UNIVERSIDADE FEDERAL DE LAVRAS

**LUCIANO DE SOUZA**

# **INSECTICIDAL POTENTIAL OF TERPENES AND PHENYLPROPANOIDS:** NATURAL SOLUTIONS AND NANOTECHNOLOGY FOR AGRICULTURAL PESTS CONTROL

**LAVRAS-MG**
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agroquímica, área de concentração em Química/ Bioquímica, para obtenção do título de Doutor.

Profa. Dra. Maria das Graças Cardoso
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**LUCIANO DE SOUZA**

# **POTENCIAL INSETICIDA DE TERPENOS E FENILPROPANOIDES:** SOLUÇÕES NATURAIS E NANOTECNOLOGIA PARA O CONTROLE DE PRAGAS AGRÍCOLAS

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APROVADO em 19 de fevereiro de 2025.

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## RESUMO

A agricultura é uma das principais atividades econômicas do Brasil. Anualmente a produtividade sofre perdas por vários fatores, incluindo a ação de insetos-praga. A principal forma de controle é o uso de inseticidas químicos sintéticos, normalmente feito de forma abusiva, o que gera problemas de contaminação de recursos naturais, seleciona indivíduos resistentes e coloca em risco a integridade de ecossistemas. Como alternativa ao uso desses compostos, produtos naturais têm ganhado destaque, especialmente terpenos e fenilpropanoides que são constituintes de óleos essenciais de espécies vegetais. Os produtos naturais são considerados ecologicamente corretos e empregados em práticas sustentáveis de produção. Objetivou-se nesse trabalho avaliar a atividade inseticida de terpenos e fenilpropanoides sobre os insetos-praga *Drosophila suzukii* e *Sitophilus zeamais*; utilizar os compostos de maior potencial na síntese de nanopartículas para contornar as limitações impostas pelas propriedades físico-químicas dessas moléculas. Adicionalmente, avaliar o efeito de doses subletais nos organismos de interesse e em espécies não-alvo, como *Doru luteipes*, *Palmistichus elaeisis* e *Tetrastichus howardi*. Para *D. suzukii*, L-(-)-carvona, carvacrol, (*E*)-anetol e (*E*)-cinamaldeído, causando alta mortalidade e deformações nos adultos. Esses compostos também alteraram a atividade de enzimas detoxificantes, como catalase (CAT), superóxido dismutase (SOD) e glutationa-S-transferase (GST), além de provocarem alterações histológicas no exoesqueleto, intestino e corpo gorduroso dessa praga. Os compostos nanoparticulados apresentaram efeito inseticida prolongado, especialmente PCL-carvacrol. Os compostos não afetaram significativamente os inimigos naturais *D. luteipes* e *P. elaeisis*, sugerindo seletividade biológica. Para *S. zeamais*, carvacrol foi composto mais eficaz, seguido por (*E*)-cinamaldeído e *p*-anisaldeído. Além disso, (*E*)-anetol demonstrou forte sinergia em misturas binárias. Os compostos ativaram enzimas antioxidantes e aumentaram a peroxidação lipídica, promovendo estresse oxidativo. Também inibiram enzimas digestivas, como $\alpha$-amilase, e reduziram a espessura de fibras musculares e intestinais, além da reserva lipídica e carboidratos no corpo gorduros. O (*E*)-cinamaldeído mostrou maior compatibilidade com *T. howardi*, parasitoide de pragas. Os resultados indicam que esses compostos naturais são potenciais bioinseticidas seletivos, com aplicações promissoras no controle sustentável de pragas agrícolas.

**Palavras-chave:** toxicologia; estresse oxidativo; alterações morfológicas; inibição de enzimas digestivas; mosca-da-asa-manchada; gorgulho-do-milho; ecofriendly.

## ABSTRACT

Agriculture is one of the main economic activities in Brazil. Annually, productivity suffers losses due to various factors, including the action of pest insects. The primary control method is the use of synthetic chemical insecticides, which is often done abusively and without awareness, causing issues such as contamination of natural resources, selection of resistant individuals, and posing risks to ecosystem integrity and human health. As an alternative to these compounds, natural products have gained attention, especially terpenes and phenylpropanoids, which are constituents of essential oils from plant species. Natural products are considered environmentally friendly and are used in sustainable production practices. This study aimed to evaluate the insecticidal activity of terpenes and phenylpropanoids on pest insects *Drosophila suzukii* and *Sitophilus zeamais*; to use the most potent compounds in nanoparticle synthesis to overcome limitations imposed by the physicochemical properties of these molecules. Additionally, the effects of sublethal doses on target organisms and non-target species, such as *Doru luteipes*, *Palmistichus elaeisis*, and *Tetrastichus howardi*, were assessed. For *D. suzukii*, L-(-)-carvone, carvacrol, (*E*)-anethole, and (*E*)-cinnamaldehyde caused high mortality and deformities in adults. These compounds also altered the activity of detoxifying enzymes, such as catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST), and induced histological changes in the exoskeleton, intestines, and fat body of this pest. Nanoparticled compounds showed prolonged insecticidal effects, especially PCL-carvacrol. The compounds did not significantly affect natural enemies *D. luteipes* and *P. elaeisis*, suggesting biological selectivity. For *S. zeamais*, carvacrol was the most effective compound, followed by (*E*)-cinnamaldehyde and *p*-anisaldehyde. Furthermore, (*E*)-anethole demonstrated strong synergy in binary mixtures. The compounds activated antioxidant enzymes and increased lipid peroxidation, promoting oxidative stress. They also inhibited digestive enzymes, such as $\alpha$-amylase, and reduced muscle fiber and intestinal thickness, as well as lipid reserves and carbohydrates in the fat body. (*E*)-cinnamaldehyde showed the greatest compatibility with *T. howardi*, a pest parasitoid. The results indicate that these natural compounds are potential selective biopesticides with promising applications for the sustainable control of agricultural pests.



**Keywords:** toxicology; oxidative stress; morphological changes; inhibition of digestive enzymes; spotted wing fly; corn weevil; ecofriendly.

## INDICADORES DE IMPACTO

O presente estudo investigou o potencial inseticida de terpenos e fenilpropanoides contra pragas agrícolas, avaliando sua eficácia tanto em formas isoladas quanto incorporadas a nanopartículas de poli($\varepsilon$-caprolactona) (PCL). Foram analisados os efeitos de compostos como carvacrol, L-(-)-carvona, (*E*)-anetol, (*E*)-cinamaldeído e *p*-anisaldeído sobre *Drosophila suzukii* e *Sitophilus zeamais*, bem como sua seletividade para organismos não-alvo, incluindo *Doru luteipes*, *Palmistichus elaeisis* e *Tetrastichus howardi*. Os resultados demonstraram alta toxicidade dos compostos isolados e nanoparticulados para as pragas-alvo. O uso de nanopartículas prolongou a atividade inseticida e potencializou os efeitos de estresse oxidativo nos insetos, elevando a atividade de enzimas como catalase, glutationa-S-transferase e glutationa peroxidase. Adicionalmente, as exposições subletais resultaram em alterações histopatológicas e no caso de *S. zeamais*, observou-se que *p*-anisaldeído promoveu forte inibição da enzima $\alpha$-amilase e aumento da atividade de lipases, enquanto as misturas binárias com (*E*)-anetol demonstraram interação sinérgica. Importante ressaltar que os compostos testados não afetaram negativamente a sobrevivência e o desenvolvimento dos organismos não-alvo, indicando sua segurança para o uso em programas de manejo integrado de pragas. Os impactos do estudo são amplamente positivos, abrangendo aspectos ambientais, tecnológicos e econômicos. O desenvolvimento de potenciais inseticidas naturais contribui para a redução da dependência de agrotóxicos sintéticos, mitigando riscos à saúde humana e aos ecossistemas. Do ponto de vista econômico, a utilização de produtos naturais como alternativa sustentável pode beneficiar agricultores ao reduzir perdas na produção e minimizar impactos negativos do uso indiscriminado de pesticidas convencionais. Além disso, os resultados se alinham diretamente com os Objetivos de Desenvolvimento Sustentável (ODS) da ONU, especialmente o ODS 2 (Fome Zero e Agricultura Sustentável), ODS 12 (Consumo e Produção Responsáveis) e ODS 15 (Vida Terrestre), reforçando o compromisso do estudo com a Agenda 2030 e a promoção de práticas agrícolas sustentáveis.

## IMPACT INDICATORS

This study investigated the insecticidal potential of terpenes and phenylpropanoids against agricultural pests, evaluating their effectiveness both in isolated forms and incorporated into poly($\varepsilon$-caprolactone) (PCL) nanoparticles. The effects of compounds such as carvacrol, L-(-)-carvone, (*E*)-anethol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde on *Drosophila suzukii* and *Sitophilus zeamais* were analyzed, as well as their selectivity for non-target organisms, including *Doru luteipes*, *Palmistichus elaeisis*, and *Tetrastichus howardi*. The results showed high toxicity of both isolated and nanoparticulate compounds to target pests. The use of nanoparticles extended insecticidal activity and enhanced oxidative stress effects in insects, increasing the activity of enzymes such as catalase, glutathione-S-transferase, and glutathione peroxidase. Additionally, sublethal exposures resulted in histopathological changes, and in the case of *S. zeamais*, it was observed that *p*-anisaldehyde strongly inhibited $\alpha$-amylase enzyme activity and increased lipase activity, while binary mixtures with (*E*)-anethol exhibited synergistic interaction. Importantly, the tested compounds did not negatively affect the survival and development of non-target organisms, indicating their safety for use in integrated pest management programs. The study's impacts are broadly positive, covering environmental, technological, and economic aspects. The development of potential natural insecticides contributes to reducing dependence on synthetic pesticides, mitigating risks to human health and ecosystems. From an economic perspective, the use of natural products as a sustainable alternative can benefit farmers by reducing production losses and minimizing negative impacts from the indiscriminate use of conventional pesticides. Furthermore, the results align directly with the UN's Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger and Sustainable Agriculture), SDG 12 (Responsible Consumption and Production), and SDG 15 (Life on Land), reinforcing the study's commitment to the 2030 Agenda and the promotion of sustainable agricultural practices.

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## SUMÁRIO

# PRIMEIRA PARTE

# 1. INTRODUÇÃO GERAL

A agricultura desempenha papel fundamental na sociedade brasileira, sendo a principal base da economia nacional. O setor é responsável pela maior parte das exportações do Brasil, com destaque para produtos como soja, milho, café, cana-de-açúcar e algodão, que colocam o país entre os maiores produtores e exportadores globais dessas commodities. Além disso, a agricultura é responsável por milhões de empregos diretos e indiretos, tanto no campo quanto na indústria de transformação, transporte e comércio. A produção agrícola garante o abastecimento interno e a segurança alimentar da população brasileira, além de contribuir significativamente para a geração de divisas, fortalecendo a balança comercial do país e estimulando o desenvolvimento de novas tecnologias voltadas para o aumento da produtividade e sustentabilidade da produção.

Diversos fatores podem impactar negativamente a produtividade agrícola, comprometendo o desempenho deste setor e sua contribuição para a economia. Entre os principais desafios estão as condições climáticas adversas, como secas prolongadas, chuvas excessivas e eventos extremos, que afetam diretamente o desenvolvimento das culturas no campo. A degradação dos solos, causada pelo uso inadequado de práticas agrícolas, também compromete a fertilidade e a capacidade produtiva das terras. Problemas estruturais, como a deficiência em logística e infraestrutura de transporte, aumentam os custos e dificultam o escoamento da produção. Além disso, pragas e doenças agrícolas representam uma ameaça constante, especialmente quando associadas ao uso ineficaz de defensivos ou à falta de manejo adequado.

