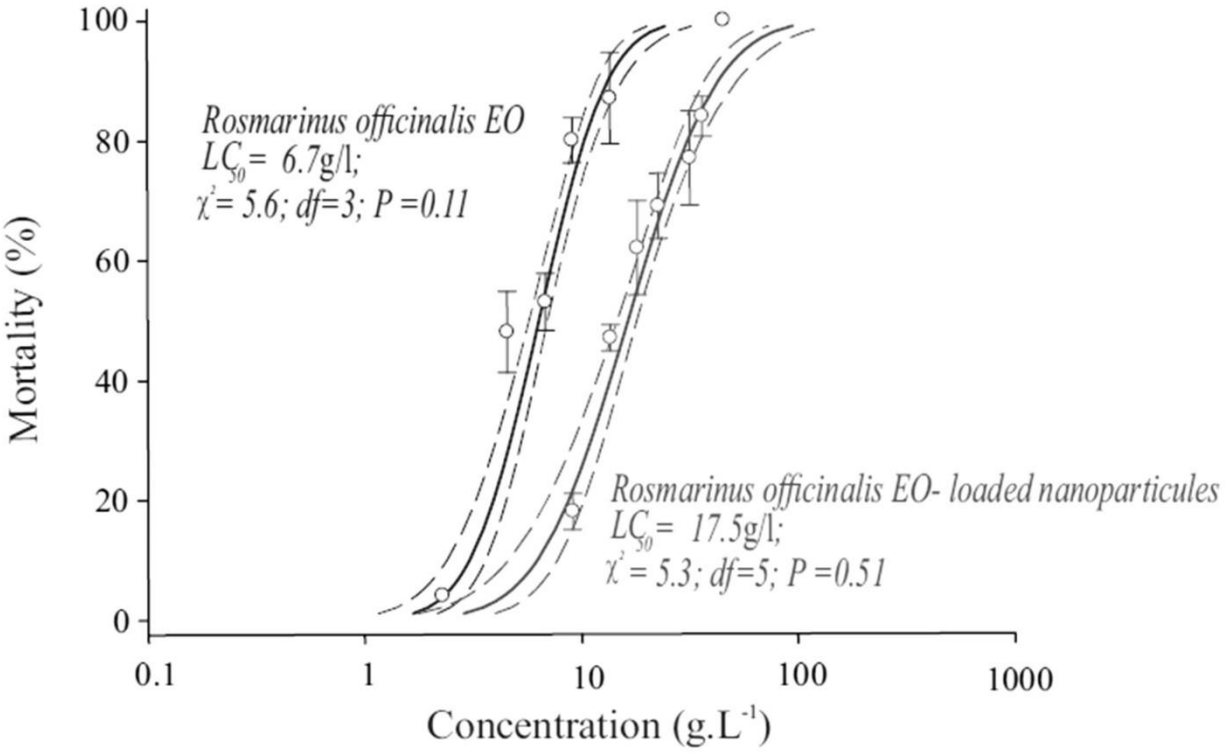

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Fig. 3

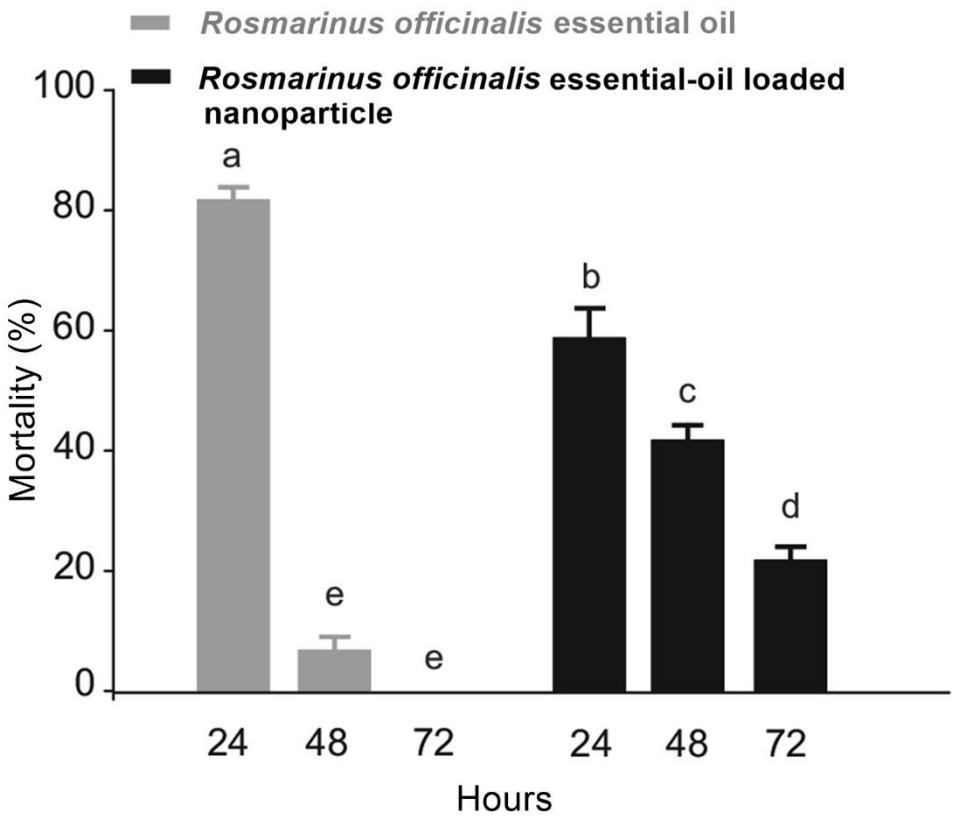

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Fig. 4

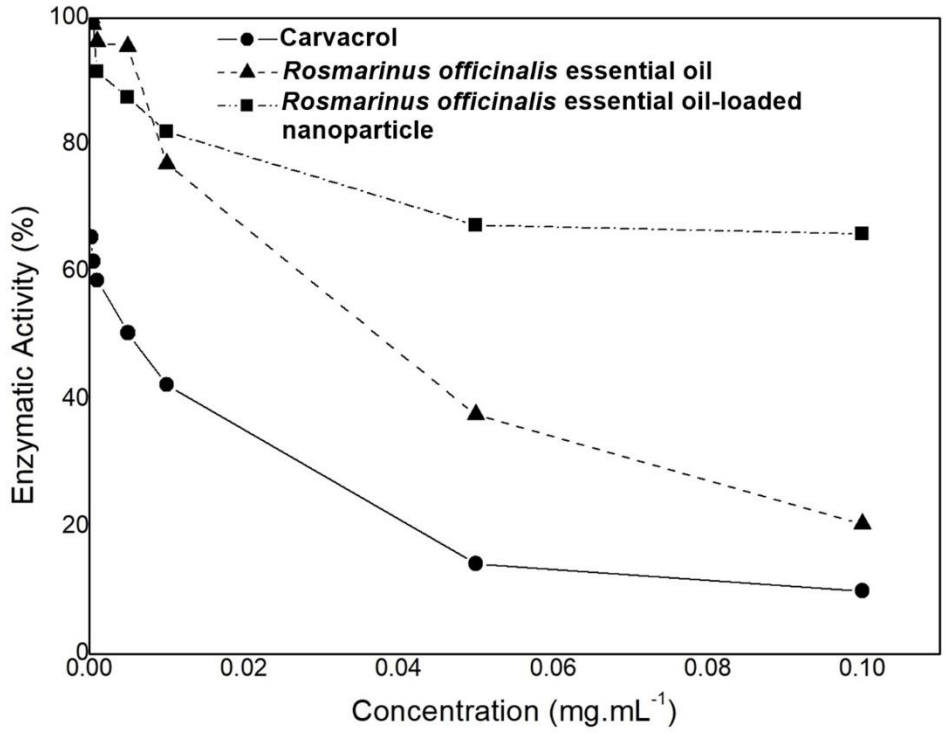

Figure 4. Anticholinesterase activity of free and nanoencapsulated *Rosmarinus officinalis* essential oil.

ARTIGO 2 - Antifungal activity of poly(ε-caprolactone) nanoparticles incorporated with *Eucalyptus* essential oils against *Hemileia vastatrix*. Data de publicação: 02/07/2022.

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Antifungal activity of poly(ε-caprolactone) nanoparticles incorporated with *Eucalyptus* essential oils against *Hemileia vastatrix*

Running Head: Antifungal action of bioactive nanoparticles

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Significance and impact of the study: The fungus *Hemileia vastatrix* Berkeley and Broome is one of the main vectors responsible for the decrease in coffee production by causing economic losses to the producers of this commodity. This work details the potential for the application of biodegradable poly(ε-caprolactone) nanoparticles containing essential oils from *Eucalyptus* to combat the fungus that causes coffee rust (*Hemileia vastatrix*). Promising results were found in this work, which indicate the feasibility of applying nanostructured systems for controlled release of essential oils to combat phytopathogenic fungi in coffee cultures.

Abstract

Coffee (*Coffea* L.) is one of the main crops produced globally. Its contamination by the fungus *Hemileia vastatrix* Berkeley and Broome has been economically detrimental for producers. The objective of this work was to extract and characterize the essential oils from *Eucalyptus citriodora* Hook, *Eucalyptus camaldulensis* Dehn and *Eucalyptus grandis* Hill ex Maiden, produce and characterize nanoparticles containing these essential oils, and evaluate the *in vivo* and *in vitro* antifungal activity of free and nanoencapsulated essential oils. The principal constituents of the essential oil from *E. citriodor*a was citronellal; that from *E. grandis* was α-pinene; and that from *E. camaldulensis* was 1,8-cineol. The *in vitro* antifungal activity against the fungus *H. vastatrix* was 100% at a concentration of 1000 μl l^{-1} for all the oils and nanoparticles containing these natural products. The sizes of the nanoparticles produced with the essential oils from *E. citriodora*, *E. camaldulensis* and *E. grandis* were 402.13 nm, 275.33 nm and 328.5 nm, respectively, with surface charges of -11.8 mV, -9.24 mV and -6.76 mV, respectively. Fourier transform infrared analyses proved that the encapsulation of essential oils occurred in the polymeric matrix of poly(ε-caprolactone). The incorporation of essential oils into biodegradable poly(ε-caprolactone) nanoparticles increased their efficiency as

biofungicides in the fight against coffee rust, decreasing the severity of the disease by up to 90.75% after treatment with the nanoparticles conaining the essential oil from *E. grandis*.

Keywords: *Eucalyptuys grandis*, *Eucalyptus camaldulensis*, *Eucalyptus citriodora*, Polymeric nanoparticles, Coffee tree rust, Microbiological activity.

Introduction

Coffee (*Coffea* L.) is one of the most important cultures in the world. Its grains are processed to produce popular beverages that are consumed on all the continents. The world production of coffee beans in the year 2021 was 176.1 million kg (Lu *et al.* 2022). More than 50% of the total production comes from four countries, which include Brazil, Vietnam, Colombia and Indonesia. Some aspects of coffee growing can influence the quality of the final product, the price of the product on the market, and, consequently, the world economy (Vogt 2020).

Coffee growing can be affected by several factors, plant diseases being one of the principal factors. Coffee rust caused by the fungus *Hemileia vastatrix* Berkeley and Broome can be considered to be one of the main diseases that affect this crop and drastically reduce its productivity (Resende *et al*. 2021).

Traditionally, coffee rust management is achieved with the aid of systemic fungicides such as triazoles, strobilurins and copper hydroxide. These agrochemicals are applied according to a fixed schedule on a predetermined date (prevention) or by regular monitoring of the phytopathology (Costa *et al.* 2019; Liebig *et al.* 2019).

Coffee farming is currently influenced by new consumer markets and globalization that increasingly demand sustainable products and processes (Lemeilleur *et al.* 2020). Natural fungicides with low toxicity and high efficiency and that do not generate residues in the environment have been studied with the objective of obtaining a sustainable production of coffee (Caetano *et al.* 2020). Many research activities have shown that essential oils can be used

for the management of pests and fungal diseases (Antonioli *et al.* 2020). For example, essential oils from *Eucalyptus* have a pleasant odor, a high extraction yield and an activity against various microorganisms because of their composition, which is rich in oxygenated monoterpenes and, mainly, a high concentration of 1,8-cineol and citronellal (Sabo and Knezevic 2019; Salehi *et al.* 2019; Galan *et al.* 2020).

Eucalyptus is one of the most economically important genera of the Myrtaceae family (Lin *et al.* 2019). They are composed of about 700 species of large trees and evergreen shrubs. The aerial parts of trees are a rich source of essential oils that are used commercially in the food and pharmaceutical industries (Nwabor *et al.* 2019; Yadav *et al.* 2019). Several phytochemical studies have reported the antifungal potential of Eucalyptus essential oils against a wide range of economically important phytopathogenic fungi, including *Alternaria alternate*, *Hemileia vastatrix*, *Botrytis cinerea*, *Rhizopus stolonifera*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Fusarium culmorum* (Caetano *et al*. 2020; Elgat *et al*. 2020; Silva *et al*. 2020; Pedrotti *et al*. 2022).

In recent years, biodegradable nanoparticles have been increasingly used as controlled release systems for different hydrophobic molecules (Hao *et al.* 2020). Biodegradable nanoparticles have unique advantages over other controlled-release colloidal systems because of their high surface area, low cost and ease of production (Soppimath *et al.* 2001; Grossen *et al.* 2017).

The effects of biodegradable poly(ε-caprolactone) (PCL) nanoparticles incorporated with essential oils from *Eucalyptus* plants on coffee rust have not yet been studied. These particles are potentially effective against this disease, even in coffee plantations where coffee rust has been identified close to the harvest period, a phase in which the use of fungicides is not advisable. In the current study, the essential oils from three botanical sources (*Eucalyptus citriodora* Hook, *Eucalyptus camaldulensis* Dehn and *Eucalyptus grandis* Will ex Maiden)

were extracted and chemically characterized. They were encapsulated in biodegradable polymeric nanoparticles, and the *in vitro* and *in vivo* antifungal activities against *H. vastatrix* were evaluated.

Results and Discussion

Chemical composition of essential oils extracted from *Eucalyptus* leaves

The chemical compositions of the essential oils from the three species of *Eucalyptus* were different for all the samples (Table 1). However, the constituents (1,8-cineole, α-pinene, limonene, *p*-cymene and α-terpineol) were common to the essential oils from *E. grandis* and *E. camaldulensis*

(Table 1).

Ten constituents of the 13 quantified in the essential oil from *E. grandis* were identified, and the principal components were α-pinene (55.21%), 1,8-cineole (16.52%) and α-terpineol (11.05%). Eight constituents were identified and quantified in *E. camaldulensis* oil, the principal components being 1,8-cineol (58.71%), limonene (27.87%) and terpinolene (3.59%). Four chemical constituents were identified and quantified in *E. citriodora* oil. The principal component was citronellal (93.97%).