Os insetos-praga são espécies de insetos que causam danos significativos aos cultivos, prejudicando a produtividade e a qualidade das colheitas. Eles podem atacar diferentes partes das plantas, como folhas, caules, raízes, flores e frutos, afetando tanto o desenvolvimento vegetativo quanto a produção agrícola. Exemplos comuns incluem lagartas desfolhadoras, pulgões, moscas-das-frutas, besouros e percevejos. Esses insetos podem se multiplicar rapidamente em condições favoráveis, como temperaturas elevadas e monoculturas, o que exige estratégias de controle eficazes. O manejo de insetos-praga inclui o uso de defensivos químicos, práticas culturais, controle biológico com inimigos naturais e técnicas integradas que visam minimizar impactos ambientais e econômicos, garantindo a sustentabilidade da produção agrícola.

Dentre as espécies praga, pode-se destacar *Drosophila suzukii*, uma espécie nativa da Ásia conhecida como mosca-da-asa-manchada. É uma espécie de inseto-praga que causa sérios prejuízos à fruticultura em diversas regiões do mundo, incluindo o Brasil. Diferente de outras moscas-das-frutas, *D. suzukii* tem a capacidade de atacar frutas ainda imaduras ou em processo de amadurecimento, como morango, cereja, amora, mirtilo e uva, o que aumenta o seu potencial de dano econômico. Os machos são facilmente identificados pelas manchas escuras nas asas, enquanto as fêmeas possuem aparelho ovipositor serrilhado que perfura a superfície das frutas para depositar os ovos. As larvas desenvolvem-se dentro dos frutos, causando apodrecimento e perda de valor comercial.

Outro exemplo de inseto praga é *Sitophilus zeamais*, conhecido como o gorgulho-do-milho. É um inseto de grande importância econômica, especialmente no armazenamento de grãos como milho, arroz, trigo e sorgo. Essa espécie se destaca por sua capacidade de infestar grãos armazenados, causando danos diretos pela alimentação e perdas indiretas devido à redução da qualidade do produto, contaminação por excrementos e aumento da suscetibilidade dos grãos ao ataque de outros insetos-praga, fungos e microrganismos. Os adultos perfuram os grãos para alimentação e oviposição, e as larvas se desenvolvem internamente, tornando os danos muitas vezes imperceptíveis até que o problema esteja avançado. A alta taxa de reprodução e adaptação ao ambiente de armazenamento torna o manejo dessa praga desafiador.

O controle de insetos-praga pode ser realizado de diversas formas, incluindo métodos químicos, biológicos, culturais e físicos, frequentemente integrados em estratégias de manejo integrado de pragas (MIP). Os inseticidas químicos sintéticos são a principal forma de controle utilizada por sua eficiência e ação rápida, mas seu uso indiscriminado traz sérios problemas. Entre os principais desafios está o desenvolvimento de resistência por parte das pragas, que reduz a eficácia dos produtos e exige doses maiores ou o uso de compostos mais tóxicos. Além disso, os inseticidas podem causar impactos ambientais significativos, como a contaminação do solo, água e atmosfera, bem como efeitos adversos sobre organismos não-alvo, incluindo polinizadores e inimigos naturais das pragas. Há também preocupações com a saúde humana devido à exposição a resíduos químicos nos alimentos e no ambiente. Por isso, alternativas como o controle biológico, o uso de agentes naturais (como parasitoides e predadores), biopesticidas, práticas culturais e tecnologias mais sustentáveis têm ganhado destaque na busca por uma agricultura mais equilibrada e menos dependente de químicos sintéticos.

A utilização de produtos naturais derivados de plantas, como óleos essenciais, ou seus constituintes isolados que são os terpenos e fenilpropanoides, apresenta diversas vantagens no controle de insetos-praga, especialmente em sistemas agrícolas que buscam maior sustentabilidade. Esses compostos possuem propriedades repelentes, inseticidas e reguladoras de crescimento, podendo atuar de forma específica sobre as pragas, reduzindo os impactos sobre organismos não-alvo e o meio ambiente. Óleos essenciais demonstram eficácia sobre diversas espécies de insetos, muitas vezes interferindo em sua alimentação, reprodução e comunicação química. Os terpenos e fenilpropanoides, que são metabólitos secundários constituintes de óleos essenciais, também têm mostrado potencial para interromper processos fisiológicos dos insetos, como a síntese hormonal ou a atividade enzimática. Além disso, por serem biodegradáveis, esses compostos minimizam riscos de contaminação ambiental e de resíduos nos alimentos. O uso desses produtos também favorece a diversificação de estratégias empregadas no MIP e reduz a dependência de inseticidas químicos sintéticos, contribuindo para o desenvolvimento de práticas agrícolas menos nocivas. Entretanto, os produtos naturais tem seu uso em campo limitado por sua baixa estabilidade em condições ambientais, devido à rápida degradação por fatores como radiação solar, altas temperaturas e umidade, o que reduz sua eficácia e persistência.

O uso de nanopartículas de polímeros biodegradáveis, como a policaprolactona (PCL), tem se mostrado uma estratégia promissora para melhorar o desempenho de inseticidas derivados de plantas em condições de campo. Essas nanopartículas atuam como sistemas de liberação controlada, protegendo os compostos ativos, como óleos essenciais e seus constituintes, da rápida degradação. Além disso, a encapsulação em PCL permite a liberação gradual e direcionada dos inseticidas, aumentando sua eficiência e reduzindo a necessidade de aplicações frequentes. A biodegradabilidade do polímero garante que não haja acúmulo de resíduos tóxicos no meio ambiente, tornando essa abordagem mais sustentável e compatível com práticas agrícolas ecológicas. Essa tecnologia também pode minimizar os efeitos adversos em organismos não-alvo, ao permitir concentrações mais eficazes no local de ação e a redução do impacto ambiental gerado pela agricultura.

O presente trabalho objetivou-se a avaliar a toxicidade dos terpenos L-(-)-carvona, *p*-cimeno, 1,8-cineol, *β*-citronelol e carvacrol e dos fenilpropanoides (*E*)-cinamaldeído, *p*-anisaldeído, (*E*)-anetol e eugenol sobre *D. suzukii*, (a estrutura química desses compostos é apresentada no Apêndice I); avaliar o efeito de doses subletais de terpenos e fenilpropanoides selecionados na mortalidade, estresse oxidativo e histopatologia de larvas de 3º instar de *D. suzukii* e sobre o organismo não-alvo *Doru luteipes*; bem com desenvolver, caracterizar e avaliar o efeito de nanopartículas preparadas com material polimérico contendo os terpenos e fenilpropanoides selecionados sobre a morfofisiologia de adultos de *D. suzukii* e sobre o organismo não-alvo *Palmistichus elaeisis*; bem como avaliar a toxicidade dos terpenos L-(-)-carvona, *β*-citronelol e carvacrol e dos fenilpropanoides (*E*)-cinamaldeído, *p*-anisaldeído, (*E*)-anetol e eugenol sobre *S. zeamais* e selecionar terpenos e fenilpropanoides que apresentarem melhor desempenho para avaliar os efeitos na morfofisiologia de *S. zeamais* e o organismo não-alvo *Tetrastichus howardi*.

# 2. OBJETIVOS

## 2.1. Objetivo geral

Avaliar o potencial inseticida de terpenos e fenilpropanoides, desenvolver e caracterizar novos materiais poliméricos contendo esses produtos naturais para manejo de pragas agrícolas.

## 2.2. Objetivos específicos

- Avaliar a toxicidade letal dos terpenos L-(-)-carvona, *p*-cimeno, 1,8-cineol, *β*-citronelol e carvacrol e dos fenilpropanoides (*E*)-cinamaldeído, *p*-anisaldeído, (*E*)-anetol e eugenol sobre adultos de *Drosophila suzukii*; avaliar a toxicidade letal e sub-letal, o estresse oxidativo e alterações histológicas em larvas de 3º instar de *D. suzukii*; avaliar a toxicidade letal e sub-letal sobre adultos do organismo não alvo *Doru luteipes*.
- Sintetizar, caracterizar e avaliar a toxicidade letal de nanopartículas de poli(ε-caprolactona) contendo os terpenos e os fenilpropanoides selecionados sobre fêmeas adultas *D. suzukii*; avaliar o desempenho das nanopartículas ao longo do tempo; avaliar os efeitos de doses subletais em enzimas relacionadas ao estresse oxidativo e histopatologia de fêmeas adultas de *D. suzukii*; avaliar a toxicidade letal e sub-letal dos terpenos e fenilpropanoides selecionados sobre adultos do organismo não alvo *Palmistichus elaeisis*.
- Avaliar a atividade inseticida dos terpenos L-(-)-carvona, *β*-citronelol e carvacrol e dos fenilpropanoides (*E*)-cinamaldeído, *p*-anisaldeído, (*E*)-anetol e eugenol sobre adultos de *Sitophilus zeamais*; avaliar a toxicidade pela interação entre pares dos compostos em estudo sobre adultos do inseto-praga; avaliar o efeito de doses subletais sobre a atividade de enzimas relacionadas ao estresse oxidativo e digestiva e na histopatologia desta espécie; avaliar o efeito de doses subletais no organismo não-alvo *Tetrastichus howardi*.

# 3. REFERENCIAL TEÓRICO

## 3.1. Metabólitos secundários de plantas

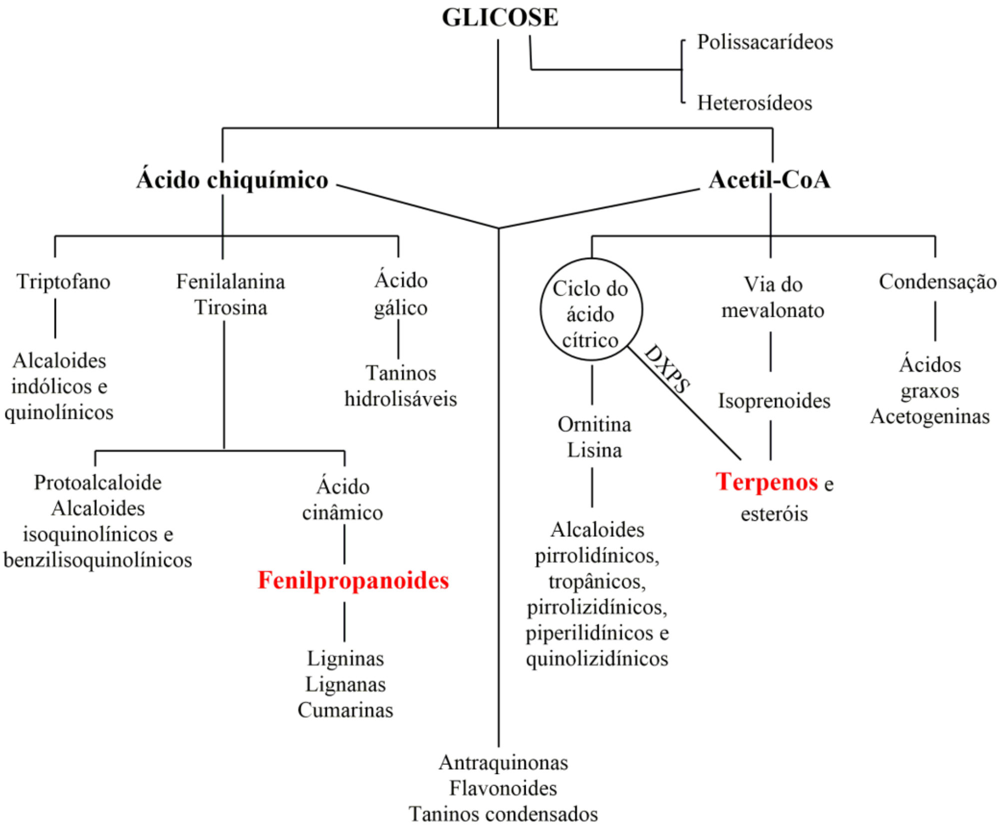
Metabolismo pode ser compreendido como a totalidade das reações químicas que ocorrem no interior das células de organismos vivos, com a finalidade de prover energia e sintetizar moléculas indispensáveis à manutenção das condições ideais para sobrevivência de indivíduos. Tais reações são catalisadas por enzimas específicas que garantem a ocorrência dessas transformações de forma ordenada, de tal modo que o conjunto das etapas envolvidas nesses processos é chamado de rota metabólica. Os compostos químicos obtidos como produto nessas reações são denominados de metabólitos, que podem ser classificados como primários e secundários. Os metabólitos primários correspondem aos carboidratos, proteínas, lipídeos, ácidos nucleicos e outros compostos que são responsáveis pela nutrição e participam de vias metabólicas fundamentais à vida do organismo (Simões et al., 2016).

Em espécies vegetais, os metabólitos secundários são produzidos e armazenados como misturas normalmente complexas, apresentando finalidade diversificada. Atuam principalmente na defesa sobre a herbivoria ou ataque de patógenos, como sinalizadores químicos para atração de polinizadores ou animais dispersores de sementes ou mediadores em relações simbióticas com outras plantas e microorganismos. Esses metabólitos se subdividem em várias classes, como os alcaloides, flavonoides, taninos, cumarinas, óleos essenciais (terpenos e fenilpropanoides), entre outros (Wink, 2008).

Os metabólitos secundários apresentam importante fonte de compostos de interesse econômico e tecnológico, sendo amplamente empregados em alimentos, cosméticos, medicamentos e preparações farmacêuticas. Em decorrência disso, os metabólitos secundários das plantas têm despertado grande interesse, devido ao seu crescente uso, o que tem implicado em um aumento no número de estudos que buscam elucidar as rotas bioquímicas que sintetizam esses metabólitos nos vegetais (Tiwari; Sangwan; Sangwan, 2016).

Os metabólitos secundários são originados a partir do metabolismo da glicose, por meio dos intermediários ácido chiquímico e acetil-CoA, como esquematizado na Figura 1. A partir do ácido chiquímico, são formados taninos hidrolisáveis, flavonoides, cumarinas, alcaloides derivados dos aminoácidos aromáticos e fenilpropanoides. A partir do acetil-CoA, são originados os aminoácidos alifáticos e alcaloides derivados deles, terpenos, esteroides, ácidos graxos e triglicerídeos. Os terpenos podem ser sintetizados também pela via da 1-desoxi-D-xilulose-5-fosfato (DXPS) (Al-Khayri et al., 2023; Pereira; Cardoso, 2012; Qaderi; Martel; Strugnell, 2023).

**Figura 1 -** Esquema da rota de biossíntese de metabólitos secundários em plantas.




Fonte: adaptado de Simões *et al*. (2016).

## 3.2. Óleos essenciais

Óleos essenciais são definidos segundo a *International Standard Organization* (ISO) como "produtos obtidos de partes de plantas por meio da hidrodestilação ou por destilação a arraste a vapor d'água, bem como os produtos obtidos por expressão dos pericarpos de frutos cítricos". Recebem também a nomenclatura de óleos voláteis, etéreos ou essências, em decorrência de suas propriedades físico-químicas, que englobam o aspecto de serem líquidos oleosos, insolúveis em água, voláteis e, na maioria dos casos, possuírem aroma intenso e agradável. Apresentam como características a alta instabilidade à luz, calor ou presença de oxigênio; são lipossolúveis e possuem coloração normalmente variando de incolor a ligeiro amarelo, mas óleos ricos em azuleno apresentarão coloração azulada, como o óleo essencial de camomila (Aleksic; Knezevic, 2014; Simões et al., 2016).

Os óleos essenciais são conhecidos e utilizados pelos humanos desde a Idade Média e, atualmente, continuam a ser de extrema importância para as áreas industrial e científica. Podem ser extraídos, principalmente, de plantas aromáticas, a partir de raízes, cascas, caules, botões florais, flores, frutos e sementes (Bakkali et al., 2008).

Os constituintes dos óleos essenciais são biossintetizados em estruturas secretoras, em que o metabólito é formado em glândulas endógenas que eventualmente se rompem e liberam as substâncias na cavidade dessas estruturas. São armazenados em células secretoras e epidérmicas, cavidades, canais secretores e tricomas glandulares (Bakkali et al., 2008).

Esses óleos podem participar de forma ativa nas interações ecológicas das plantas com outros indivíduos, sendo esses animais, microorganismos ou outras plantas. Desempenham a função de promover a manutenção da sobrevivência das plantas, conferindo a elas a capacidade de adaptação às condições do ambiente em que se encontram, como maior proteção contra herbívoros, funções ecológicas (atração de polinizadores e dispersores de sementes) e efeitos alelopáticos com outras plantas (Castro et al., 2008).

A composição química dos óleos essenciais e a proporção de cada constituinte são determinadas pelos genes da espécie vegetal em questão. Contudo, fatores edafoclimáticos, representados pelas variações das estações do ano, índice de chuvas, radiação solar, altitude, poluição, entre outros, podem interferir na biossíntese dos metabólitos secundários. Podem ocorrer, também, variações promovidas por fatores decorrentes do processo de colheita e manuseio do material vegetal, como o método de cultivo, idade da planta, horário de colheita, secagem, estocagem, entre outros (Blank et al., 2010; Gobbo-Neto; Lopes, 2007).

### 3.2.1. Biossíntese dos constituintes dos óleos essenciais

Os compostos químicos que formam os óleos essenciais estão divididos em duas classes principais, os terpenos, normalmente monoterpenos e sesquiterpenos, e fenilpropanoides (Huang; Ho; Wang, 2021; Simões et al., 2016). Os constituintes apresentam funções orgânicas muito variadas, podendo ser hidrocarbonetos terpênicos, aldeídos, cetonas, fenóis, ésteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas e compostos sulfurados. O óleo essencial de determinada espécie vegetal pode conter poucos ou vários constituintes, dos quais apenas pouquíssimos apresentarão concentração elevada, sendo chamados de constituintes majoritários; os demais em baixa concentração são chamados de minoritários e os de baixíssima concentração denominados de traços (Simões et al., 2016).

### 3.2.1.1. Terpenos

Os terpenos são formados a partir de dois intermediários básicos, o isopentenil pirofosfato (IPP) e o dimetilalil pirofosfato (DMAPP). Os compostos terpênicos compreendem uma grande variedade de metabólitos secundários de origem vegetal. São classificados de acordo com o número de unidades isoprênicas que possuem. Aqueles com 10 átomos de carbonos, que contêm duas unidades isoprênicas, são classificados como monoterpenos, os de 15 átomos de carbono (três unidades isoprênicas) são os sesquiterpenos e aqueles de 20 átomos de carbono (quatro unidades isoprênicas) são os diterpenos (Simões et al., 2016; Taiz et al., 2017). As estruturas do isopreno, IPP e DMAPP são apresentadas na Figura 2.

**Figura 2 -** Estruturas do isopreno, isopentenil e dimetilalil pirofosfato.


Fonte: do autor (2025).

Os terpenos podem ser biossintetizados a partir de metabólitos primários, partindo-se de duas rotas metabólicas distintas. A primeira via consiste na rota de metabolismo do mevalonato, é apresentada na Figura 3. Nessa via três moléculas de acetil-CoA são unidas por uma série de reações enzimáticas para formar o ácido mevalônico. Esse intermediário é, então, pirofosforilado, descarboxilado e desidratado, formando o isopentenil pirofosfato (IPP), sempre por meio da ação de enzimas específicas. Essa é a unidade prenilada ativa para a formação dos terpenos, que se interconverte por isomerização em dimetilalil pirofosfato (DMAPP). As moléculas de IPP e seu isômero DMAPP reagem para formar terpenos maiores, em reações do tipo “cabeça-cauda”. A adição eletrofílica de unidades de IPP e DMAPP, pela ação da enzima prenil-transferase, forma o intermediário geranil pirofosfato (GPP, $C_{10}$), que condensa com outra unidade IPP, fornecendo o farnesilpirofosfato (FPP, $C_{15}$). Por fim, a junção de FPP com outra unidade de IPP leva à produção de geranilgeranila pirofosfato (GGPP, $C_{20}$), precursor dos diterpenos (Böttger et al., 2018; Dewick, 2009a; Roba, 2020).

**Figura 3 -** Biossíntese de terpenos pela via do mevalonato (continua).

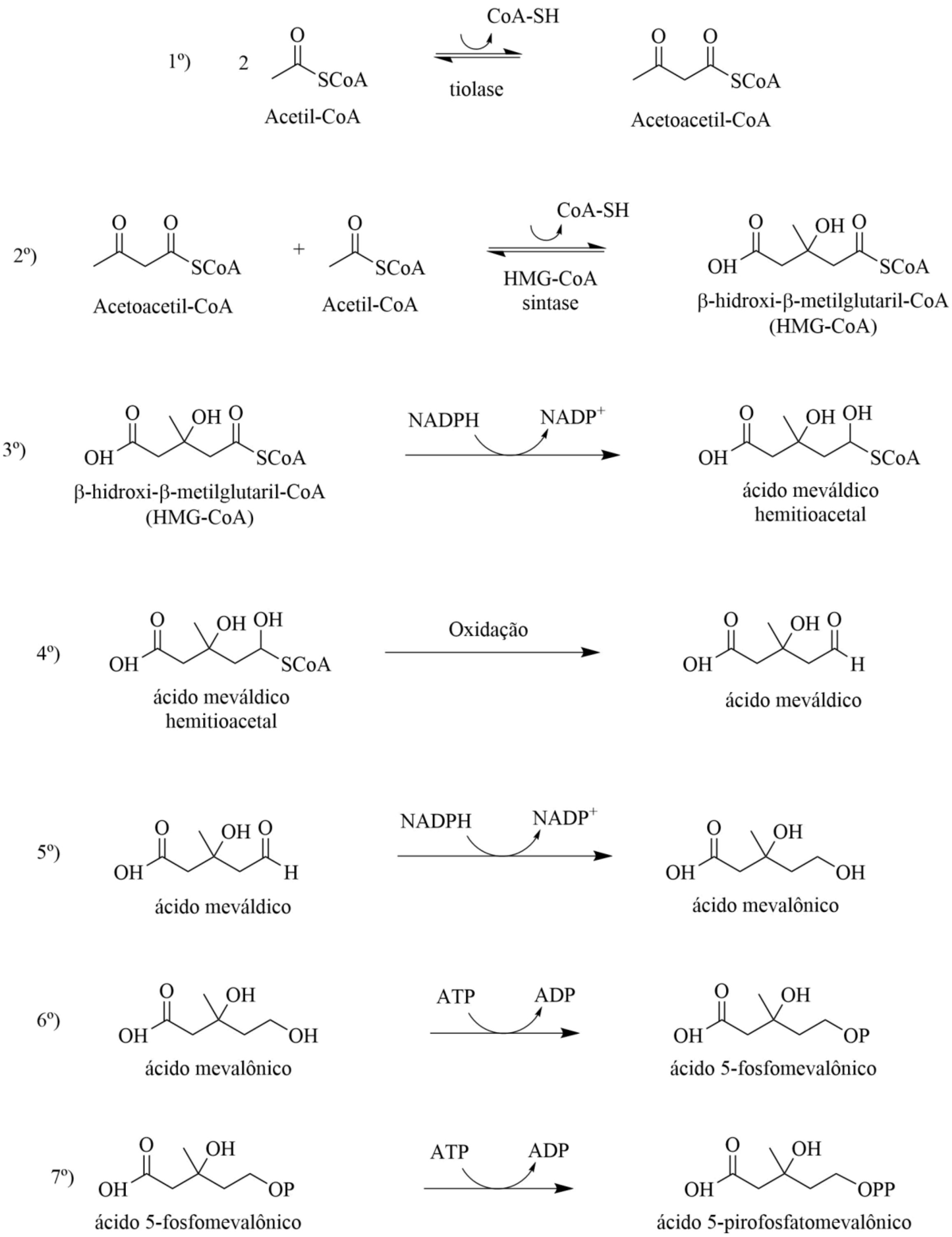
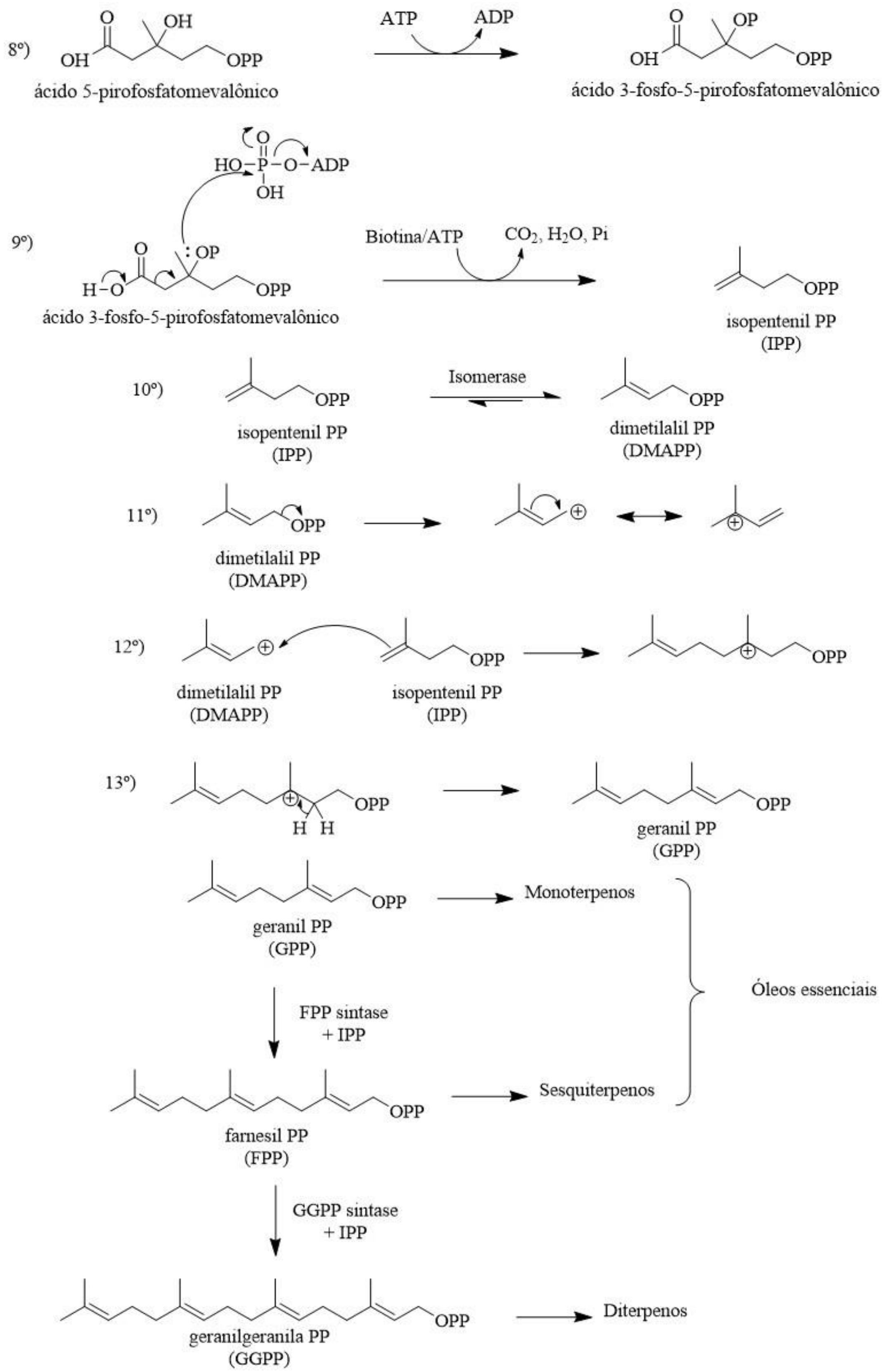