The composition of an essential oil of the same species of plant can vary because of several factors such as temperature, ultraviolet radiation, altitude, availability of water, attack by insects and microorganisms, among others. This fact explains the difference in the composition and concentration of each substance in the essential oils of this work when compared with those found in the literature (Gobbo-Neto and Lopes 2007).

Infrared Analysis

The spectra of the samples analyzed by FTIR are of paramount importance in the characterization of nanoparticles because they prove that the essential oils were incorporated into the polymer and that there were no undesirable chemical reactions between the polymer matrix and the constituents of the essential oils to form undesirable products. The spectra of the samples are shown in Figure 1.

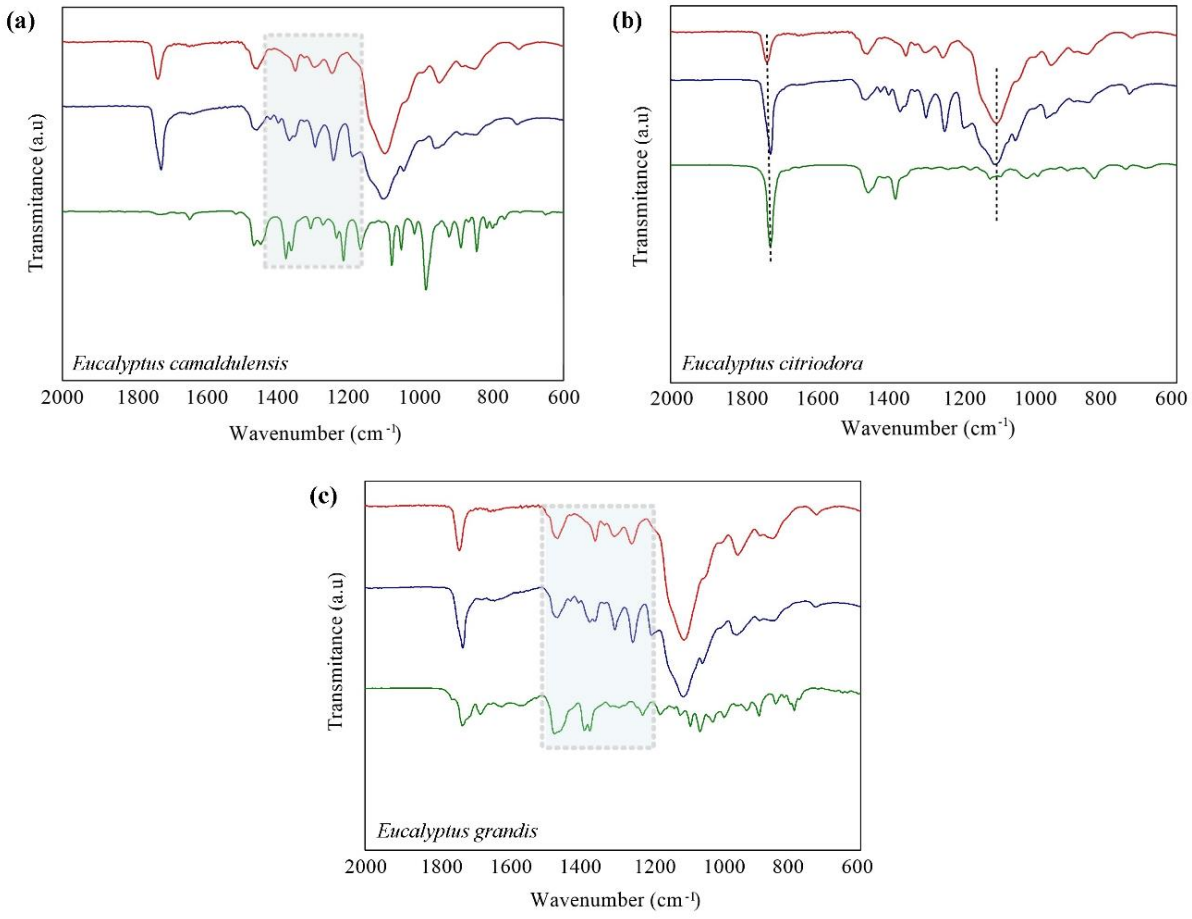

Fig.1

Figure 1. Fourier transform infrared analysis of essential oil free and nanoencapusulated. (A): (—) poly(ε-caprolactone) nanoparticles, (—) Essential oil (*E. camaldulensis*), (—) *E. camaldulensis essential* essential oil-loaded nanoparticle; (B): (—) poly(ε-caprolactone) nanoparticles, (—) Essential oil (*E. citriodora*), (—) *E. citriodora essential* essential oil-loaded nanoparticle *E. citriodora*; (C): (—)poly(ε-caprolactone) nanoparticles, (—) Essential oil (*E. grandis*), (—) *E. grandis essential* essential oil-loaded nanoparticle.

On the basis of the spectra presented in Figure 1, the PCL analysis is in accordance with data found in the literature. Strong bands that appear at 1720 cm^{-1} are characteristic of the stretching vibrational mode of the carbonyl group (C=O). The 1294 cm^{-1} band is characteristic of the stretching mode of the main chain (C-C) and (C-O) of the PCL and has been used to investigate changes in the crystallinity of this polymer. The intense, wide band that appears at 1100 cm^{-1} is characteristic of the vibrations of the ester bonds of Tween 80, which is used as an emulsifier in the production of PCL nanoparticles (Zanetti *et al.* 2019).

The incorporation of the essential oils into the polymeric nanoparticles is demonstrated by the fact that the bands present in the spectra of the essential oils at approximately 1720 cm^{-1}, 1500 cm^{-1}, 1350 cm^{-1} and 1100 cm^{-1} also appeared in the nanoparticle spectra. These bands in the spectrum of nanoparticles containing essential oils have undergone elongation relative to those of the spectrum of nanoparticles of PCL. This effect can be due to intermolecular interactions between the constituents of essential oils and between these constituents and the polymer matrix.

In the FTIR spectrum obtained for the essential oil from *E. citriodora* and for the nanoparticles containing this oil, an intense band at 1720 cm^{-1}, which corresponds to the stretching vibration of the carbonyl bond (C=O), can be observed. This band is probably due to the carbonyl of the principal citronellal constituent present in this oil (Ribeiro *et al.* 2014). The bands that appear in the spectrum at 1350-1500 cm^{-1} correspond to unsaturation (C=C) present in the constituents of the essential oils.

An intense, wide band can be observed at 1100 cm^{-1} in the spectra obtained for the essential oil from *E. grandis* and the nanoparticles containing this oil. This band is characteristic of the vibrations of the ester bonds of Tween 80, as well as the ether bond (C-O-C) of one of the principal constituents (1,8-cineol) in this oil. The bands that appear at 1350 - 1500 cm^{-1}

correspond to the stretching of the olefinic bonds of the camphene, limonene and *p*-cymene constituents.

Bands that appear in 1350 - 1500 cm^{-1} can be observed in the spectra obtained for the essential oil from *E. camaldulensis* and for the nanoparticles containing this oil. These bands are characteristic of the stretching of the double bonds in the constituents of the essential oil. The strong band that appears in the nanoparticle spectrum at 1720 cm^{-1} and that does not appear in the oil spectrum is characteristic of the stretching vibrational mode of the carbonyl group of the PCL. The intense, wide band that appears at 1100 cm^{-1} in the spectrum obtained for nanoparticles and for this oil correspond to the vibrations of the ester bonds of PCl and Tween 80 and the ether bond of one of the principal constituents (1,8-cineol) present in this essential oil. Both organic functions vibrate at the same wavelength (Pant *et al.* 2014).

Zeta Potential

The incorporation of essential oils into the PCL nanoparticles changed the surface charge of the polymer, particle size, and influenced the polydispersity index. The particles incorporated with the essential oils were larger than the PCL particles (186.86±0.61 nm = 100%) without the oil. This increase in particle size can be due to the encapsulation effect. Essential oils, when incorporated into PCL nanoparticles, can form nanocapsules, which are vesicular systems in which the oil is confined to a cavity surrounded by a single polymeric membrane, or nanospheres, which are matrix systems in which the oil is physically and uniformly dispersed. This incorporation results in an increase in the particle size (Soppimath *et al.* 2001). In a study performed by Jummes *et al.* (2020), the authors state that PCL nanoparticles that did not contain essential oils are smaller because of their empty interior.

The sizes of the particles containing the essential oils from *E. citriodora*, *E. grandis* and *E. camaldulensis* varied from 402.13±42.0 (94.7%) to 4117±1248 (5.3%) nm, from

328.5±17.31 (8%) to 3773±108.6 (92%) nm, and from 275.33±13.4 (86.2%) to 3546±511.8 (13.8%) nm, respectively. Particles with diameters smaller than 410 nm differed statistically in relation to this parameter, whereas particles with a size scale greater than 1000 nm were statistically equal ($p > 0.05$). The variation in particle size was confirmed by the values of the polydispersity index, which were 0.43, 0.46, and 0.40 for the particles containing the oils from *E. citriodora*, *E. grandis* and *E. camaldulensis*, respectively, and 0.098 for PCL nanoparticles.

The results obtained for the zeta potential differed statistically for all samples ($p<0.05\%$). The zeta potential obtained for the PCL particles was -8.39±0.52 mV, a negative potential. The nanoparticles incorporated with the essential oils from *E. citriodora*, *E. camaldulensis* and *E. grandis* exhibited a zeta potential of -11.8±30.18, -9.24±0.23 and -6.76±0.34 mV, respectively. According to literature data found, higher values for the zeta potential (positive or negative) correspond to a smaller number of particles and a lower tendency for aggregation (Souza *et al.* 2012; Abriata *et al.* 2019). Thus, we can infer that the most stable emulsion was prepared with the essential oil from *E. citriodora*.

The incorporation of essential oils into the PCL nanoparticles tends to make the zeta potential more negative because the essential oil can contain chemical constituents that have hydroxyl groups, which can ionize to form negative anions when they are adsorbed on the surface of the particles.

Encapsulation Efficiency

The encapsulation efficiency percentage (EE%) for the essential oils from *E. citriodora, E. camaldulensis* and *E. grandis* were 78.08±0.54, 93.38±0.94 and 93.16±0.61%, respectively. The fact that the values obtained for the essential oils from *E. camaldulensis* and *E. grandis* were grearer than those obtained for the essential oil from *E. citriodora* might be related to a

greater hydrophilic-lipophilic balance of these oils because the essential oil from *E. citriodora* contains mostly citronellal (93.97%).

Results similar to those of this work were found in the literature for EE%. Ahsaei *et al*. (2020) studied the essential oils from *R. officinalis* and *Z. multiflora* incorporated into poly(ε-caprolactone) nanoparticles. The authors observed EE%s of 75.8% and 84.4%, respectively. Jummes *et al.* (2020) studied the same system of polymeric nanoparticles of poly(ε-caprolactone) containing the essential oil from *C. martinii* and found an EE% of 99.54%. On the basis of the results found in this work and after comparing with the data found in the literature, one can conclude that the EE% can vary according to the essential oil used, as well as the polymer matrix, surfactant and the technique for preparing the nanoparticles.

In vitro* antifungal activity of essential oils and nanoparticles against *Hemileia vastatrix

The results for the *in vitro* activity against *H. vastatrix* of essential oils and nanoparticles containing these oils are presented in Table 2. According to these results, antifungal activity against *H. vastatrix* was observed for all the essential oils and all the nanoparticles containing these oils. There was a dose-dependent correlation; that is, the antifungal activity increased with an increase in concentration. No antifungal activity was observed with the treatments performed using culture medium (2% agar/water), Tween 80 1% v/v and PCL nanoparticles. The treatment performed with the Opera® fungicide inhibited 100% of the spore germination at all the concentrations tested.

Table 2.

Greater antifungal activity for the essential oil from *E. citriodora* in its free form was observed at a lower concentration (125 μl l^{-1}) than those of the other oils, a result similar to that

found for the commercial fungicide Opera®. The nanoparticles with the greatest antifungal activity were those containing the essential oils from *E. citriodora* and *E. camaldulensis*. In the treatment using free and nanoencapsulated *E. grandis* essential oil, damage to the morphological structure of the fungus was observed, such as deformation of the spore structure and uniform growth of hyphae.

The mechanism of antifungal activity of essential oils is related to inhibition of the synthesis and biological function of ergosterol and inhibition of spore germination. Other possible mechanisms of antifungal activity of the essential oils made up of terpenes are related to inhibition of the electron transport chain and inhibition of the ATPase enzyme in mitochondria. These mechanisms are only possible because of the lipophilic characteristics of the chemical constituents of essential oils, in which they interact or penetrate the cell membrane of fungi in a united or synergistic way (Kisová *et al*. 2020).

Some constituents of essential oils, such as α-pinene, found in the essential oils from *E. grandis* (55.21%) and *E. camaldulensis* (5.59%) in this study, have already had their antifungal mechanisms elucidated They caused damage to the integrity of the cell membrane, morphological changes in the conidia, vacuolization and disorganization of the cytoplasm, and rupture of the plasma and mitochondrial membrane, in addition to causing changes in the permeability of the fungal membrane (Rguez *et al*. 2020).

The activity of citronellal, the principal constituent (93.97%) of the essential oil extracted from *Eucalyptus citriodora* in this study, has already been studied against several fungi, and it has shown interference in membrane integrity and reduction in ergosterol levels as a mechanism of antifungal activity. This substance negatively regulates the ergosterol genes responsible for the conversion of lanosterol to ergosterol (Yang *et al*. 2021). The same mechanisms of antifungal action described above for the chemical constituents of essential oils

might have occurred with the fungus *Hemileia vastatrix* after treatments with *Eucalyptus* essential oils because these oils also contain these same substances.