Figura 3 – Biossíntese de terpenos pela via do mevalonato (conclusão).




Fonte: adaptado de Dewick, (2009a).

A segunda via de biossíntese de terpenos é a 1-deoxi-D-xilulose-5-fosfato (DXPS), apresentada na Figura 4. Essa rota metabólica ocorre nos plastídios e nela o piruvato e o D-gliceraldeído-3-fosfato formam o 1-deoxi-D-xilulose-5-fosfato que, por sua vez, dá origem ao 2-C-metil-D-eritritol-4P (MEP). Posteriormente, são formados por sucessivas reações o isopentenil pirofosfato (IPP) e o dimetilalil pirofosfato (DMAPP) (Dewick, 2009a).

Originadas através de um precursor comum, o acetil-CoA, as estruturas terpênicas sintetizadas pelas rotas do mevalonato e DXPS podem sofrer diversas modificações por meio de reações de redução, oxidação e ciclização catalisadas por enzimas, formando vários derivados terpênicos, que constituem um dos maiores grupos de metabólitos secundários vegetais (Dewick, 2009a).

**Figura 4 -** Biossíntese de terpenos pela via DXPS (continua).

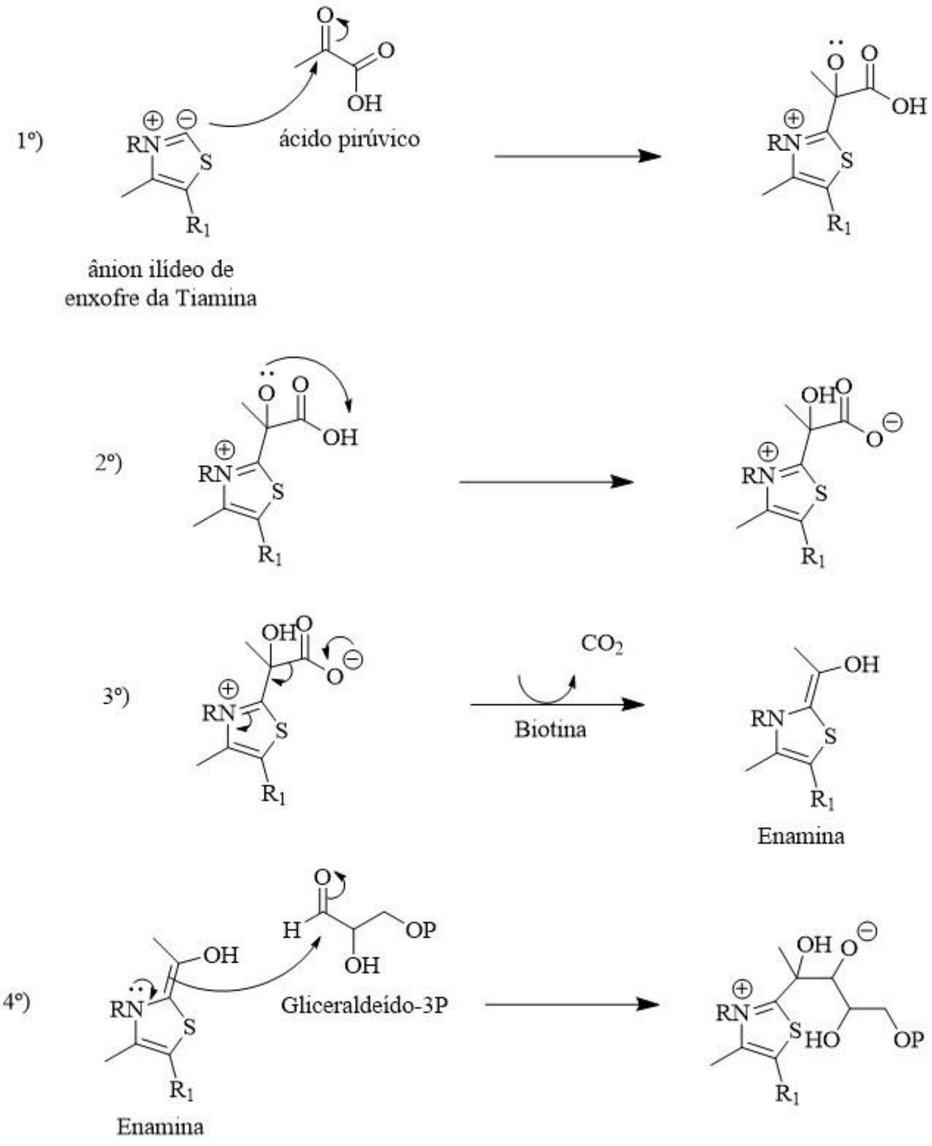


Figura 4 – Biossíntese de terpenos pela via DXPS (continua).

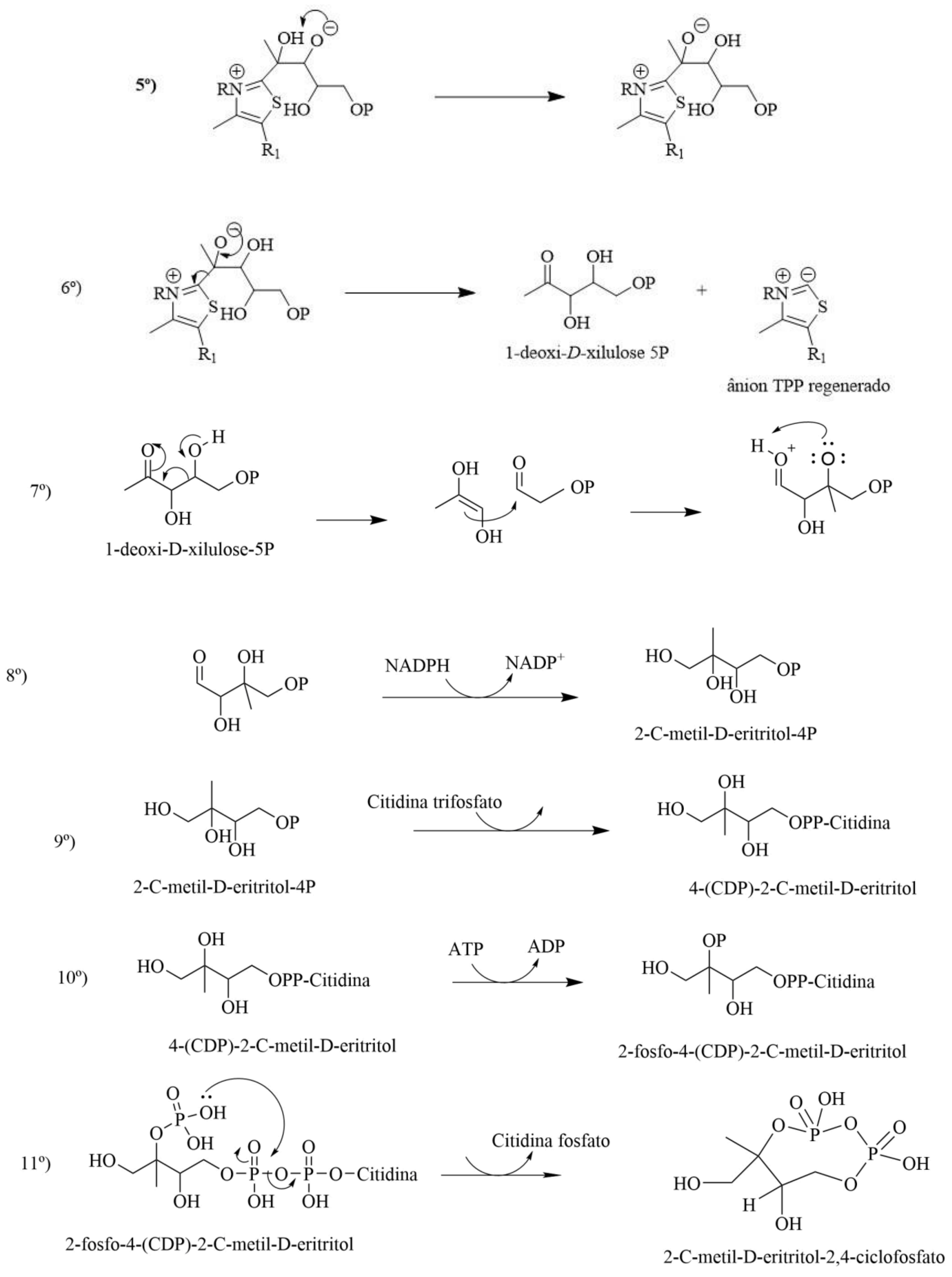
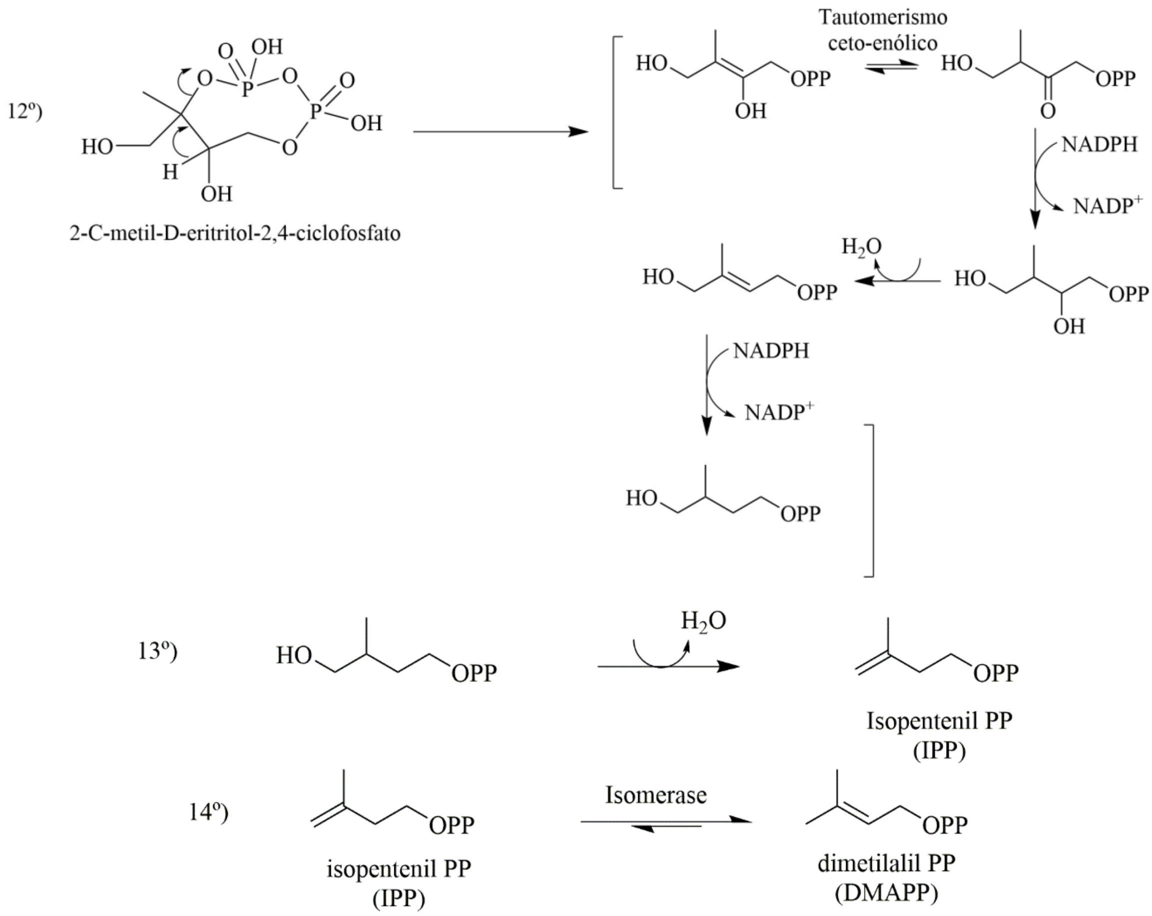