In vivo antifungal activity of essential oils and nanoparticles against *Hemileia vastatrix:*

Preventive effect

According to the results presented in Figure 2, the active ingredients that exhibited antifungal activity against *H. vastatrix* were the free and nanoencapsulated essential oil from *E. camaldulensis* and *E. grandis*, the nanoparticles incorporated with the essential oil from *E. citriodora* and the Opera® fungicide. Greater activities were observed for the nanoencapsulated essential oils from *E. grandis* and *E. camaldulensis* than for their free forms. The activity was similar to that of the fungicide Opera®, whose application delayed the appearance of the disease for eight weeks. The delay in the onset of the disease was three weeks for the free essential oil from *E. grandis*. No activity was observed for the essential oil from *E. camaldulensis* in its free form, a fact that might be related to its high volatility. The development of the disease was delayed for three weeks and for one week, respectively, in the presence of the free and nanoencapsulated forms of the essential oil from *E. citriodora.*

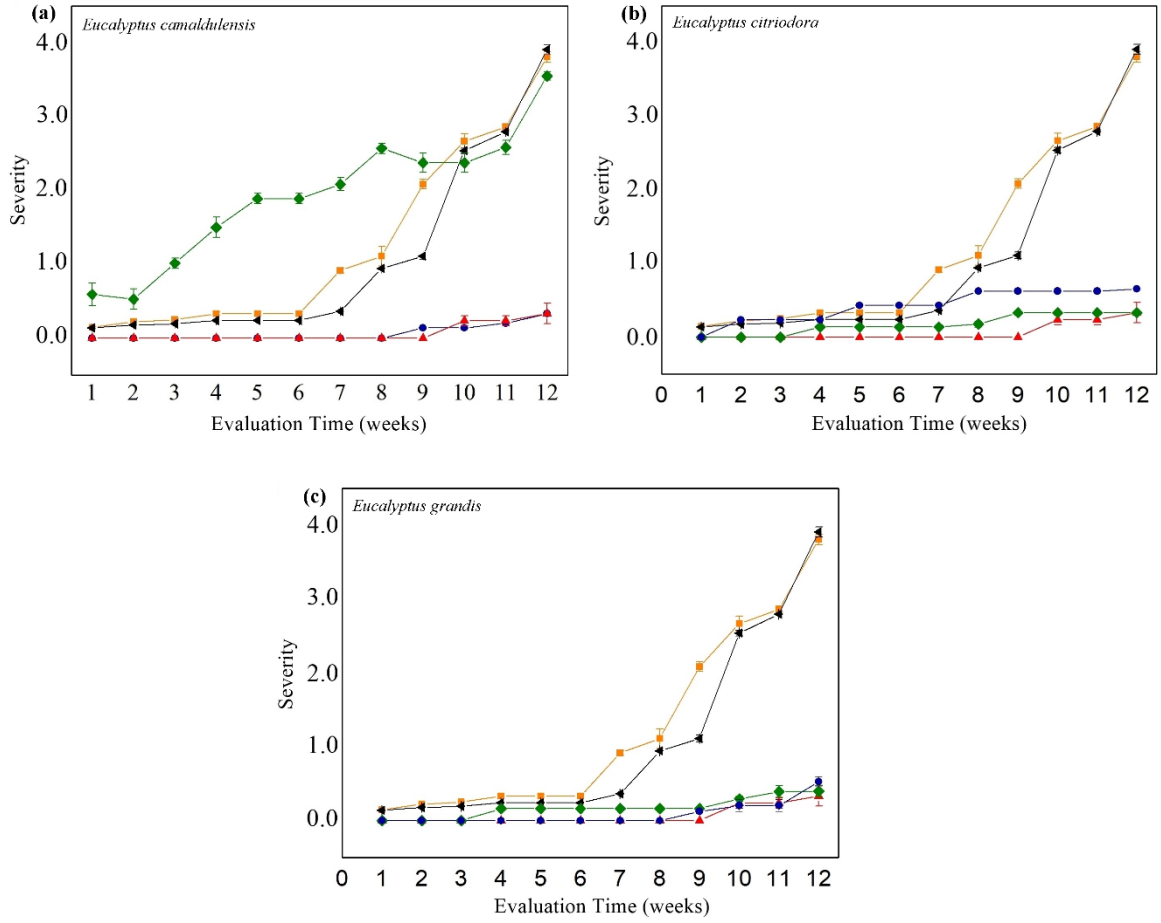

Fig.2

Figure 2. Preventive activity of the free and nanoencapusulated essential oils against coffee rust. (A) (—) poly(ε-caprolactone) nanoparticles, (—) fungicide Opera®, (—) Water, (—) Essential oil (*E. camaldulensis*), (—) *E. camaldulensis essential* essential oil-loaded nanoparticles; (B) (—) poly(ε-caprolactone) nanoparticles, (—) fungicide Opera®, (—) Water, (—) Essential oil (*E. citriodora*), (—) *E. citriodora essential* essential oil-loaded nanoparticle. (C) (—) poly(ε-caprolactone) nanoparticles, (—) fungicide Opera®, (—) Water, (—) Essential oil (*E. grandis*), (—) *E. grandis essential* essential oil-loaded nanoparticles.

It can be inferred that the nanoencapsulation of essential oils from *E. grandis* and *E. citriodora* lead to an increase in their preventive antifungal activities. This increase can be related to the gradual release of the constituents of these oils, as well as the prevention of their degradation by external factors. This protection can extend the antifungal activities for a longer

period. As for the essential oil from *E. camaldulensis*, nanoencapsulation might not have been as effective because the oil can be mostly contained in the nucleus of the nanoparticles. Therefore, the volatilization rate is low, which results in a low antifungal activity.

The use of essential oils as active principles for preventing the attack of pathogens is related to their eliciting properties; that is, they induce the increase in phytoalexins, which are directly linked to plant defense mechanisms. The application of essential oils such as *Cymbopogon nardus, Cinnamomum zeylanicum* and *Syzygium aromaticum* in plants can increase the production of enzymes related to systemic acquired resistance (RSA), such as peroxidase and chitinase, as well as increase the production of phenolic compounds, which are directly related to the defense mechanism of plants against various pathogens (Werrie *et al*. 2020). The application of free and nanoencapsulated *Eucalyptus* essential oils might have increased the production of these compounds, thereby increasing and prolonging the plant's defense mechanism against coffee rust.

Several studies have shown that essential oils, in addition to having direct antifungal activities, can promote plant growth. They can also activate the defense mechanisms related to antioxidant activity via the production of phenylpropanoids and proteins related to pathogenesis to overcome infection by pathogens (Rguez *et al*. 2020).

Healing Effect

The antifungal activity related to the curative effect was observed for all the samples, except those made with PCL nanoparticles and water. The results are shown in the Figure 3.

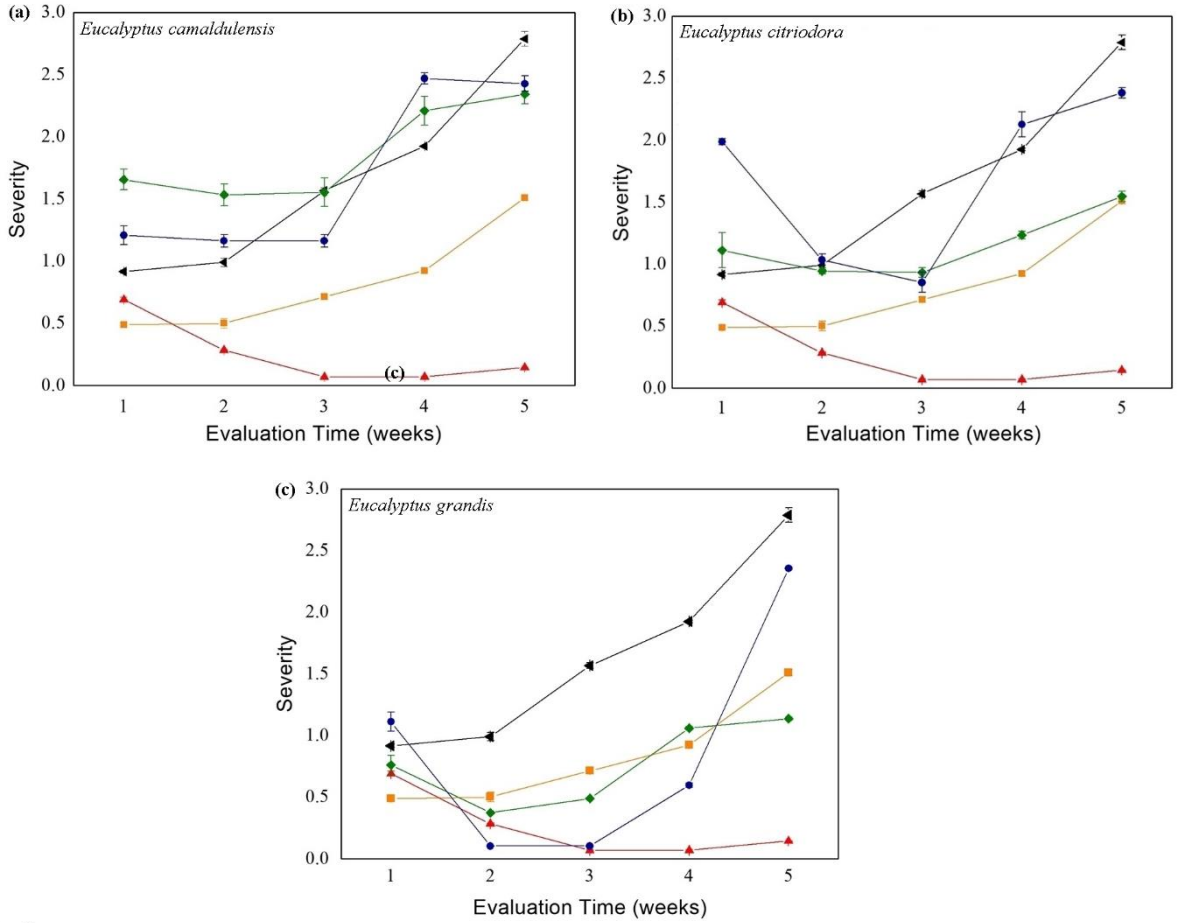

Fig.3

Figure 3. Curative effect of the free and nanoencapusulated essential oils against coffee rust. (A): (—) poly(ε-caprolactone) nanoparticles, (—) fungicide Opera®, (—) Water, (—) Essential oil (*E. camaldulensis*), (—) *E. camaldulensis essential* essential oil-loaded nanoparticles; (B): (—) poly(ε-caprolactone) nanoparticles, (—) fungicide Opera®, (—) Water, (—) Essential oil (*E. citriodora*), (—) *E. citriodora essential* essential oil-loaded nanoparticle. (C): (—) poly(ε-caprolactone) nanoparticles, (—) fungicide Opera®, (—) Water, (—) Essential oil (*E. grandis*), (—) *E. grandis essential* essential oil-loaded nanoparticles.

The greatest *in vivo* antifungal activity was observed for the free essential oil from *E. grandis.* The severity of the disease decreased from 0.76 to 0.37 after the first week of application, that is, a decrease of 49.09%. The increase in severity was observed in the third

week of evaluation, where the severity increased from 0.37 to 0.48 and then to 1.05 and 1.13 in the fourth and fifth week of evaluation, respectively.

The activity of the nanoencapsulated essential oil from *E. grandis* was greater than those for this oil in its free form. After its application, the severity of the infection decreased by 90.75%, from 1.11 to 0.10, in the second week of evaluation, and this value remained constant until the third evaluation. The increase in severity was only observed in the fourth assessment, increasing from 0.10 to 0.59, and from 0.59 to 2.35 in the fifth assessment.

The *in vivo* activity against *H. vastatrix* was observed for the free essential oil from *E. citriodora.* The severity decreased from 1.11 to 0.94 after its application, and this value remained constant until the third evaluation, where it decreased by 15%. The increase in severity was observed in the fourth evaluation, where it increas from 0.93 to 1.23 and later to 1.54 in the fifth evaluation. Better results were obtained for this oil in its nanoencapsulated form than in its free form. The severity of the disease decreased from 1.98 to 1.03 after the first application, which represented a decrease of 52.0%. This value continued to decrease to 0.85 until the third assessment. The increase in severity was only observed in the fourth evaluation, where it increased from 0.85 to 2.12, and later from 2.12 to 2.38 in the fifth assessment.

The behavior of the antifungal activity of the nanoparticles containing the essential oils from *E. citriodora* and *E. grandis* was similar to that of the fungicide Opera®. This commercial fungicide reduced the severity from 0.68 to 0.28 after application, and this percentage continued to decrease to 0.06 until the third evaluation, that is, decreases of 58.84 and 91.18%, respectively. It remained constant until the fourth evaluation. The increase in severity was only observed in the last assessment, increasing from 0.06 to 0.14. It can be inferred that the nanoparticles promoted a gradual release of the essential oils from *E. grandis* and *E. citriodora*, which lead to a stability of action after their applications.

The treatments performed with the free and nanoencapsulated essential oil from *E. camaldulensis* resulted in the same behavior. After its application, the severity of the disease decreased by from 1.65 to 1.53 and from 1.21 to 1.16 and remained constant until the third week of evaluation. Thus, a decrease of 92.6 and 96.24% was observed for the free and nanoencapsulated oil, respectively. The increase in severity was observed in the fourth and fifth weeks of evaluation, reaching a value of from 2.2 to 2.34 and 2.4 to 2.42 in the fifth evaluation for the free and nanoencapsulated oil, respectively. *E. camaldulensis* oil appears to act more as a fungistat than as a fungicide, andt the amount of oil present on the surface of the nanoparticles was sufficient to maintain the fungistatic activity during the two weeks of evaluation. The maintenance of the antifungal activity observed for nanoencapsulated essential oils after their applications might be related to the gradual release of the oil constituents from the nanoparticles, which ensures an antifungal activity for a longer period of time.

In vitro and *in vivo* antifungal activity against the fungus *H. vastatrix* were observed for the systems investigated in this study. The volatility of the essential oils incorporated into nanostructures decreased. and their *in vivo* antifungal activity was prolonged. The results indicate the feasibility of applying nanostructured systems for controlled release of essential oils to combat phytopathogenic fungi in coffee cultures.

Material and Methods

Obtaining plant material and extracting essential oils

The leaves of the *E. citriodora, E. grandis* and *E. camaldulensis* trees were chosen for the extraction of essential oils. These trees were grown in the Department of Forest Engineering of the Federal University of Lavras, MG, Brazil (DEF/UFLA/MG) and received the registration numbers 10150, 10533 and 10266, respectively (48). The leaves that were selected for collection exhibited young, healthy characteristics, and no contamination by microorganisms

or mechanical damage caused by insects was observed. The leaves were collected from branches of *Eucalyptus* trees of approximately 10 years of age. The leaves were collected at a distance of 5 m above the ground. The leaves were collected in March 2019, cleaned, chopped, weighed and added to a 5 l round bottom flask. To this flask, 2.5 l of water was added, and the flask was subjected to hydrodistillation for a period of 2 hours using a modified Clevenger apparatus (Pimentel *et al.* 2008; Anvisa 2010).