Figura 4 – Biossíntese de terpenos pela via DXPS (conclusão).

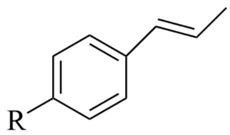



Fonte: adaptado de Dewick, (2009a).

### 3.2.1.2. Fenilpropanoides

Os fenilpropanoides são metabólitos secundários presentes em um grande número de espécies vegetais. São caracterizados por apresentarem uma estrutura básica formada por um anel benzênico unido a uma cadeia lateral insaturada com três átomos de carbono, conforme mostrado na Figura 5 (Aleksic; Knezevic, 2014; Dewick, 2009b).

**Figura 5 -** Estrutura básica de um fenilpropanoide.



R = H ou OH

Fonte: do autor (2025).

Os fenilpropanoides são produzidos a partir da via do ácido chiquímico e apresentam menor abundância do que os terpenos. Quando apresentam uma hidroxila no anel benzênico na posição *para* em relação à cadeia carbônica lateral, são derivados do aminoácido tirosina, e quando a hidroxila não está presente no composto, são derivados da fenilalanina (Deng; Lu, 2017; Dewick, 2009b). Os fenilpropanoides também podem conter em suas estruturas outros grupos funcionais oxigenados. Nessa rota metabólica ocorre, inicialmente, a formação do ácido chiquímico por meio da condensação aldólica de dois metabólitos da glicose, o fosfoenolpiruvato (via glicolítica) e a eritrose-4-fosfato (via das pentoses). Após ser formado, o ácido chiquímico sofre várias reações, até ser convertido nos aminoácidos fenilalanina e tirosina. Pela ação da enzima fenilalanina amonialiase (FAL), os aminoácidos perdem uma molécula de amônia, formando os ácidos cinâmico a partir da fenilalanina e *p*-cumárico a partir da tirosina. Por fim, por meio de reações de redução, oxidação e ciclização, os ácidos cinâmico e *p*-cumárico originam diversos fenilpropanoides (Simões et al., 2016; Vogt, 2010). Essa via metabólica é apresentada na Figura 6.

**Figura 6 -** Biossíntese de fenilpropanoides (continua).

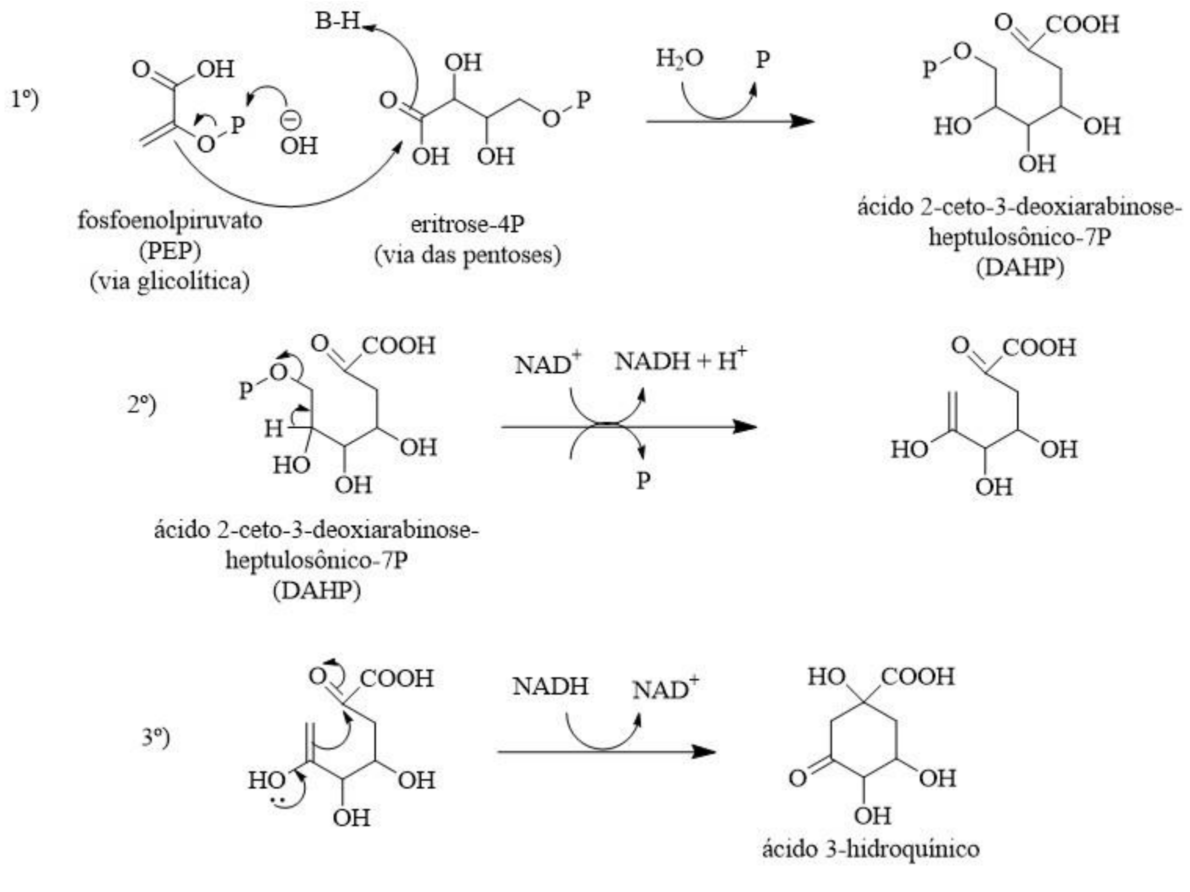


Figura 6 – Biossíntese de fenilpropanoides (continua).

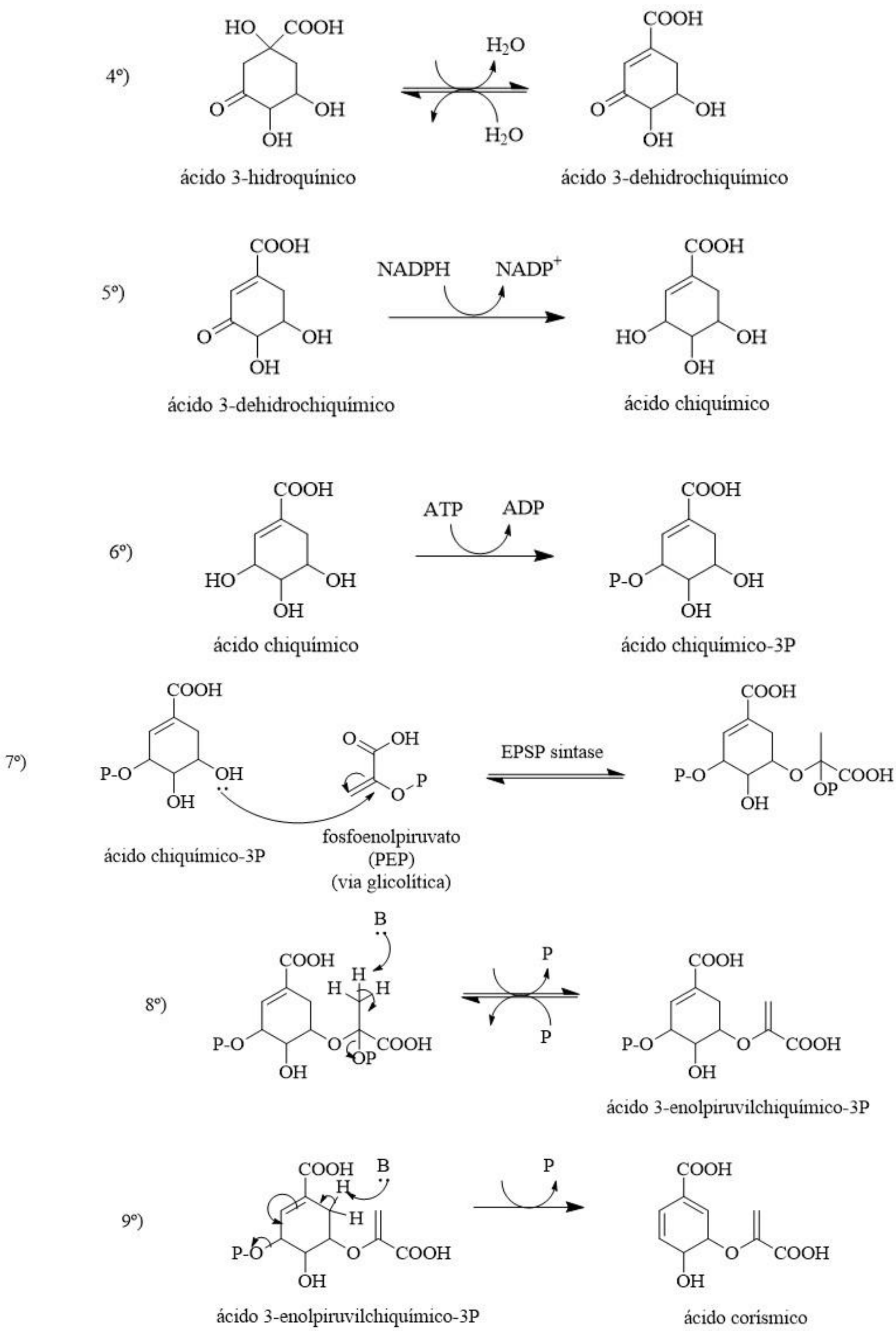


Figura 6 – Biossíntese de fenilpropanoides (continua).

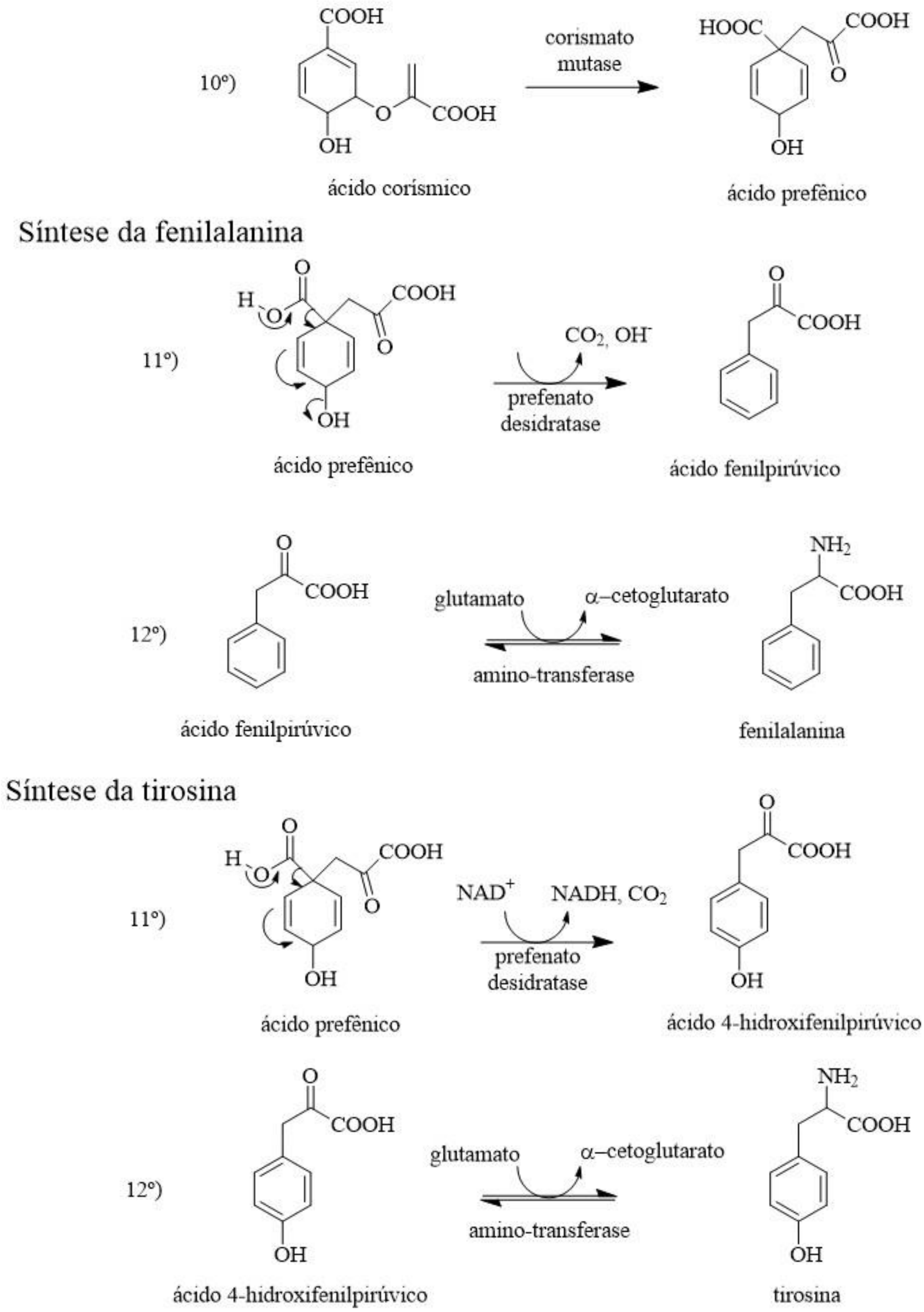
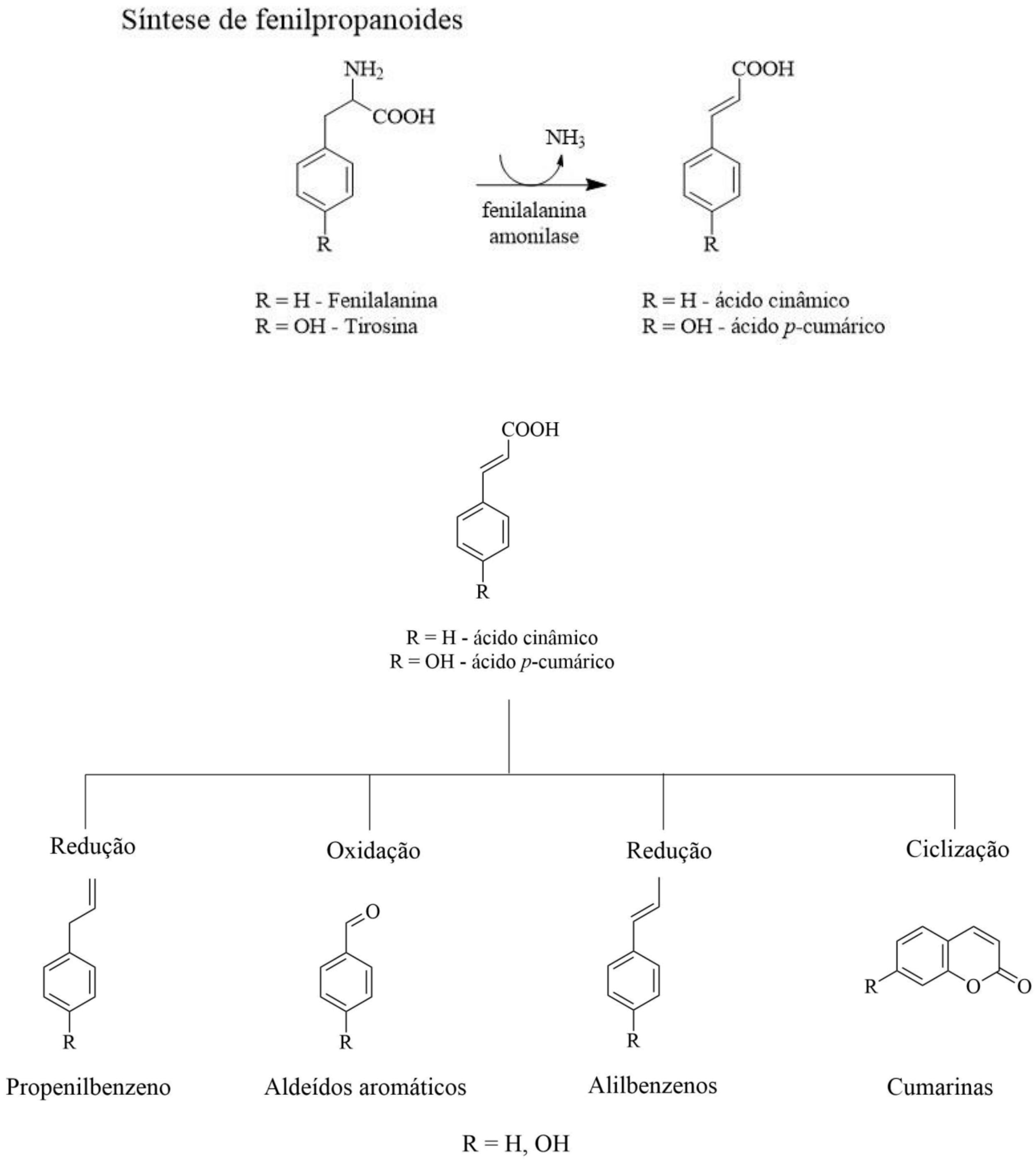


Figura 6 – Biossíntese de fenilpropanoides (conclusão).




Fonte: adaptado de Simões et al. (2016).

### 3.3. Potencialidades biológicas de óleos essenciais e seus constituintes

Há séculos os seres humanos conhecem e exploram as mais diversas atividades biológicas dos óleos essenciais e seus constituintes. Estes têm sido amplamente utilizados com finalidade bactericida, antiviral, fungicida, antiparasitária, inseticida, além de outras aplicações medicinais e cosméticas. São de extrema valia para as indústrias farmacêutica, sanitária, cosmética, agrícola e alimentícia (De Sousa et al., 2023; Hou et al., 2022; Jugreet et al., 2020).

Esse grande espectro de atividades biológicas está relacionado, entre outros fatores, à diversidade química dos constituintes dos óleos essenciais. Hidrocarbonetos, como *p*-cimeno, $\alpha$-terpineno, limoneno, farneseno e mirceno, apresentam atividade antibiótica, antiviral, antitumoral, descongestionante e estimulante. Óxidos, como óxidos de bisaboleno e linalool, e 1,8-cineol, estão relacionados à atividade antiinflamatória, expectorante e estimulante. Ésteres, como acetatos de geranila, bornila e linalila, apresentam atividade antiinflamatória, fungicida, antiespasmódica, sedativa e anestésica. Álcoois, como mentol, linalool e $\beta$-citronelol, apresentam potencial anestésico, antimicrobiano, antiinflamatório e antiséptico. Fenóis, como eugenol, carvacrol, timol e chavicol, estimulam o sistema imune, são antimicrobianos, espasmolíticos e anestésicos. Cetonas, como L-(-)-carvona, tujona e pulegona, são mucolíticos, sedativos, analgésicos, digestivos e antivirais. Por fim, aldeídos, como *p*-anisaldeído, (*E*)-cinamaldeido, neral, geranial e citronelal, são antimicrobianos, antipiréticos, antivirais, vasodilatadores e sedativos (Djilani; Dicko, 2012).

São vastos os relatos na literatura de publicações que comprovam as atividades biológicas de óleos essenciais, de terpenos e fenilpropanoides isolados, como pode ser visto a seguir: atividade antioxidante (Ferreira et al., 2023; Teixeira et al., 2022; Yildiz et al., 2021), atividade antifúngica (Brandão et al., 2023; Niu et al., 2022; Scariot et al., 2021), atividade bactericida (Auezova et al., 2020; Guimarães et al., 2019; Nogueira et al., 2021), atividade antiinflamatória (Araruna et al., 2020; Saldanha et al., 2021), atividade inibitória enzimática (Lunguinho et al., 2021; Tavares et al., 2022), atividade inseticida (Caetano et al., 2022; Souza et al., 2022, 2021), entre diversas outras atividades biológicas e publicações.

### 3.3.1. Atividade inseticida de óleos essenciais e seus constituintes

Inúmeras pesquisas têm investigado o potencial inseticida de óleos essenciais e seus constituintes isolados como uma alternativa aos inseticidas sintéticos. O uso de produtos naturais de origem vegetal é normalmente visto como uma opção ambientalmente correta. Como consequência, metabólitos secundários de plantas podem servir como compostos promissores para o desenvolvimento de inseticidas com novos modos de ação (Haddi et al., 2020; Isman, 2020; Prakash et al., 2021).