Chemical characterization of the essential oils

The constituents of the essential oils were identified using a Shimadzu GC-17A gas chromatograph equipped with a model QP 5050A mass detector and a capillary column of fused silica (30 m x 0.25 mm) with a DBS bound stationary phase (film thickness, 0.25 μm). The carrier gas was helium at a flow rate of 1.18 ml min^{-1}. The temperature was programmed from 60°C, increasing to 240°C at 3°C min^{-1}. The column temperature was then raised at 10°C min^{-1} to 300°C, where the temperature was maintained for 7 min. The injector temperature was 220°C, and the detector (or interface) temperature was 240°C. To perform the analysis, an aliquot of 0.1 μl of the sample diluted 1:100 in hexane was injected onto the column. A mixture of hydrocarbons (C_7H_{16}, C_8H_{18}, ..., $C_{29}H_{60}$, $C_{30}H_{62;}$ Merck®) at a concentration of 1000 μg ml^{-1} was analyzed. Each component in hexane was also injected. The impact energy was 70 eV.

The quantification of the constituents was performed using a Shimadzu CG-17A gas chromatograph equipped with a flame ionization detector. The experimental parameters for the analyses were the same as those used in the identification of the chemical constituents by GC-MS. However, the carrier gas was synthetic air, and the temperature of the detector was 300°C.

The constituents were identified by comparing their retention indices with retention indices from the literature (Adams 2017). The retention indices were obtained using the homologous series of alkanes (C_8H_{18}, C_9H_{20}…, $C_{20}H_{42;}$ Merck®) at a concentration of 40 mg l^{-}

[1] for each constituent, calculated according to the method of Van Den Dool and Kratz (1963). The data were also compared with the two NIST107 and NIST2 libraries available on the instrument. The spectra of the samples were compared with those of the literature (Nist 2010).

Production of nanoparticles containing the essential oils from *Eucalyptus sp.*

The poly(ε-caprolactone) polymer, PCL, (M_n = 50.000 g mol^{-1}) was obtained from Perstorp (Warrington, United Kingdom). Acetone (CAS number 67-64-1) and Tween 80 (CAS number 9005-65-6) were purchased from Synth (São Paulo, Brazil) and used to prepare the biodegradable polymeric nanoparticles used in this study.

The biodegradable polymeric nanoparticles were obtained by the solvent emulsification/evaporation technique, with some modifications (Reis *et al.* 2006; Rao and Geckeler 2011). For this purpose, a PCL-acetone solution was prepared in which 130 mg of PCL polymer was added to 27 ml of acetone and subjected to magnetic stirring at 37°C until complete solubilization of the polymer. To maintain the stability of the system and the interaction between the ionic phases of the liquid-particle interface of the particles that will be formed, 4 ml of Tween 80 surfactant was added, and the solution was stirred at room temperature for 15 min until complete homogenization. With the aid of an injection pump, the suspension containing PCL-acetone-Tween 80 was dripped at a rate of 300 µl min^{-1} into 53 ml of water with magnetic stirring. After dripping, the solution composed of PCL-acetone-Tween 80-distilled water was stirred at room temperature until complete evaporation of the acetone. The volume of acetone lost by evaporation was replaced by the addition of distilled water to the system.

The encapsulation of *Eucalyptus* essential oils in PCL nanoparticles was achieved in a manner similar to the nanoparticle synthesis described above. However, it differed with respect to the fact that 4 ml of *E. citriodora, E. camaldulensis* and *E. grandis* oils were added for the

preparation of the respective encapsulated systems after dissolving the polymer (PCL) in the solvent (acetone). Thus, a 50 ml l^{-1} concentration of essential oil in the colloidal suspension was obtained. The following systems were obtained: PCL nanoparticles (PCL), PCL nanoparticles containing encapsulated *E. citriodora* oil (NC), *E. camaldulensis* oil (NCM) and *E. grandis* oil (NG).

Infrared Analysis

Fourier transform infrared data were recorded on an IRAffinity-1 FTIR spectrophotometer (Shimadzu, Kyoto, Japan). The FTIR spectrophotometer was continuously purged with nitrogen. A total of 64 scans (400 - 4000 cm^{-1}) were collected with a resolution of 2 cm^{-1}. Infrared spectra were recorded in the transmission mode using 5 µl of nanoparticle colloids that were deposited on KBr pellets (Fraj *et al.* 2019).

Measurements of particle size, zeta potential and encapsulation efficiency

Zeta potential and mean particle diameter were determined by dynamic light scattering in a Zetasizer Nano ZS (Malvern Instruments Inc., Worcestershire, UK). Milli-Q water was used as a dispersant to avoid the effects of multiple dispersion, dispersion and interactions between nanoparticles. The zeta potential was measured by electrophoretic mobility considering the dielectric constant of water of 78.5. The parameters used in the analyses were the refractive index (173º), wavelength (633 nm), geometry (spherical), emulsion volume (1.5 ml), analysis temperature (25ºC), number of runs (11) and analysis time (2 min). The average cumulative diameter (z-average) and the polydispersity index (PdI) were used to describe the size and distribution of nanoparticles, respectively (Fraj *et al*. 2019).

Encapsulation Efficiency

The Encapsulation Efficiency (EE%) was pdetermined according to the method proposed by Ahsaei *et al*. (2020) with some modifications.

EE% = (Initial oil -Free oil)/ (Initial oil) x 100%

The initial oil corresponds to the amount of oil used in the production of nanoparticles, and the free oil refers to the amount of oil discharged into the lower chamber of the Chromafil® Xtra PTFE-20/25 membranes; pore size, 0.20 µm; Filter Ø, 25 mm. After filtration through the membrane, the filtrate was centrifuged for 20 min and then 3 ml of the supernatant was removed with a pipette. The amount of essential oil was determined by measuring the absorbance of the essential oils collected using a UV/VIS spectrophotometer (UV-1601PC, SHIMADZU) at 225 nm for the essential oils from *E. citriodora* ($y = 0.0002x + 0.3005$ $R^2 = 0.9658$) and *E. camaldulensis* ($y = 0.0003x + 0.7844$ $R^2 = 0.939$), and at 315 nm for the essential oil from *E. grandis* ($y = 0.0003x + 0.1916$ $R^2 = 0.9957$).

Determination of *in vitro* antifungal activity of essential oils and nanoparticles incorporated with essential oils against *H. vastatrix*.

The percentage of germination of *H. vastatrix* was determined in triplicate in the treatments with the active ingredients and compared with the treatment with the negative control using culture medium (2% aqueous agar) and PCL and the positive control (Opera®). The microdilution test was employed, in which inoculants were collected in the field, diluted in water to a concentration of 10^6 UFC ml^{-1}, and counted in a Neubauer camera. Volumes of 300 µl of the inoculum were transferred to 6-cm-diameter Petri dishes containing a mixture of 5 ml of 2% agar culture medium. Aliquots of the essential oils (0.65, 1.25, 2.5, 5.0, 7.5, 10.0, and 15.0 µl) and of the nanoparticles containing these essential oils (12.5, 25, 50, 100, 150, 200,

and 300 µl) were added to the culture medium contained in the Petri dishes to yield the final concentrations of 125, 250, 500, 1000, 1500, 2000 and 3000 µl l^{-1} for each treatment. The negative control was achieved using 5 ml of 2% aqueous agar culture medium and 300 µl of inoculum. A solution of PCL nanoparticles without the essential oils was also evaluated in the microorganism under study using the same method as that of the PCL nanoparticles containing the essential oils. The positive control was performed using 15.0 µl of the control fungicide (Opera®) in a recommended dose for 5 ml of medium, as described by the manufacturer. The plates were incubated in a BOD at 25ºC for a period of 24 h. The upper right part of the Petri dish was standardized for performing microscope readings to count the number of spores (Silva *et al.* 2014).

Obtaining the *H. vastatrix* inoculum and inoculation of the microorganisms in coffee seedlings.

The *H. vastatrix* spores used in the inoculation of this fungus in coffee seedlings were collected in the field from naturally infected coffee leaves of the cv. Mundo novo. The spores were collected by scraping the sporulated pustules and placed in test tubes. For inoculation on the coffee leaves, a solution at a concentration of 1.0 x 10^6 urediniospores per mL of aqueous agar (0.1 w/v) containing Tween 20 (0.05 v/v) was prepared. In the inoculation stage, the abaxial part of the coffee leaves was sprayed with the solution of urediniospores with the aid of a hand pump. The application was performed on the abaxial part of the coffee leaves because that is where the urediniospores penetrate the stomata to contaminate the leaf (Rayner 1961). Subsequently, the seedlings were stored in a chamber at a temperature of 23°C in the absence of light, with a relative humidity of 50%. After 72 hours of inoculation, the coffee seedlings were transferred to a greenhouse at a temperature of 30ºC and a relative humidity of 47%. The seedlings were kept at this location until the end of the experiments (Salustiano *et al.* 2008).

Determination of *in vivo* antifungal activity against *H. vastatrix* for essential oils and nanoparticles containing essential oils.

The determination of *in vivo* antifungal activity was accomplished by assessing the curative and preventive effects. The evaluations of the curative and preventive effects were performed using a diagrammatic scale developed by Cunha *et al.* (2001) to assess the severity of coffee rust. The scores on this scale range from 0 to 5. The score 0 corresponds to absence of disease; 1, < 3% severity; 2, from 3 to 6% severity; 3, from 6 to 12% severity; 4, from 12 to 25% severity; and 5, from 25 to 50% severity (Figure 4).

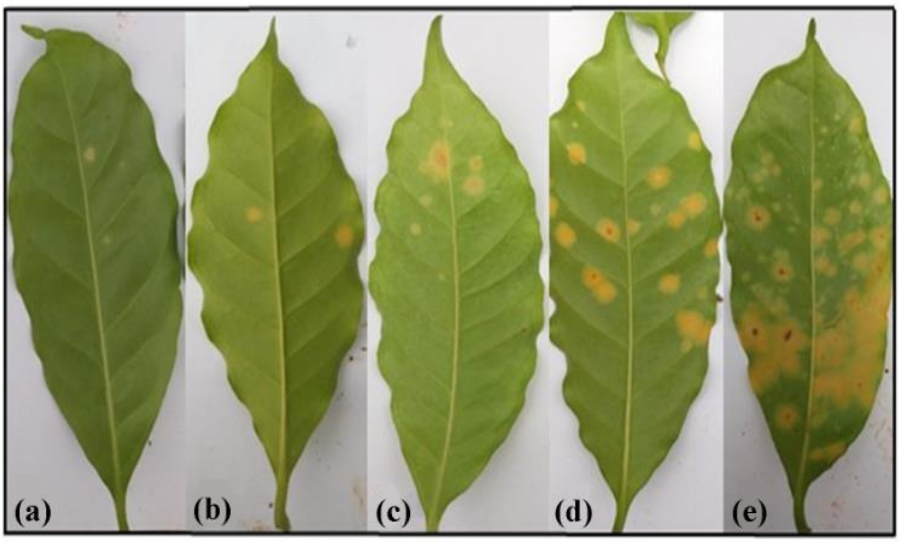

Fig.4

Figure 4. Diagrammatic scale for evaluating the severity of rust in coffee.

*The score 0 corresponds to absence of disease; (a) score 1: < 3% severity; (b) score 2: from 3 to 6% severity; (c) score 3: from 6 to 12% severity; (d) score 4: from 12 to 25% severity and (e) score 5: from 25 to 50% severity.

The treatments used in both experiments were water (negative control), poly(ε-caprolactone), Opera® (fungicide control), *E. camaldulensis* essential oil, *E. citriodora* essential oil, *E. grandis* essential oil, nanoparticle with the essential oil from *E. camaldulensis*,

nanoparticle with the essential oil from *E. citriodora* and nanoparticle with essential oil from *E. grandis*. The concentrations of essential oils used to evaluate the curative and preventive effects was 1500 μl l^{-1}, that is, a concentration higher than the MIC found in the *in vitro* tests.

Determination of the curative effect of essential oils and nanoparticles on *H. vastatrix*

The curative effect was determined by spraying the active ingredients, with the aid of a hand pump, on the abaxial part of the coffee leaves contaminated with *H. vastatrix* until the saturation of the leaves, that is, until the solutions began dripping from the leaves. The inoculation of the *H. vastatrix* fungus was performed according to the method described above. The time interval between the inoculation of *H. vastatrix* spores and the application of treatments was 30 days, during which time the severity of the disease could already be observed. After seven days of application of the treatments, four leaves from each coffee tree were chosen at random, marked and evaluated; one evaluation was performed per week for five weeks (Cunha *et al.* 2001; Pereira *et al.* 2012).

Determination of the preventive effect of essential oils and nanoparticles on *H. vastatrix.*

The preventive effect was determined by spraying the treatments with the aid of a hand pump, on the abaxial part of the healthy leaves of the coffee Seven days after the application of the treatments, the coffee seedlings were inoculated with the *H. vastatrix* fungus, according to the method mentioned in topic above. Twenty days after the inoculation with *H. vastatrix*, four leaves of each coffee seedling were chosen at random, marked and evaluated. The evaluations were performed weekly until the moment when spores of rust began to appear on the leaves of the seedlings submitted for treatment with the Opera® control fungicide (Cunha *et al.* 2001; Pereira *et al.* 2012).

Statistical analysis

The *in vitro* tests were conducted in a completely randomized design, and the analyses were performed in triplicate. The *in vivo* tests were performed using a randomized block design (RBD) consisting of nine treatments and twelve repetitions, totaling 108 experimental units for each experiment (curative and preventive). The results obtained for the different treatments were analyzed using analysis of variance and multiple comparisons of the means by the Scott-Knott test ($p < 0.05$) using the statistical software "Rstudio®", version 4.0.2.

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Conflicts of interest: The authors declare that no conflict of interests exists.

Availability of data and material: All data generated or analyzed during this study are included in this article.

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Author´s contributions

Alex Rodrigues Silva Caetano: Developed the study concept; designed research experiments; performed experiments, and wrote the manuscript, conceptualization, methodology and writing - (review and editing);

Maria das Graças Cardoso: Conceptualization, methodology, supervision, formal analysis, writing - (review and editing), project administration and funding acquisition.

Gabriela Aguiar Campolina: Conceptualization, methodology and visualization

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Graphical abstract:

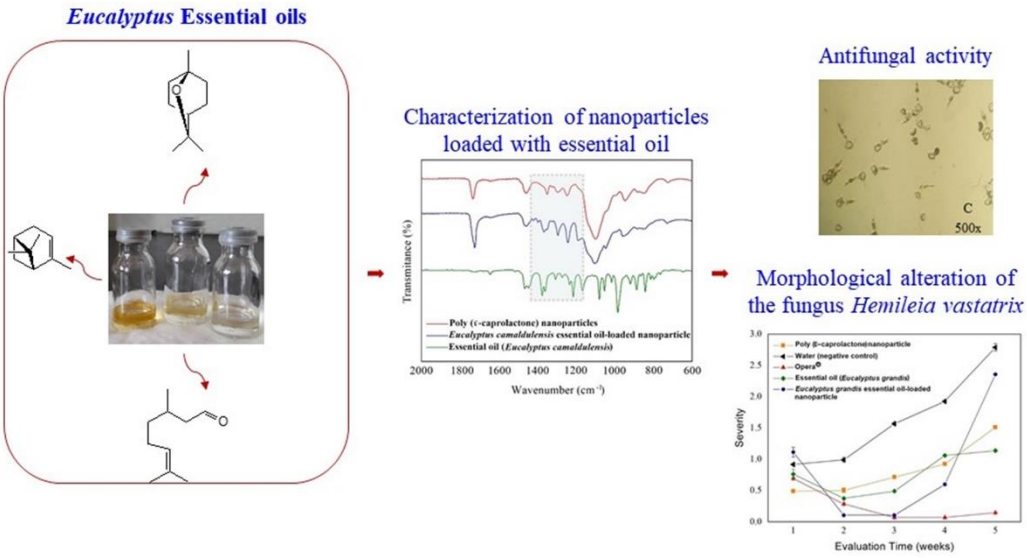

Table 1. Chemical composition of the essential oils extracted from the leaves of *Eucalyptus citriodora, Eucalyptus grandis* and *Eucalyptus camaldulensis*.

Peak	Compounds[a]	RI	RL[b]	Area (%)		
				E. grandis	*E. camaldulensis*	*E. citriodora*
1	α-pinene	933	932	55.21	5.59	-
2	camphene	950	946	0.78	-	-
3	*p*-cymene	1024	1020	0.67	0.39	-
4	limonene	1029	1024	4.49	27.87	-
5	1,8-cineole	1032	1026	16.52	58.71	-
6	terpinolene	1085	1086	-	3.59	-
7	α-fenchol	1120	1118	1.16	-	-
8	*trans*-pinocarveol	1142	1135	0.65	-	-
9	isopulegol	1149	1145	-	-	2.00
10	citronellal	1152	1148	-	-	93.97
11	isoisopulegol	1159	1155	-	-	1.45
12	isoborneol	1172	1155	3.83	-	-
13	α-terpineol	1195	1186	11.05	1.22	-
14	citronellol	1226	1223	-	-	2.58
15	α-salinene	1438	1498	-	0.48	-
16	viridiflorol	1586	1592	-	2.15	-
	Total identified			94.81%	100%	100%

RI: calculated retention index. RL: literature retention index.

[a] The components are listed in order of elution on the apolar capillary column of fused silica with a DBS bound stationary phase.

[b] Mass spectrum retention index library (Adams 2017).

Table 2. *In vitro* antifungal activity of essential oils and nanoparticles against *Hemileia vastatrix.*

	Percent inhibition								
Concentration (μl l^{-1})	W	PCL	O	OC	NC	OG	NG	OCM	NCM
125	0Ga	0Ga	100Aa	99.18Aa	17.16Fc	57.59Cb	23.04Ed	69.36Bc	36.19Dc
250	0Da	0Da	100A	99.33Aa	95.46Bb	96.03Ba	47.12Cc	94.69Bb	95.46Bb
500	0Da	0Da	100A	100Aa	100Aa	98.86Aa	91.65Cb	95.63Bb	100Aa
1000	0Ba	0Ba	100A	100Aa	100Aa	100Aa	100Aa	100Aa	100Aa

The means followed by the same uppercase letter in the rows and the same lowercase letter in the columns do not differ from one another by the Scott-Knott test at the 5% probability level. W: culture medium (2% agar/water). PCL: [poly(ε-caprolactone)]; O: Opera®; OC: *E. citriodora* essential oil; NC: nanoparticle with essential oil of *E. citriodora*; OG: *E. grandis* essential oil; NG nanoparticle with essential oil from *E. grandis*; OCM: *E. camaldulensis* essential oil; NCM: nanoparticle with essential oil from *E. camaldulensis*.

ARTIGO 3 - Antifungal activity of poly(lactic acid) nanofibers containing the essential oil from *Corymbia citriodora* or the monoterpenes *β*-citronellol and citronellal against mycotoxigenic fungi.

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Antifungal activity of poly(lactic acid) nanofibers containing the essential oil from *Corymbia citriodora* or the monoterpenes *β*-citronellol and citronellal against mycotoxigenic fungi

Running Head: Antifungal action of bioactive nanofibers

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Significance and impact of the study: Food contamination by mycotoxigenic fungi of the genus *Aspergillus* is one of the main factors that causes food loss worldwide. This work is highly relevant because it details the potential of the application of poly(lactic acid) nanofibers incorporated with essential oil from *Corymbia citriodora*, *β*-citronellol and citronellal in inhibiting the growth of these fungi and also in the production of mycotoxins. In addition to detailing the morphological changes caused in these microorganisms after treatments with the active ingredients. Promising results were found in this work, which indicate the feasibility of applying nanofibers incorporated into monoterpenes in the control of these fungi.

Abstract

Food contamination by mycotoxigenic fungi is one of the principal factors that cause food loss and economic losses in the food industry. The objective of this work was to incorporate the essential oil from *Corymbia citriodora* Hook and its constituents citronellal and *β*-citronellol into poly(lactic acid) nanofibers; to characterize the nanofibers by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC); to evaluate the antifungal activity by the fumigation method; to evaluate the antimycotoxigenic activity in *Aspergillus carbonarius, Aspergillus ochraceus, Aspergillus westerdijkiae, Aspergillus flavus* and *Aspergillus parasiticus*; and to evaluate the morphology of these microorganisms. All the nanofibers had a regular, smooth and continuous morphology, with an average diameter ranging from 114.15 to 264.47 nm. FTIR analyses confirmed that the active ingredients were incorporated into the polymer matrix. The incorporation of active ingredients into poly(lactic acid) changed its glass transition temperature (Tg) and crystallization temperature (Tc), but it did not change the melting temperature (Tm). All the samples exhibited antifungal and ochratoxigenic inhibitory activities up to 100 and 99%, respectively. However, 100% inhibition of the production of aflatoxin B1 and B2 was not

observed. The images obtained by SEM indicated that the nanofibers caused damage to the hyphae, caused a decrease in the production of spores, and caused deformation, rupture and non-formation of the conid head. The incorporation of the essential oil from *Corymbia citriodora*, *β*-citornellol and citronellal into poly(lactic acid) nanofibers increased their efficiencies and might be an alternative for the control of mycotoxigenic fungi.

Keywords: Antifungal activity, *Corymbia citriodora*, *Aspergillus* sp., mycotoxins; nanofibers.

Introduction

Food contamination by mycotoxigenic fungi such as *Aspergillus carbonarius, Aspergillus ochraceus, Aspergillus westerdijkiae, Aspergillus flavus* and *Aspergillus parasiticus* is one of the main causes of food loss. When these microorganisms contaminate foods such as cereals, coffee, cocoa, spices, grapes, dried fruits and animal feed, they cause undesirable changes, altering the flavor, aroma and nutritional content. In addition, these microorganisms are producers of mycotoxins, which are undesirable in food because they are dangerous (Boonmee *et al*. 2020; Brandão *et al*. 2020; Silva *et al*. 2020).

Among the mycotoxins, ochratoxin A (OTA) is produced especially by *A. carbonarius*, *A. ochraceus* and *A. westerdijkiae.* It stands out in terms of collateral damage to human health because of its high carcinogenic potential and agroeconomic consequences. Other mycotoxins, such as aflatoxins B1 and B2, are produced by *A. flavus* and B1, B2, G1 and G2 by *A. parasiticus*. Alfotoxin B1 is considered to be the most toxic among the aflatoxins because it possesses hepatotoxic, teratogenic and mutagenic properties (IARC 1993; IARC 2002; Boonmee *et al*. 2020).

Mycotoxins, once present in food, are difficult to eliminate. These substances are relatively thermally stable because they have a complex chemical structure. Some contain aromatic rings, hydroxyl and carbonyl groups, hydrogen bonds and intermolecular dipole-

dipole bonds. Therefore, currently available industrial processes are insufficient to eliminate them. The safest way to prevent food contamination by these substances is to prevent the development of fungi during the cultivation and storage processes. Currently, the most effective way to combat these microorganisms is to use synthetic fungicides. However, these compounds are limited in terms of the period of application, and, when applied incorrectly, they can be harmful to human health and the environment, in addition to selecting resistant species (Abd-Elsalam *et al*. 2017; Boonmee *et al*. 2020).

Fungicides with the GRAS designation of the American Food and Drug Administration (FDA) are safe products that can be added to foods and that do not result in collateral damage to humans and the environment. Essential oils have been the subject of research that seeks to produce alternative fungicides. Essential oils are secondary metabolites produced by plants. They have a complex chemical composition and consist of terpenes and phenylpropanoids. Among the various biological properties conferred by their chemical constituents, the antifungal activity is very important (Abd-Elsalam *et al*. 2017; Caetano *et al*. 2020).

The essential oil obtained from the leaves of *Corymbia citriodora* Hook is one of the most widely used in the pharmaceutical, cosmetic, and the food industries. Its chemical composition consists mainly of citronellal, among other minor constituents such as β-citronellol and 1,8-cineole. The antifungal activity of this essential oil has already been reported in the literature regarding *P. oxalicum*, *A. fumigatus*, *A. nidulans*, *H. vastatrix*, *T. rubrum*, and *M. canis*, among other microorganisms (Javed *et al*. 2012; Tolba *et al*. 2015; Caetano *et al*. 2020; Javaid *et al*. 2020).

To enhance the biological activity of essential oils, mitigate their high volatility and make their industrial application possible, these substances are being incorporated into nanostructured polymeric matrices. Poly(lactic acid) (PLA) is a biodegradable, thermoplastic, semi-crystalline aliphatic polyester. PLA is synthesized from lactic acid or obtained from natural resources such

as starch. This polymer is biodegradable and biocompatible, making it a candidate for the production of films, fibers and smart packaging (Jonoobi *et al*. 2010; Brito *et al*. 2011).

Among the most widely used nanomaterials in the development of fungicidal products, polymeric nanofibers can be underscored. These are nanostructures that are used as vehicles for the controlled release of active principles. They also have a high porosity and a high surface area, and their morphological structures can be modified. Polymeric nanofibers can be produced by the "Solution Blow Spinning" (SBS) technique. This technique consists of using a pump and a syringe to feed concentric nozzles into which the polymeric solution containing the active ingredient and a pressurized gas are simultaneously injected. The pressure difference and the shear at the gas/solution interface produce filaments of polymer solution directed towards the collector. During the flight to the collector, the solvents evaporate, and the nano- and microfibers are formed (Nepomuceno *et al*. 2017; Brandão *et al*. 2022).

The objective of this work was to evaluate the antifungal activity, inhibition of mycotoxin production and morphological changes in *A. carbonarius, A. ochraceus, A. westerdijkiae, A. flavus* and *A. parasiticus* resulting from the treatment with PLA nanofibers incorporated with *C. citriodora* essential oil and with the monoterpenes *β*-citronellol and citronellal.

Results and Discussion

Chemical characterization of the essential oil from *Eucalyptus citriodora*

The essential oil from *C. citriodora* was characterized by Caetano *et al*. (2020) and contained citronellal (88.83%) as the principal chemical constituent, whereas isoisopulegol (4.73%), *β*-citronellol (3.39%) and isopulegol (2.12%) were minor constituents. The chemical structures of the constituents present in this essential oil are presented in Figure 1.

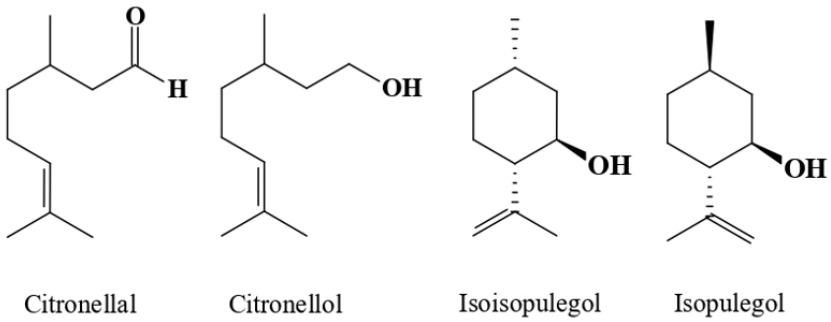

Figure 1. The chemical constituents of the essential oil from *Corymbia citriodora*.

Characterization of the Nanofibers

Fourier transform infrared analysis of nanofibers embedded with active ingredients

The spectra obtained in the Fourier transform infrared (FTIR) analysis for the nanofibers incorporated with the active ingredients are shown in Figure 2.

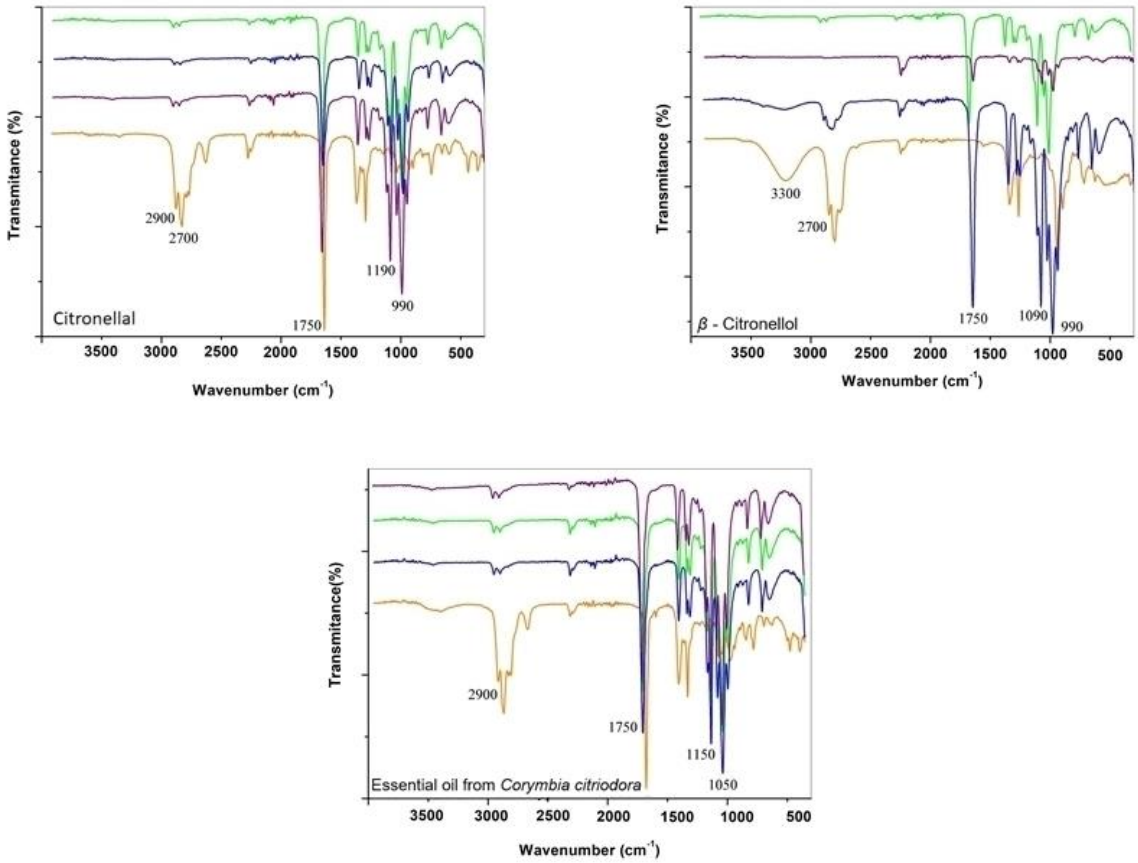

Figure 2. Fourier transform infrared analysis of essential oil and monoterpenes free and incorporated into nanofibers. Citronellal: (—) poly(lactic acid) nanofibers, (—) citronellal, (—) nanofiber with 20% of citronellal, (—) nanofiber with 30% of citronellal. *β*-citronellol: (—) poly(lactic acid) nanofibers, (—) *β*-citronellol, (—) nanofiber with 20% of *β*-citronellol, (—) nanofiber with 30% of *β*-citronellol. Essential oil from *Corymbia citriodora*: (—) poly(lactic acid) nanofibers, (—) essential oil from *Corymbia citriodora*, (—) nanofiber with 20% of essential oil from *Corymbia citriodora*, (—) nanofiber with 30% of essential oil from *Corymbia citriodora*.