Caetano et al. (2022) demonstraram que o óleo essencial de *Rosmarinus officinalis* em sua forma livre e nanoencapsulada foram eficientes para promover o controle de *D. suzukii* em testes *in vitro*. Além disso, os autores mostraram que o óleo essencial promoveu significativa inibição da enzima acetilcolinesterase, indicando um provável mecanismo de ação dessa classe de produtos naturais. Por fim, a técnica de nanoencapsulação prolongou o período da atividade inseticida do óleo essencial, o que significa que essa técnica foi eficiente para contornar a alta volatilidade dos óleos. De Souza et al. (2022) estudando os óleos essenciais de *Illicium verum*, *Myristica fragrans* e *Schinus molle* observaram apreciável atividade inseticida sobre *D. suzukii*, com destaque para o óleo de *Illicium verum*, rico em (*E*)-anetol, que apresentou baixíssima concentração letal 50. Os óleos em estudo inibiram a enzima acetilcolinesterase, com destaque para o óleo de *Schinus molle*, rico em hidrocarbonetos mono e sesquiterpênicos. Os autores demonstraram através de análise histológica que o óleo de *Illicium verum* causou desbalanço energético nos insetos, uma vez que as reservas de lipídios e glicogênio foram severamente reduzidas quando expostos a concentrações mais altas do óleo. Tal desbalanço ocorreu pela incapacidade de se obter energia através da dieta ingerida, provavelmente pela inibição de enzimas digestivas.

Outros trabalhos avaliaram a atividade dos óleos essenciais, de seus terpenos e fenilpropanoides majoritários isolados, como é o caso do óleo essencial de *Anethum graveolens*, rico em carvona (30,11%), que apresentou bom desempenho inseticida sobre *D. suzukii* pelo método de fumigação na concentração de 5% e promoveu a repelência dos insetos (Bošković et al., 2023). Anteriormente, Park et al. (2016) avaliaram a atividade inseticida por contato do óleo essencial de *Thymus zygis* e seu componente majoritário carvacrol separadamente sobre machos e fêmeas de *D. suzukii*. Os autores observaram que o carvacrol isolado foi mais tóxico para ambos os sexos. Neste mesmo ano, Kim et al. (2016) demonstraram que o óleo de

*Cinnamomum cassia*, rico em (*E*)-cinamaldeído (81,6%), foi eficiente em promover a mortalidade de *D. suzukii* na exposição por contato na dose de 5 µg.

Trabalhos de Haddi et al. (2015b), com os óleos essenciais de *Syzygium aromaticum* L., rico em eugenol, e *Cinnamomum zeylanicum* L., rico em (*E*)-cinamaldeído, apresentaram atividade inseticida semelhante sobre *Sitophilus zeamais* através da exposição por contato com resíduos secos, sendo as $CL_{95}$ = 3,96 e 3,47 µL/cm$^2$, respectivamente. Doses subletais promoveram efeito estimulante no tempo médio de sobrevivência, alterou sua capacidade de mobilidade e reduziram a taxa de respiração dos insetos. Além disso, o óleo de *S. aromaticum* em dose subletal aumentou o número de larvas de *S. zeamais*. Nesse mesmo sentido, Brito et al. (2021) relataram que o óleo de *Illicium verum*, composto majoritariamente por (*E*)-anetol (77,4%), apresentou $CL_{95}$ igual a 609,75 µL/L de ar em teste de toxicidade por fumigação sobre essa mesma praga. Adicionalmente, Rodríguez et al. (2022) avaliaram a atividade de compostos fenólicos sobre *S. zeamais*, incluindo os terpenos carvacrol e timol, que são isômeros. Os autores concluíram que os compostos destacados apresentaram o melhor desempenho no teste de toxicidade por contato com resíduos secos, carvacrol ($CL_{50}$ = 221 µmol/cm$^2$) e timol ($CL_{50}$ = 196 µmol/cm$^2$). Ambos os compostos inibiram a enzima acetilcolinesterase, com valores de $CI_{50}$ de 0,019 e 0,96 mM, para o carvacrol e timol, respectivamente. Além disso, o timol causou a maior repelência do gorgulho, 63% a 40 µM. Recentemente, Moutassem et al. (2024) demonstraram que o óleo essencial de *Thymus pallescens*, rico em carvacrol (56,6%), apresentou $CL_{50}$ igual a 17,7 µL/mL em teste de toxicidade por contato e 15 µL/L de ar em teste de toxicidade por fumigação sobre adultos de *S. zeamais*. Além disso, os autores relataram que esse óleo essencial reduziu o conteúdo de proteínas e carboidratos nos insetos e promoveu elevação do teor de lipídeos.

Embora promissores, esses constituintes presentes nos óleos essenciais apresentam baixa estabilidade frente a oxidação, baixa solubilidade em água e alta volatilidade, dificultando aplicações em campo (Mossa, 2016; Pavela; Benelli, 2016). A nanotecnologia é uma ferramenta que ao ser utilizada junto aos terpenos e fenilpropanoides permite superar as limitações impostas por suas propriedades físico-químicas (Giunti et al., 2023), principalmente o uso de nanopartículas, que são estruturas de tamanho nanométrico que apresentam características distintas dos materiais em escala macroscópica, devido à sua grande área superficial (Lee; Yun; Park, 2015).

## 3.4. Insetos

Os insetos pertencem à classe Insecta, subordinada à superclasse Hexapoda, que é caracterizada por indivíduos em fase adulta com corpo dividido em três principais regiões (cabeça, tórax e abdômen) e seis pernas. Dentro da classe Insecta, há a subdivisão em ordens representadas por linhagens que divergem entre si, sendo conhecidas por um conjunto de características bem distintas que permitem, quase sempre, fácil identificação. Como exemplos de ordens, têm-se: Coleoptera (besouros), Diptera (moscas), Lepidoptera (borboletas e mariposas), entre outras. Essa classe compreende muitas espécies, sendo a maioria ainda não identificada (Brown, 2001; Eggleton, 2020; Resh; Cardé, 2009).

Os insetos vivem em ambientes variados, aquáticos ou terrestres, sobre e sob o solo, durante a totalidade de sua vida ou parte dela. O ciclo de vida dos insetos permite a sua sobrevivência em uma grande variedade de condições, com extremos de frio ou calor, em períodos úmidos ou secos, ou ainda, em climas muito variáveis em curtos intervalos de tempo. Atuam em variados ecossistemas promovendo a reciclagem de nutrientes por meio da degradação de materiais vegetais ou de origem animal; promovem a propagação de plantas, incluindo a polinização e dispersão de sementes; servem de alimento para espécies de vertebrados, como aves, mamíferos, répteis e anfíbios (Gullan; Cranston, 2014).

Esses seres vivos são de extrema importância em função da sua grande diversidade, pelo papel ecológico que desempenham, pela influência que exercem na agricultura e por serem vetores de transmissão de doenças aos seres humanos e outras espécies (Scudder, 2009).

### 3.4.1. Insetos-praga

Uma população de inseto pode receber o *status* de praga em função da abundância de indivíduos e os efeitos negativos que causam. Normalmente, esses efeitos decorrem da prática alimentar dos insetos sobre a fisiologia do hospedeiro, que podem ser plantas, animais ou humanos. O nível de dano varia de acordo com a espécie praga e com o hospedeiro. Por exemplo, moscas da fruta, mesmo em baixos índices populacionais, promovem danos estéticos que comprometem a comercialização do produto. Já gafanhotos causam danos em pastagens quando atingem altas densidades populacionais (Gullan; Cranston, 2014).

Especificamente em plantas, os danos podem ser variados e atingir todos os órgãos vegetais. Serão considerados danos diretos quando a parte comprometida é a de interesse comercial e, indireto, quando a estrutura atingida não possuir valor econômico, mas ser importante para a fisiologia vegetal, comprometendo a sua produtividade. Além disso, os insetos podem atuar indiretamente ao transmitir vírus, bactérias, fungos e outros patógenos, ou injetar substâncias toxicogênicas durante o processo alimentar (Gallo et al., 2002; Sharma; Kooner; Arora, 2017).

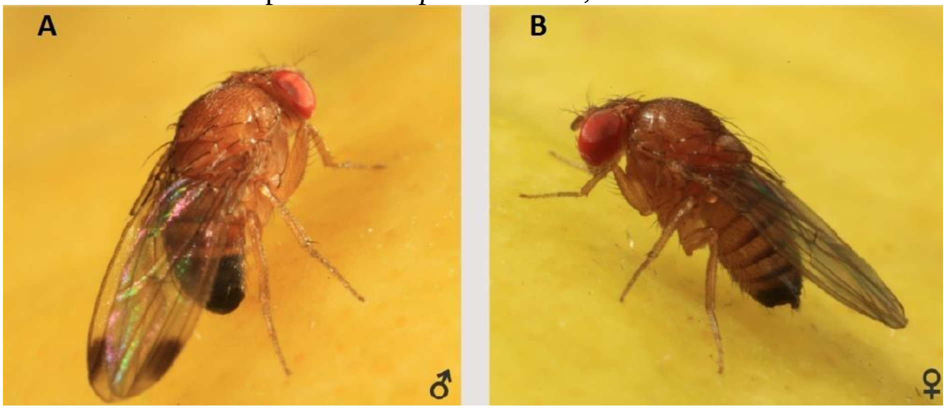
Muitos fatores colaboram para que insetos se tornem praga. Podem-se destacar a inserção de espécies exóticas em ambientes que não possuem predadores naturais específicos. Adicionalmente, um inseto pode ser inofensivo até se tornar o vetor de doenças, ou ainda, insetos nativos podem se tornar praga para plantas exóticas inseridas em seu habitat. Não menos importante, a prática da monocultura favorece a proliferação de insetos especialistas ou espécies generalistas em razão do grande aglomerado de recursos alimentares e, por fim, o uso indiscriminado de inseticidas favorece a seleção de indivíduos resistentes (Gullan; Cranston, 2014).

#### 3.4.1.1. *Drosophila suzukii*

*Drosophila suzukii*, (Figura 7), é uma espécie de mosca, (Diptera: Drosophilidae), que apresenta grande potencial para causar danos econômicos, pois as fêmeas possuem ovipositor serrilhado que lhes permitem infestar frutos saudáveis e não danificados antes da colheita, apresentando grande variedade de hospedeiros, como amoras, mirtilos, cerejas, uvas, morangos, framboesas e espécies selvagens, além de frutas apodrecidas caídas ao solo (Lee et al., 2011).

Nativa do leste asiático, os primeiros relatos de ocorrência no Brasil, dessa praga em potencial, remontam à Região Sul do país, na cidade de Nova Veneza em fevereiro de 2013, seguida por Erechim em março, Botuverá em abril, Vila Maria e Osório em maio. É impossível determinar com precisão por onde e quando essa espécie foi inserida em território nacional, mas dados de coletas anteriores sugerem que a entrada se deu pelos estados do Sul durante o verão de 2012/2013 (Deprá et al., 2014). Em Minas Gerais os primeiros relatos de ocorrência desse inseto remontam a março de 2016 (Andreazza et al., 2016a).

**Figura 7 -** Adultos da espécie *Drosophila suzukii*, em A macho e B fêmea.




Fonte: Andreazza et al. (2016b).

Os indivíduos adultos medem de dois a três milímetros, possuem olhos vermelhos, tórax e abdômen de coloração marrom pálido. Os machos, (Figura 7A), se diferem das fêmeas, (Figura 7B), pela presença de uma mancha escura próxima à borda de cada asa. A fêmea põe de um a três ovos por local de postura, chegando até a, aproximadamente, 380 ovos durante sua vida completa. Os ovos são translúcidos, de coloração branco-leitosa e brilhante; à medida que a larva se desenvolve, o aspecto leitoso se extingue. As larvas também são branco-leitosas, com corpo cilíndrico e aparelho bucal de coloração preta, desenvolvem-se dentro da fruta hospedeira, apresentando três instares. O processo alimentar da larva cria áreas na polpa da fruta de aspecto repugnante, inviabilizando a sua comercialização. Completado o desenvolvimento larval, formam-se as pupas, que inicialmente são amarelo-acinzentadas, posteriormente ficam castanhas e, por fim, amareladas e enrijecidas (Walsh et al., 2011).