According to the spectra, all the active principles were incorporated into the poly(lactic acid) polymer matrix. Bands characteristic of the functional groups of the chemical constituents present in the active principles appear in the spectra obtained for the nanofibers.

The FTIR transmittance spectra recorded for the pure PLA nanofibers are in agreement with the data found in the literature (Oliveira *et al*. 2013). The three peaks characteristic of pure PLA were observed at approximately 1050, 1090 and 1150 cm^{-1}. These peaks refer to the symmetrical elongation of C-CH_3, the symmetrical elongation of COC and the asymmetrical rocking of CH_3, respectively. At 1750 cm^{-1}, an intense absorption can be observed, which corresponds to the aldehyde CHO functional groups present in abundance in this polymer.

For the essential oil from *C. citriodora* and for citronellal, bands related to the aldehyde functional group can be observed at approximately 1750 cm^{-1}. This functional group is present in citronellal, a major constituent of the essential oil from *C. citriodora*, and in the analytical standard for citronellal. Similar data for citronellal were observed by Adilina *et al*. (2007), where the band referring to the organic aldehyde function was observed at 1726 cm^{-1}.

Regarding the analytical standard for *β*-citronellol, it bears a hydroxyl group, which can be observed in the spectrum at 3300 cm^{-1}. This band can also be seen in the spectrum obtained for the essential oil. The alcohol band present in the essential oil refers to the alcoholic hydroxyls present in *β*-citronellol, isopulegol and isoisopulegol, chemical constituents of this oil. Adilina *et al*., (2007), studying alcoholic monoterpenes, also found a characteristic band at 3346 cm^{-1} for this functional group.

FTIR analyses of nanofibers incorporated with active principles are of paramount importance for the characterization of nanomaterials. In addition to confirmation of the incorporation of these substances into the polymer matrix, any chemical reaction between the polymer and the active principle can be identified from the spectra (Bagheri *et al*. 2021).

Scanning Electron Microscopy of Nanofibers

The results obtained for the morphology and for the average diameter of the nanofibers are shown in Figure 3. All the nanofibers possessed a regular, smooth and continuous morphology.

The average diameter of the nanofibrous samples ranged from 114.15 to 264.47 nm. The incorporation of essential oil and monoterpenes resulted in an increase in the average diameter of nanofibers for all the samples, except for that containing 20% of the essential oil, in which the average diameter (115.37 nm) was statistically equal ($p > 0.05$) to that of the nanofibers prepared with only PLA (114.15 nm).

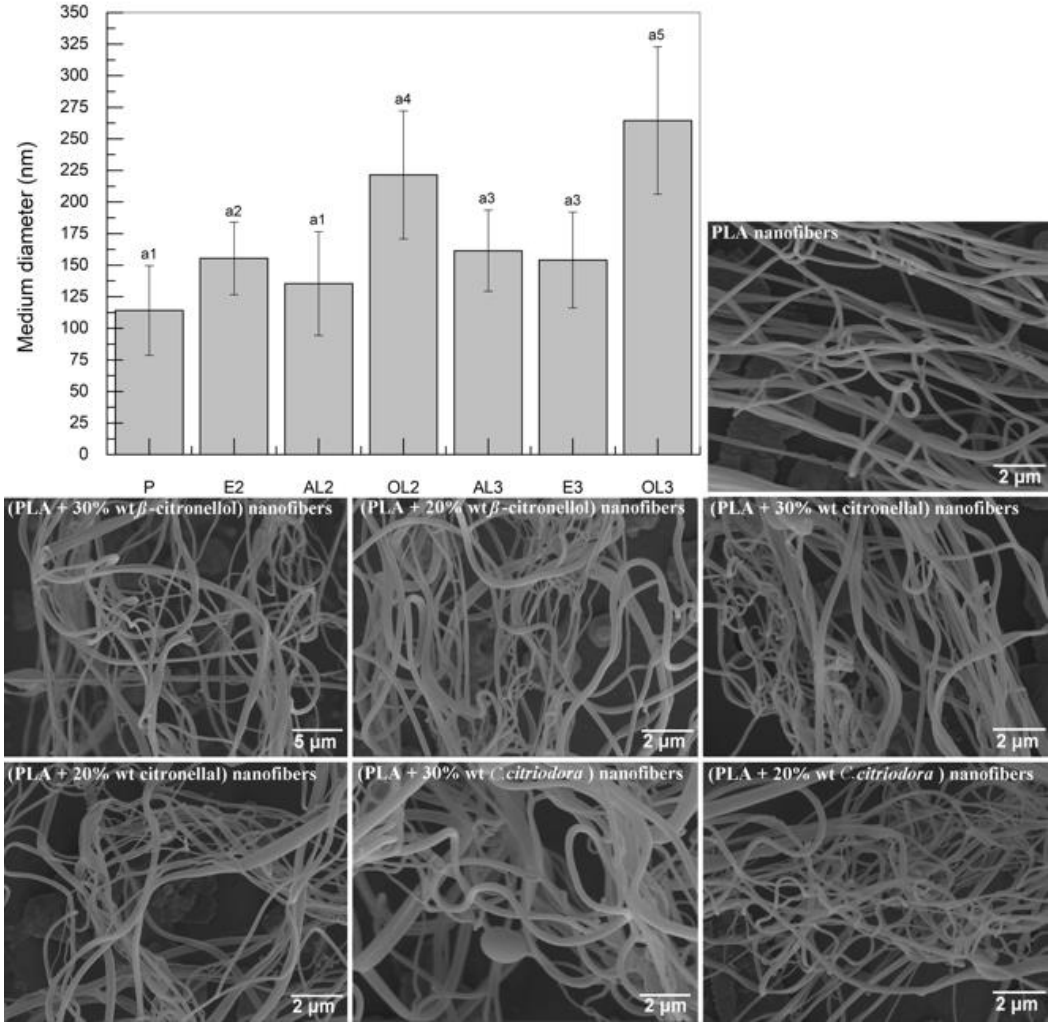

Figure 3. Morphology and measurement of the average diameter of nanofibrous mats.

P: Poly(lactic acid) nanofiber; OL3: Nanofiber loaded with *β*-citronellol (30%); OL2: Nanofiber loaded with *β*-citronellol (20%) AL3: Nanofiber loaded with citronellal (30%); AL2: Nanofiber loaded with citronellal (20%); E3: Nanofiber loaded with essential oil from *Corymbia citriodora dora* (30%); E2: Nanofiber loaded with essential oil from *Corymbia citriodora* (20%).

Scaffaro and Lopresti (2018) studied the incorporation of the monoterpene carvacrol into PLA polymeric solution and observed a slight increase in the average diameter of the nanofibers after the incorporation of this essential oil constituent into the polymer matrix. Their results corroborates that of this work.

The increase in the diameter of the nanofibers after the incorporation of the active principles in the polymeric solution can be related to the increase in the viscosity of the solution. This parameter is fundamental during the synthesis of nanofibrous mats, where more viscous solutions normally tend to form nanofibers with greater diameters than less viscous solutions (Mori *et al*. 2015; Casasola *et al*. 2016).

Differential Scanning Calorimetry

The results obtained for the thermal properties of the nanofibers containing the active principles are presented in Table 1.

Table 1.

There was a decrease in the glass transition temperature (Tg) for all the nanofibers containing the active principles in relation to the nanofiber synthesized with only PLA (Table 1). The greatest decrease was observed for the nanofibers produced with 20% *β*-citronellol, for which a Tg of 42ºC was observed. A decrease in the crystallization temperature (Tc) was also observed for all the samples. The most relevant being a decrease of 24ºC for the nanofibers incorporated with 30% of citronellal. There was no significant change in the melting temperature (Tm) in the presence of the active ingredients.

The decrease in Tg and Tc values for PLA nanofibers containing the active ingredients can be related to the intermolecular hydrogen bonds and dipole-dipole bonds of the essential

oil constituents such as citronellal and *β*-citronellol with the ester functional groups of the polymer, as well as to the low molecular weight of these compounds. These factors allow these substances to act as a chain spacer to make the polymer chain more flexible and lower the Tg and Tc. Compounds that have a low molecular weight, when incorporated into a polymeric matrix, can act as a plasticizer because they provide greater mobility to the functional groups at the end of the chain that are in free rotation (Moradkhannejhad *et al*. 2020; Brandão *et al*. 2022).

Antifungal activity

The results for the *in vitro* antifungal activity of the essential oil from *C. citriodor*a and the citronellal and *β*-citronellol monoterpenes, free and incorporated into poly(lactic acid) nanofibers, are presented in Table 2.

Table 2.

According to the results presented in the table above, *in vitro* antifungal activity against all the tested microorganisms was observed for all the active ingredients, except that no antifungal activity against *A. westerdijkae* was observed for the essential oil from *C. citriodora in its* free form. A correlation can also be observed between the increase in antifungal activity and the increase in the concentration of active principles incorporated into the PLA nanofibers. The antifungal activity for the same active ingredient varied among the microorganisms tested. No antifungal activity was observed for the poly(lactic acid) polymer matrix in which the active principles were incorporated and for the dimethyl sulfoxide (DMSO) solvent.

A 50% inhibition of mycelial growth of the microorganisms *A. ochraceus, A. flavus, A. parasiticus* and *A. carbonarius* was found for the essential oil from *C. citriodora* and for the

oxygenated monoterpenes citronellal and *β*-citronellol at a concentration of 3000 μl l^{-1}. The inhibitions of the mycelial growth of *A. westerdijkae* at the concentration of 3000 μl l^{-1} were 10.16% and 20.01% for the monoterpenes citronellal and citronellol, respectively. No antifungal activity against the fungus *A. westerdijkae* was observed for the essential oil from *C. citriodora.* This microorganism exhibited greater resistance against the active principles tested.

The incorporation of the active ingredients into the nanofibrous PLA matrix did not follow a linear pattern with the expected results for the inhibition of mycelial growth. The antifungal potential against a microorganism was lost after incorporation of some active ingredients into the polymeric matrix. However, the antifungal potential against other microorganisms increased.

A 100% inhibition of the mycelial growth of the microorganisms *A. ochraceus* and *A. carbonarius* and 75.93% inhibition of the fungus *A. westerdijkae* were observed for the monoterpene *β*-citronellol after its incorporation into the nonofibrous matrix of PLA (30%). The inhibitions of the mycelial growth of the microorganisms *A. flavus* and *A. parasiticus* were 37.23 and 20.94%, respectively. The antifungal activity of the citronellal monoterpene decreased after its incorporation into PLA nanofibers at both concentrations. Mycelial growth was inhibited by less than 20% in relation to all the microorganisms tested. For the fungus *A. ochraceus,* no antifungal activity was observed for the nanofibers containing citronellal at both concentrations.

The results found for the essential oil from *C. citriodora* incorporated into the nanofibers of PLA (30%) were encouraging in relation to the microorganisms *A. parasiticus* and *A. westerdijkae*. The inhibition of mycelial growth of *A. parasiticus* increased from 52.17 to 78.62%. No inhibition of the mycelial growth of *A. westerdijkae* was observed with the free form of the oil, but an inhibitory activity of 20.83% was observed after its incorporation into PLA nanofibers (30%). There was a decrease in antifungal activity from 55.20 to 41.44%

against *A. flavus*, from 63.14 to 21.35% against *A. ochraceus* and from 61.53 to 26.93% with *A. carbonarius*.

An inhibition of less than 20% in the mycelial growth of *A. westerdijkae* was observed for all the active principles in the free form. However, the antifungal potential against this microorganism increased after incorporation of these active principles into the nanofibrous mats, especially for citronellol, for which the increase was 55.92%.

The antifungal activities of essential oils and oxygenated monoterpenes are related to the ability of these substances to interact with the fungal cell membrane and cause damage to the cell wall, consequently causing the leakage of cellular materials and ions. These substances, when crossing the fungal cell matrix, are also responsible for inhibiting the synthesis and biological activity of ergosterol, which is a fundamental hormone necessary to maintain the vitality of fungal cells (Kedia *et al*. 2015; Caetano *et al*. 2020).

According to Kalemba and Kunicka (2003), the antimicrobial action of essential oils is related to the lipophilic character of their hydrocarbon skeletons and the hydrophilic character of their functional groups, following a suggested order of activity: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons. Based on the results observed in this work, it can be inferred that citronellol possessed a greater antifungal potential than citronellal against all the microorganisms tested by the fumigation method. Therefore, the suggested order of antifungal activity in relation to citronellol and citronellal should be restructured because greater fungicidal activity was observed for the alcohol functional group than the aldehyde functional group.

Inhibition of ochratoxin A production

The results for the inhibition of the production of OTA by the fungi *A. ochraceus*. *A. carbonarius* and *A. westerdijkae* are shown in Table 3. A significant percentage of inhibition

of OTA production ($p < 0.05$) was observed for the different concentrations of active ingredients.

Table 3.

The concentration of OTA produced by the fungal controls of *A. carbonarius, A. westerdijkae* and *A. ochraceus* was 0.45; 0.63 and 0.0108 μg g^{-1}, respectively. The productions of OTA by *A. carbonarius* and *A. westerdijkae* were generally higher than the production of OTA by *A. ochraceus*, which corroborates the data found in this work (Brandão *et al*. 2020). Promising results were found in relation to the inhibition of OTA production. This substance is not desirable in foods because it has carcinogenic properties.

Little inhibition of mycelial growth of the fungus *A. westerdijkae* was observed. However, 99% inhibition of the production of OTA was observed in the presence of nanofibers containing the essential oil from *C. citriodora* (20 and 30%) and with nanofibers containing citronellal (30%). The concentration of ochratoxin decreased from 0.63 to 0.0015 μg g^{-1}. The inhibition of the production of OTA by *A. carbonarius* by a 3000 μl l^{-1} concentration of the essential oil from *C. citriodora* and citronellal in their free forms was significant. The production of this mycotoxin was inhibited by 99.70%, and its concentration decreased from 0.45 to 0.00135 μg g^{-1}.

The greatest inhibition of the production of OTA by *A. ochraceus* occurred with the essential oil from *C. citriodora* in its free form at the concentration of 3000 μl l^{-1}. The production of OTA decreased by 86.53% from 0.00108 to 0.00015 μg g^{-1}. No inhibition of OTA production by these microorganisms was observed in the treatments performed with DMSO and with PLA nanofibers.

Rezende et al. (2021) studying the essential oil of *Myristica fragrans* H., the authors found an inhibition of OTA production by *Aspergillus ochraceus* up to 93.72% at a concentration of 3.91 μl l^{-1}. These results corroborate those of this work demonstrating the potential for inhibiting the production of OTA by essential oils in fungi of the genus *Aspergillus*.

The inhibition of mycotoxin production by essential oils is related to the effects caused by their chemical constituents on the expression levels of genes responsible for OTA biosynthesis in *Aspergillus* species. Some essential oils, such as those from fennel, cardamom, anise, chamomile, celery, cinnamon, thyme, taramira, oregano and rosemary, inhibited the production of OTA by *A. carbonarius* and caused damage to the genes acOTApks and acOTAnrps, together with the acpks gene and the two regulatory genes laeA and veA. This same mechanism of action might have occurred with the active principles used in this work (El Khoury *et al*. 2016; Chelaghema *et al*. 2021).

Inhibition of the production of Aflatoxin B1 and B2

The complete inhibition of the production of Aflatoxin B1 and B2 by *A. flavus* and *A. parasiticus* did not occur with the active ingredients because two characteristic points with retention factors (RF) and fluorescence similar to the commercial standards were observed in all the samples analyzed. The inhibition of the production of mycotoxins might be correlated with the inhibition of the number and growth of mycelia because these structures are responsible for the production of these carcinogenic substances (Hua *et al*. 2014).

In a study performed by Atanda *et al*. (2007) on the use of essential oils from *O. basilicum* and *C. sativum* for the inhibition of mycelial growth by *A. parasiticus,* the authors observed a 100% inhibition of mycelial growth and aflatoxin B1 production fby *O. bacilicum* oil and observed no results for *C. sativum* oil. Several factors can influence the variation in the results of mycotoxin inhibition by essential oils, including the chemical composition of the oil,

the species of fungi tested, the concentration applied and the duration of effect of the oil on the spores of the fungi. (Kocić-Tanackov *et al*. 2019).

Scanning Electron Microscopy of Microorganisms

The morphology of the *Aspergillus* species treated and untreated with the essential oil from *C. citriodora* and the monoterpenes *β*-citronellol and citronellal, free and incorporated into poly(lactic acid) nanofibers, are shown in Figure 4.

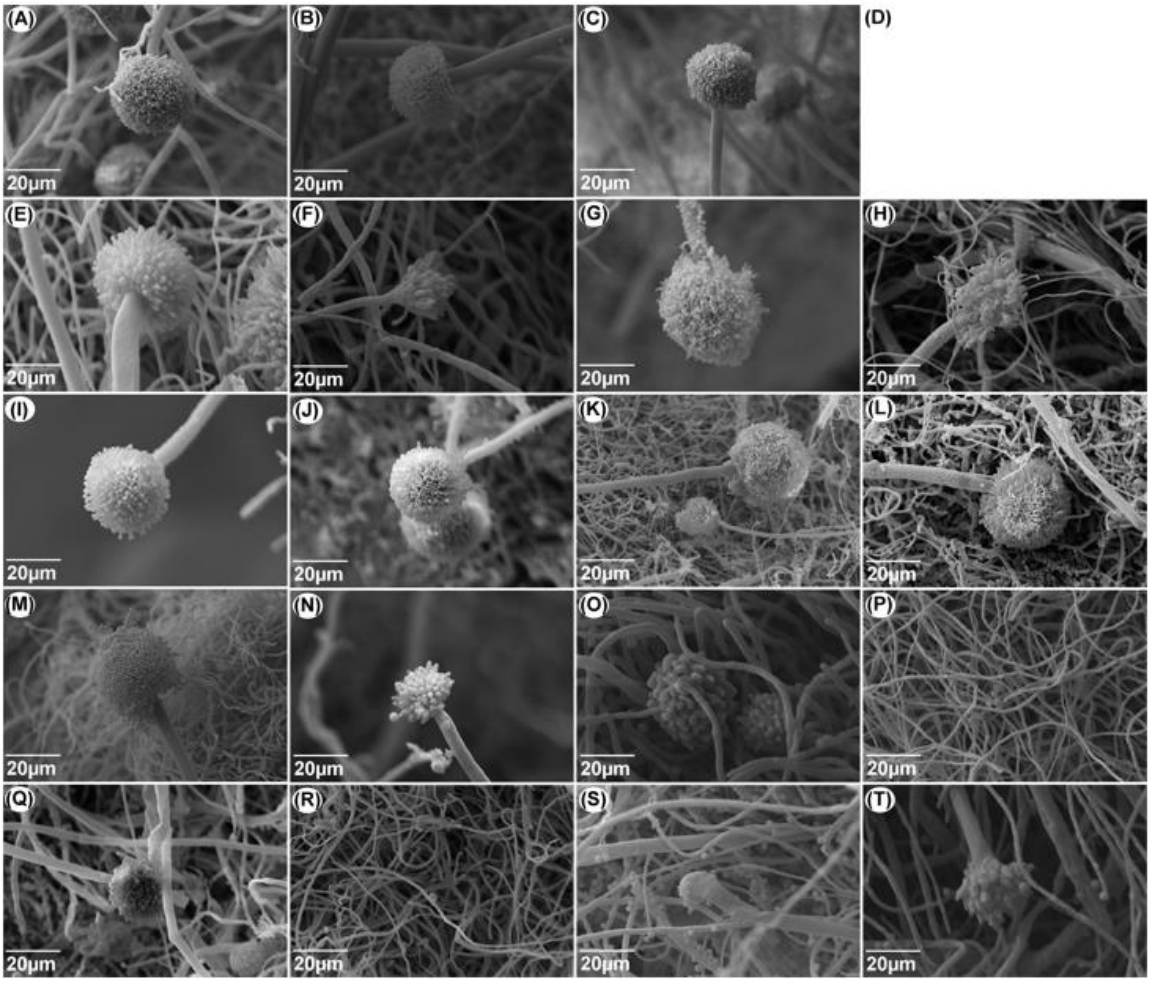

Figure 4. Scanning electron micrographs of *A. carbonarius, A. ochraceus, A. westerdijkiae, A. parasiticus* and *A. flavus* treated with nanofibers containing the essential oil from *C. citriodora*, *β*-citronellol and citronellal.

*Scanning electron micrographs of the fungal controls *A. carbonarius* (A), *A. ochraceus* (E), *A. westerdijkiae* (I), *A. parasiticus* (M) and *A. flavus* (Q). (B) and (C) *A. carbonarius* treated with nanofibers incorporated with the essential oil from *C. citriodora* and citronellal, respectively. (F), (G) and (H), *A. ochraceus* treated with nanofibers incorporated with the essential oil from *C. citriodora*, *β*-citronellol and citronellal, respectively. (J), (K) and (L), *A. westerdijkiae* treated with nanofibers containing the essential oil from *C. citriodora*, *β*-citronellol and citronellal, respectively. (N), (O) and (P) *A. flavus* treated with nanofibers containing the essential oil from *C. citriodora* and citronellal, respectively. (R), (S) and (T) *A.*

parasiticus treated with nanofibers containing the essential oil from *C. citriodora* and citronellal, respectively.

The morphologies of the fungal controls *A. carbonarius (A), A. ochraceus (E), A. westerdijkiae (I), A. parasiticus (M)* and *A. flavus* (Q), were normal, with healthy development of conidia and conidiophores (Figure 4). The hyphae were uniform with robust, smooth surfaces and constant, linear and regular diameters.

After the treatments of the microorganisms with the nanofibers containing the active principles, alterations of the hyphae were observed: a smaller diameter, wrinkled, collapsed and flattened (F), (H), (P), (R), (S) and (T). Deformation of the conid's head could be observed with ruptures and non-formation in (F), (H), (N), (S) and (T). Growth of mycelia occurred, but there was no normal production of spores in the treatments (B), (F), (H), (N), (O), (P), (R), (S) and (T).

In a study performed by Brandão *et al*. (2020) using the essential oil from *E. erythropappus* on *A. carbonarius*, *A. flavus*, and *A.ochraceus*, the authors observed morphological changes in hyphae and conidia and a decrease in production of the spores. These results corroborate those found in this work, confirming that the constituents of essential oils can cause morphological damage to microorganisms.

The application of poly(lactic acid) polymeric nanofibers incorporated with the essential oil of *Corymbia citriodora* and with the isomeric monoterpenes citronellal and *β*-citronellol caused inhibition of mycelial growth, inhibition of mycotoxin production and morphological damage in the fungi *A. carbonarius*, *A. ochraceus* , *A. westerdijkiae*, *A. flavus* and *A. parasiticus*. The innovative results indicate the feasibility of applying nanofibers mats for controlled release of essential oils and monoterpenes in the fight against mycotoxigenic fungi.

Material and Methods

Extraction of essential oil from *Corymbia citriodora* and acquisition of monoterpenes

The essential oil from *C. citriodora* was the same as that used by Caetano *et al*. (2020). The essential oil was extracted from fresh leaves of *C. citriodora* at the Laboratory of Organic Chemistry - Essential Oils of the Federal University of Lavras (UFLA). The essential oil was extracted by hydrodistillation using a modified Clevenger apparatus (Brasil 2010). The extractions were performed in triplicate, distilling each sample of plant material for two hours. After extraction, the essential oil was separated from the hydrolate by centrifugation and stored in an amber glass bottle at low temperature. Analytical standards for *β*-cironellol and citronellal were purchased from Sigma-Aldrich® with a purity greater than 95%.

Solution blow spinning (SBS)

PLA nanofibrous mats were obtained by solution blow spinning (SBS) using a modification of the method described by Miranda *et al*. (2021). This technique consists of a source of compressed air (Schulz, model 10VL/200-2HP, Santa Catarina, Brazil) at 100 kPa, an injection pump (NE-300, New Era Pump Systems, NY) equipped with a glass syringe (FLURAN F-5500-A, Ismatec, Wertheim, Germany) and operating at a feed rate of 4.0 ml h^{-1}, and a rotating nozzle with a protrusion length of 0.2 mm. The fibers were collected at 300 rpm and 12 to 20 cm away from the nozzle. The PLA solution (10% by weight) was prepared in chloroform.

The nanofiber mats incorporated with the active principles were prepared according to the method described above with some modifications. After dissolving the polymer in chloroform, the active principles (*C. citriodora* essential oil, *β*-citronellol and citronellal) were added to the solution in the proportion of 20 and 30% relative to the mass of the polymer. The solution was homogenized for 10 min, and the spinning process was performed.

Characterization of Nanofibers by Scanning Electron Microscopy

All the samples were observed by scanning electron microscopy (SEM, model Zeiss DSM960, USA) according to the method proposed by Scaffaro and Lopresti (2018). Initially, the samples were coated with gold using a SCD 050 Bal-Tec sputter applicator (BalzersAG, Liechtenstein). Mean fiber diameters were measured using Image J software (National Institutes of Health, Bethesda, MD). One hundred random measurements were made for each sample.

Fourier transform infrared spectrophotometry (FTIR)

The nanofibrous mats were characterized by Fourier transform infrared spectrophotometry (FTIR) using a Vertex 70 model Bruker spectrophotometer (Ettlingen, Germany). The spectra were recorded in attenuated total reflectance (ATR) mode, accumulating 32 scans in a spectral range from 4000 to 500 cm^{-1} at a resolution of 4 cm^{-1} (Miranda *et al*. 2020).

Differential Scanning Calorimetry (DSC)

DSC measurements were performed on a Q100 (TA Instruments) calorimeter according to the method proposed by Miranda *et al*. (2019). The 10 mg samples were placed in sealed pans and heated from 25 to 180°C at 10°C min^{-1} under a nitrogen atmosphere with a flow rate of 50 ml min^{-1}. All the samples were subjected to two temperature cycles: the first cycle was used to erase the thermal history, and the second to determine the glass transition temperatures (Tg).

Evaluation of antifungal activity by the fumigation method

The fungitoxic activity was evaluated by applying the *in vitro* fumigation test according to a modification of the method proposed by Guimarães *et al*. (2011) and Oliveira *et al*. (2019). Initially, the essential oil from *C. citriodora* and the monoterpenes *β*-citronellol and citronellal were diluted in dimethyl sulfoxide (DMSO) at concentrations that varied from 50 to 3000 µl l^{-}

[1]. Subsequently, 15 ml of culture medium was added to each Petri dish. The Czapek Yeast Extract Agar (CYA) culture medium was used to inoculate the *A. carbonarius* and *A. westerdijkiae* fungi, whereas the Yeast Extract Sucrose (YES) culture medium was used to inoculate the *A. ocrhaceus, A. parasiticus* and *A. flavus* fungi. After the culture medium solidified, 250 μl of the different concentrations of the active ingredients were added to 4-cm-diameter filter paper disks adhered to the top of the Petri dish. Then, 10 μl of a spore suspension of each fungus at a concentration of 10^6 spore ml^{-1} colony forming units (CFU) were added to a 4-mm-diameter filter paper adhered to the center of the culture medium. The plates were sealed with plastic film and placed in the growth chamber at a temperature of approximately 25°C for a period of seven days. The plates were removed from the growth chamber, and the diameter of the mycelial growth was measured by drawing two perpendicular lines in the center of the plate. The *in vitro* test to evaluate the antifungal activity of the nanofibers was performed in the same manner as that used for the free active principles; however, the nanofibers with a diameter of 4 cm were attached to the lid of the Petri dish.

Morphological analysis of microorganisms

The samples used for scanning electron microscopy (SEM) analysis were prepared from Petri dishes employed in the analysis to evaluate mycelial growth. The samples analyzed were those treated with the nanofibers incorporated with the active principles at a concentration of 30%. Three colony plugs (5 mm in diameter) were removed from the medium of each colony after 10 days of incubation and fixed in a modified Karnovsky solution (2.5% glutaraldehyde, 2% formaldehyde, 0.05 M cacodylate buffer, pH 7.2, and 0.001 M $CaCl_2$ buffer; Sigma-Aldrich®, São Paulo, Brazil). Samples were washed three times for 10 min with cacodylate buffer. The samples were dehydrated using a series of increasing concentrations of acetone (25%, 50%, 75% and 100%); they were dried in a critical point apparatus (Bal-tec CPD 030); and the

samples were coated with gold (Bal-tec CPD 050) according to the method described by Oliveira *et al*. (2017). The samples were examined under a scanning electron microscope (Leo Evo 040) to obtain the electron micrographs.

Inhibition of the production of ochratoxin A

Samples for determining the inhibition of the production of ochratoxin A (OTA) were prepared from Petri dishes used in the analysis of mycelial growth. The inhibition of OTA production by the active ingredients and by the nanofibers was tested at concentrations of 2000 and 3000 μl l^{-1} and 20 and 30%, respectively, in *A. carbonarius*, *A. ochraceus* and *A. westerdijkiae*. All the plates were incubated in biological oxygen demand (BOD) at 25°C for 10 days. OTA extraction was performed on the tenth day of the incubation period according to the method proposed by Passamani *et al*. (2014). Three culture plugs were removed from the center, middle and edge of each colony. Each plug was weighed and transferred to test tubes. One milliliter of methanol (Merck; HPLC grade) was added to each tube, which was shaken vigorously for five seconds and kept at room temperature for 60 minutes.

The quantification of OTA was performed according to the method proposed by Passamani *et al*. (2014). Extracts of *A. carbonariu*s, *A. ochraceus* and *A. westerdijkiae* were filtered through PTFE (polytetrafluoroethylene) filter units (0.22 μm, Millipore), and 20 μl of each filtrate was injected directly into the liquid chromatography system: an Efficiency HPLC (Shimadzu), equipped with two model LC-6AD high pressure pumps, and an AXL RF-10 fluorescence detector, a DGU-20A3 degasser and an auto-injector with a SIL-10AF autosampler. Separations were performed using an Agilent-Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm) equipped with an Agilent-Zorbax Eclipse XDB-C18 4-Pack (4.6 × 12.5 mm, 5 μm). Elution was achieved with an isocratic system of methanol:acetonitrile:water:acetic acid (35:35:29:1). OTA was monitored by excitation at 332 nm and emission at 476 nm, using

a flow rate of 0.8 ml min^{-1}. The retention time (TR) of OTA was 10±0.5 minutes. The analytical curve used to determine the OTA concentration was the same as that used by Brandão *et al*. (2020). Subsequently, the band area was correlated with the concentration of the respective standard solution. The coefficient of determination (R^2) was 0.9999, and the limit of detection (LOD) and limit of quantification (LOQ) were 0.0004 and 0.0016 μg g^{-1}, respectively. All the OTA samples and standard solutions were analyzed in triplicate.

Inhibitory activity in the production of aflatoxin B1 and B2

Samples used to evaluate the antimycotoxigenic activity were prepared from Petri dishes used in mycelial growth analysis. Inhibition of the production of aflatoxin B1 and B2 by *A. flavus* and *A. parasiticus* by active principles and nanofibers were tested at concentrations of 2000 and 3000 μl l^{-1} and at 20 and 30%, respectively. All the plates were incubated in BOD at 25°C for 10 days.

The identification of aflatoxin B1 and B2 was performed according to the method described by Freire *et al*. (2017), with modifications. To perform the analysis, 1-cm-diameter plugs of culture medium containing the microorganisms treated with the free active principles and incorporated into the nanofibers were added to silica gel-coated aluminum TLC plates (Merck-silica gel 60; 20 × 20 cm). A solvent mixture composed of toluene:ethyl acetate:formic acid (60:30:10) was used as the mobile phase. Parallel to the samples, 10 μl of each of the standard solutions (aflatoxin B1 at 11.1060 μg ml^{-1} and aflatoxin B2 at 11.9271 μg ml^{-1}; Sigma-Aldrich®, São Paulo, Brazil) were applied. Aflatoxin production was confirmed under ultraviolet light at 366 nm on a CAMAG Chromatovisor (Uf-Betrachter).

Statistical analysis

The tests were performed in a completely randomized design, and all the analyses were performed in triplicate. The results obtained for the different treatments were evaluated by analysis of variance and multiple comparisons of the means by the Scott-Knott test ($p<0.05$) using the statistical software Sisvar (Ferreira 2011).

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Conflict of Interest Statement

The authors have no conflicts of interest to declare

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Maria das Graças Cardoso: Conceptualization, methodology, supervision, formal analysis, writing - (review and editing), project administration and funding acquisition.

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Graphical abstract:

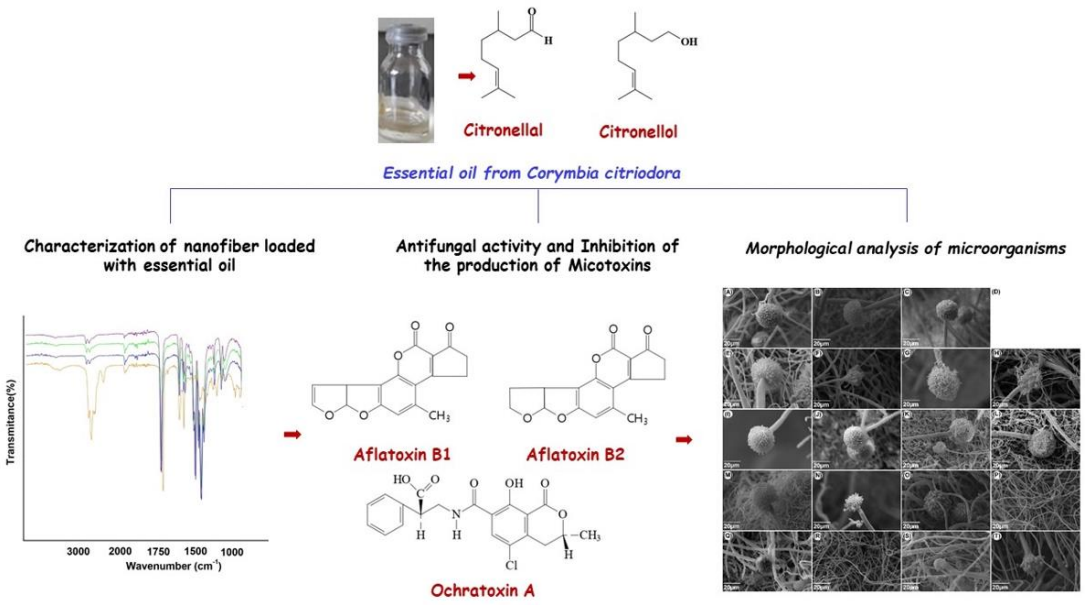

Table 1. Thermal properties of free poly(lactic acid) nanofibers and nanofibers conaining the essential oil from *Corymbia citriodora*, *β*-citronellol and citronellal.

Sample	**T_g (°C)**	**T_c (°C)**	**T_m (°C)**	**ΔH_c (J g^{-1})**	**ΔH_m (J g^{-1})**
Poly(lactic acid) nanofiber	54	92	168	4.8	15.1
(PLA + 30 wt% Citronellal) nanofibres	47	68	167	2.5	15.7
(PLA + 20 wt% Citronellal) nanofibres	45	81	167	0.8	11.7
(PLA + 30 wt% *β*-Citronellol) nanofibres	42	81	166	0.9	10.9
(PLA + 20 wt% *β*-Citronellol) nanofibres	42	84	168	0.89	13.3
(PLA + 30 wt% *C. citriodora* oil) nanofibres	51	88	167	0.51	12.7
(PLA + 20 wt% *C. citriodora* oil) nanofibres	51	88	167	0.65	12.6

Glass transition temperature (Tg), crystallization temperature (Tc), melting temperature (Tm), crystallization enthalpy (ΔH_c) and melting enthalpy (ΔH_m).

Table 2. Antifungal activities of the essential oil from *Corymbia citriodora* and the *β*-citronellol and citronellal monoterpenes, free and incorporated in poly(lactic acid) nanofibers.

	Inhibition of mycelial growth (%)				
Sample	***A. ochraceus***	***A. flavus***	***A. parasiticus***	***A. westerdijkae***	***A. carbonarius***
C. citriodora (2000 µl l^{-1})	61.53 a5 ± 2.53	35.80 a3 ± 6.66	46.60 a4 ± 0.42	-	63.99 a6 ± 2.79
C. citriodora (3000 µl l^{-1})	63.14 a5 ± 4.18	55.20 a4 ± 6.60	52.17 a4 ± 0.67	-	61.53 a6 ± 4.18
Citrolellal (2000 µl l^{-1})	55.14 a3 ± 0.69	-	49.52 a4 ± 0.36	-	55.28 a4 ± 0.48
Citrolellal (3000 µl l^{-1})	59.55 a4 ± 0.66	57.56 a4 ± 1.97	52.46 a4 ± 0.26	10.16 a2 ± 1.84	59.58 a5 ± 0.71
β-Citronellol (2000 µl l^{-1})	63.14 a5 ± 1.39	63.21 a5 ± 6.35	52.05 a4 ± 0.61	13.33 a2 ± 1.17	61.03 a6 ± 0.03
β-Citronellol (3000 µl l^{-1})	61.76 a5 ± 0.18	64.16 a5 ± 1.99	60.46 a5 ± 0.59	20.01 a3 ± 4.08	63.05 a6 ± 1.39
(PLA + 20 wt% *C. citriodora* oil) nanofibres	18.54 a1 ± 2.29	27.56 a2 ± 1.56	70.54 a6 ± 8.11	16.84 a3 ± 2.50	11,98 a2 ± 1.32
(PLA + 30 wt% *C. citriodora* oil) nanofibres	21.35 a1 ± 6.87	41.44 a3 ± 0,60	78.62 a7 ± 0.56	20.83 a3 ± 1.13	26.93 a3 ± 3.95
(PLA + 20 wt% citronellal) nanofibres	-	6.250 a1 ± 0.04	1.630 a1 ± 1.30	3.740 a1 ± 0.11	6.410 a1 ± 0.20
(PLA + 30 wt% Citronellal) nanofibres	-	21.80 a2 ± 3.77	2.710 a1 ± 0.08	15.28 a2 ± 1.13	11.21 a2 ± 1.31
(PLA + 20 wt% *β*-Citronellol) nanofibres	44.67 a2 ± 0.13	35.10 a1 ± 1.94	7.450 a2 ± 0.11	54,82 a4 ± 3.34	100.0 a7 ± 0.00
(PLA + 30 wt% *β*-Citronellol) nanofibres	100.0 a6 ± 0.00	37.23 a3 ± 1.76	20.94 a3 ± 0.61	75.93 a5 ± 7.04	100.0 a7 ± 0.00

***** The means followed by the same lower case letter in the columns not differ from one another by the Scott-Knott test at the 5% probability level.

(-) Showed no significant inhibition activity.

Table 3. Inhibition of ochratoxin A production by *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Aspergillus westerdijkae* fungi treated with *Corymbia citriodora* essential oil, citronellal and *β*-citronellol, free and incorporated into poly(lactic acid) nanofibers.

Inhibition of ochratoxin A production (%)			
Sample	***A. ochraceus***	***A. carbonarius***	***A. westerdijkae***
C. citriodora (2000 µl l^{-1})	28.97 a3 ± 5.61	81.83 a5 ± 0.16	94.67 a7 ± 0.05
C. citriodora (3000 µl l^{-1})	86.53 a6 ± 2.13	99.56 a7 ± 0.04	95.19 a8 ± 0.03
Citronellal (2000 µl l^{-1})	0.00 ± 0.00	77.25 aa ± 0.002	76.73 a3 ± 0.33
Citronellal (3000 µl l^{-1})	0.00 ± 0.00	99.70 a7 ± 0.002	49.81 a2 ± 0.69
β-Citronellol (2000 µl l^{-1})	21.90 a2 ± 3.27	88.66 a6 ± 0.009	88.47 a6 ± 0.04
β-Citronellol (3000 µl l^{-1})	21.40 a1 ± 0.73	61.43 a3 ± 0.38	81.29 a4 ± 0.11
(PLA + 20 wt% *C. citriodora* oil) nanofibres	0.00 ± 0.00	0.00 ± 0.00	99.75 a9 ± 0.002
(PLA + 30 wt% *C. citriodora* oil) nanofibres	43.08 a4 ± 0.71	0.00 ± 0.00	99.58 a9 ± 0.02
(PLA + 20 wt% Citronellal) nanofibres	0.00 ± 0.00	10.62 a1 ± 0.62	34.16 a1 ± 0.38
(PLA + 30 wt% Citronellal) nanofibres	48.26 a5 ± 0.31	58.84 a2 ± 0.55	99.82 a9 ± 0.003
(PLA + 20 wt% *β*-Citronellol) nanofibres	0.00 ± 0.00	-	0.00 ± 0.00
(PLA + 30 wt% *β*-Citronellol) nanofibres	-	-	86.84 a5 ± 0.44

***** The means followed by the same lower case letter in the columns not differ from one another by the Scott-Knott test at the 5% probability level.

3 CONSIDERAÇÕES GERAIS

A incorporação dos óleos essenciais de *Corymbia citriodora*, *Eucalyptus grandis*, *Eucalyptus camaldulensis*, *Rosmarinus oficinallis* e dos monoterpenos *β*-citronellol e citronellal em matrizes poliméricas de nanopartículas e nanofibras se mostraram eficazes no controle da volatilização dessas substâncias, além de potencializar e prolongar a atividade antifúngica e inseticida sobre os fungos *Hemileia vastatrix*, *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, *Aspergillus flavus* e *Aspergillus parasiticus*, e sobre a mosca *Drosophila suzukii*. Com base nos resultados obtidos neste trabalho, pode-se inferir que os sistemas nanopoliméricos incorporados com esses princípios ativos mostram-se promissores no desenvolvimento de novos produtos biocompatíveis e biodegradáveis que podem ser aplicados à indústria agrícola e de alimentos.

Para que essa tecnologia possa chegar às indústrias e, posteriormente, ao consumidor final, é necessário que ela passe por um processo de validação da Agencia Nacional de Vigilância Sanitária (Anvisa) e do Ministério de Agricultura Pecuária e Abastecimento (Mapa). Alguns testes podem ser realizados para se ter maior conhecimento sobre a tecnologia, como: avaliar o tempo de prateleira das formulações; construir uma curva cinética de volatilidade para os princípios ativos incorporados nas matrizes poliméricas e acompanhar a cinética de degradação desses materiais no meio ambiente.