Estudos apontam que as perdas econômicas podem superar US$ 2,35 milhões por ano em pequenas regiões produtoras (DiGiacomo et al., 2021), ou valores superiores a US$ 420 milhões em grandes áreas produtoras de frutas vermelhas (Bolda; Goodhue; Zalom, 2010). O controle dessa espécie é um desafio contínuo, realizado principalmente por métodos químicos (Van Timmeren; Isaacs, 2013), controle biológico (Wang et al., 2020), controle das condições de cultivo (Tochen et al., 2016), uso de plantas melhoradas geneticamente (Murphy et al., 2016) e pelo uso de produtos naturais (Caetano et al., 2022; De Souza et al., 2022, 2024; Keesey et al., 2019).

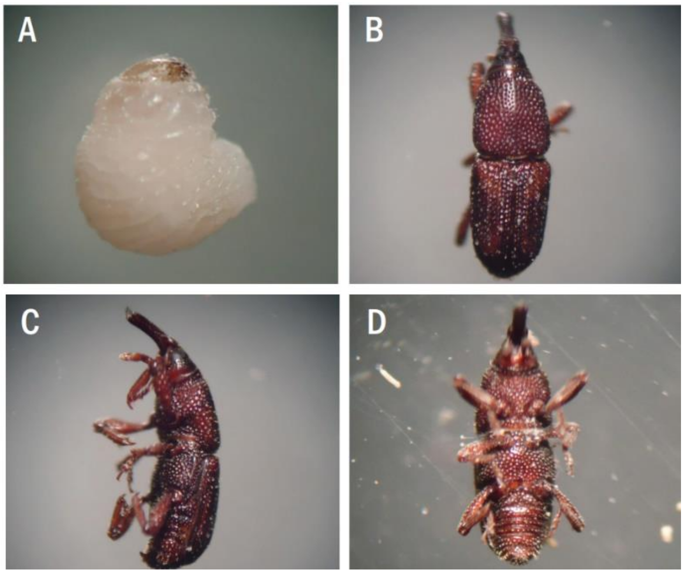
### 3.4.1.2. *Sitophilus zeamais*

*Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), Figura 8, popularmente conhecido como gorgulho-do-milho, é uma das principais espécies primárias de inseto-praga que se desenvolvem em milho armazenado (Napoleão et al., 2015). Além do milho, esta peste se desenvolve em outros cereais como arroz, trigo e sorgo e em produtos industrializados como biscoitos e macarrão (Athanassiou; Kavallieratos; Campbell, 2017; Milosavljević et al., 2024). Esta espécie apresenta distribuição global, entretanto gera grande preocupação nas regiões tropicais, subtropicais e neotropicais do planeta (Banga et al., 2020; Corrêa et al., 2013). A ação dessa praga compromete a qualidade nutricional do grão e a eficiência de germinação de sementes, pois as larvas se alimentam das reservas de amido e do gérmen do milho (De Araújo et al., 2017; Napoleão et al., 2015). Adicionalmente, a ação de *S. zeamais* abre caminho para a ação de pragas secundárias, como *Tribolium castaneum* (Coleoptera: Tenebrionidae), e microorganismos patogênicos (Yun et al., 2018).

Os adultos são gorgulhos que medem entre 2,0 mm e 3,5 mm de comprimento, apresentando coloração castanho-escura com manchas mais claras nos élitros, perceptíveis logo após a emergência. Possuem a cabeça projetada para frente em forma de rostro curvado, sendo mais curto e robusto nos machos e mais longo e fino nas fêmeas. As larvas têm coloração amarelo-clara, com a cabeça marrom-escura, enquanto as pupas apresentam coloração branca. O período de oviposição pode durar 104 dias, com uma média de 282 ovos por fêmea. A longevidade das fêmeas é de aproximandamente 140 dias. O período de incubação varia de 3 a 6 dias, e o ciclo completo, do ovo até a emergência dos adultos, é em torno de 34 dias (Lorini et al., 2015).

O controle desse inseto-praga é feito principalmente pela utilização de inseticidas químicos sintéticos pelo potencial que esses compostos têm de dizimar grandes populações rapidamente (Haddi et al., 2015a; Moutassem et al., 2024). Entretanto, poucos compostos aprovados para o manejo dessa espécie, aliado ao uso indiscriminado e não racional, contribuíram para o desenvolvimento de resistência em *S. zeamais* (Correa et al., 2014; Haddi et al., 2015b). Há relatos de resistência desse gorgulho a piretróides, indoxacarb, malation, pirimifós metílico, fenitrotiona e fosfina (Corrêa et al., 2011; Haddi et al., 2015a; Machuca-Mesa; Turchen; Guedes, 2024; Pereira et al., 2009).

**Figura 8 -** *Sitophilus zeamais*, em A larva, em B adulto dorsal, em C adulto lateral e em D adulto ventral.




Fonte: Lorini et al. (2015).

### 3.4.2. Insetos inimigos naturais

São considerados inimigos naturais os insetos que promovem a predação ou parasitam outros insetos, particularmente insetos-praga de plantas. Por meio desse tipo de alimentação, os inimigos naturais contribuem para um tipo de regulação de pragas conhecido como controle biológico natural. Inimigos naturais podem promover cerca de 33% do controle natural de pragas em sistemas de cultivo. Estes insetos benéficos podem suprimir ou atrasar o crescimento populacional de pragas, contribuindo para a mortalidade de espécies que são mais vulneráveis. Quando populações diversas de inimigos naturais estão presentes, o controle de pragas se torna mais eficaz devido a fenologias diferentes (Getanjaly; Sharma; Kushwaha, 2015).

Predadores são animais que devem consumir mais de um indivíduo de outra espécie para completar seu ciclo de vida. A partir dessa definição, o hábito predatório ocorre em uma ampla gama de grupos de insetos. Algumas ordens, como Odonata e Neuroptera, são totalmente ou predominantemente predadoras. Outras ordens contêm grande número de espécies que são predadoras, como Hemiptera, Coleoptera, Mecoptera, Diptera e Hymenoptera. Até mesmo algumas espécies nas ordens Ephemeroptera, Orthoptera, Plecoptera, Thysanoptera, Trichoptera e Lepidoptera. A predação por insetos pode ocorrer por meio de várias estratégias, destacando-se a caça, perseguição e emboscada, captura, provisionamento, alimentação do hospedeiro, forrageamento em massa, entre outros (Hajek; Eilenberg, 2018; Souza et al., 2019).

Os insetos parasitoides possuem um ou mais estágios larvais que parasitam outros artrópodes, desenvolvendo-se em seu interior promovendo a morte do hospedeiro antes de completar seu ciclo de vida. Provavelmente existem milhões de espécies de parasitoides, distribuídas em diversas ordens, como Neuroptera, Lepidoptera, Coleoptera, Diptera e Hymenoptera. Neste último grupo, é estimado a existência de cerca de 250 mil espécies, muitas das quais ainda não foram descritas. O comportamento dos parasitoides varia em aspectos como a forma de localizar o hospedeiro, o estágio de vida parasitado, o ciclo de vida e outros fatores, o que os torna altamente adaptados e preparados para colonizar quase todos os ambientes. Esses organismos desempenham um papel crucial na regulação biológica de numerosos insetos herbívoros, muitos deles de grande relevância econômica para a agricultura, pecuária e silvicultura (Hajek; Eilenberg, 2018; Silveira et al., 2019).

#### 3.4.2.1. *Doru luteipes*

*Doru luteipes* (Dermaptera: Forficulidae), (Figura 9), é um inseto predador onívoro popularmente chamado de tesourinha. Os insetos adultos medem entre 8 e 15 mm, corpo delgado e alongado com pinças no final do abdome, coloração marrom avermelhada, antenas finas, com ou sem asas, quando presentes, as asas externas são curtas e endurecidas, de coloração amarelo-alaranjada, que não cobrem o abdome. Os ovos apresentam formato ovalado, de coloração branco-amarelada, as posturas são feitas em grupos, em locais úmidos e protegidos, como entre as folhas, no cartucho ou entre as palhas da espiga do milho e no solo. As ninfas são semelhantes aos adultos, porém menores e sem asas. Os adultos e ninfas são importantes predadores de diversas pragas agrícolas (Michereff Filho et al., 2019). Essa característica torna esse inseto um potencial agente para uso em práticas de controle biológico (Haas, 2018; Silva et al., 2023).

Esta espécie possui grande capacidade predatória, alimentando-se de potenciais insetos-praga de diversas ordens como Lepidoptera, Hemiptera e Thysanoptera. A tesourinha alimenta-se de pólen quando há baixa disponibilidade de presas. Além disso, seu hábito tigmotático permite que tenha acesso a insetos de hábito críptico, que muitas vezes são difíceis de manejar com métodos tradicionais de controle (Rodrigues-Silva et al., 2024; Silva et al., 2021, 2023).

**Figura 9 -** Adulto da espécie *Doru luteipes*.

Fonte: Michereff Filho et al. (2019).

O uso indiscriminado de pesticidas sintéticos afeta *D. luteipes*. Estudos tem demonstrado que esses compostos afetam a sobrevivência e capacidade de predação desse inseto. A mistura dos compostos imidacloprida e β-ciflutrina foi altamente tóxica, promovendo mortalidade total da amostragem do predador, pela via de exposição por contato ou ingestão para ninfas e adultos. A metaflumizona causou mortalidade maior que 95% e 45% de ninfas e adultos, respectivamente. Adicionalmente, a metaflumizona reduziu o consumo de ovos pelas ninfas e a capacidade de locomoção de adultos de *D. luteipes* (Moreira et al., 2023).

#### 3.4.2.2. *Palmistichus elaeisis*

*Palmistichus elaeisis* Delvare & LaSalle, 1993 (Hymenoptera: Eulophidae), Figura 10, é um inimigo natural promissor. É um parasitoide de pupas de diversas espécies de lagartas desfolhadoras. É uma espécie de endoparasitoide gregário e generalista com importante papel no controle de insetos no setor florestal (Da Silva Camilo et al., 2016). As fêmeas de *P. elaeisis* depositam ovos em pupas do hospedeiro e, após a emergência, suas larvas parasitas se alimentam de órgãos e tecidos do hospedeiro. É nativo da região Neotropical e possui hábitos polífagos, alimentando-se de lepidópteros de importância econômica, como *Anticarsia gemmatalis*, *Diaphania hyalinata*, *Psorocampa denticulata*, *Spodoptera frugiperda*, *Thyrinteina arnobia*, entre outros (Rolim et al., 2020).

Relatos de Cruz et al., (2017) demonstram que pesticidas sintéticos como o malathion promovem a mortalidade de fêmeas adultas de *P. elaeisis* antes que elas possam parasitar seus hospedeiros e o diflubenzuron reduz a proporção sexual a partir da segunda geração. Posteriormente, Pereira Costa et al., (2020) citam que o inseticida deltametrina afeta consideravelmente o parasitismo e emergência desse inimigo natural, o que impede a combinação dessas duas formas de controle no MIP.

**Figura 10 -** Fêmea adulta de *Palmistichus elaeisis* parasitando pupa de *Tenebrio molitor*.

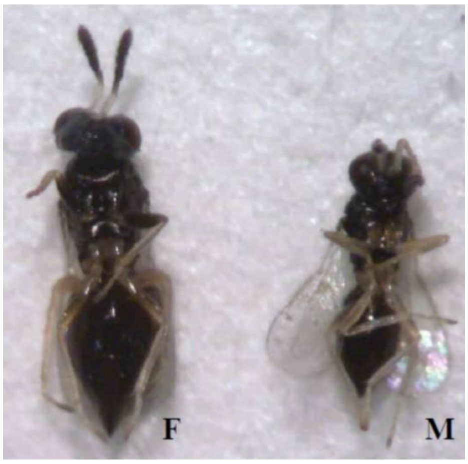
Fonte: Silveira et al. (2019).

### 3.4.2.3. *Tetrastichus howardi*

*Tetrastichus howardi* (Olliff, 1893) (Hymenoptera: Eulophidae), (Figura 11) é um endoparasitoide com elevado potencial para ser empregado no controle biológico de lepidópteros-praga. Esta espécie é polifaga, parasita de pragas agrícolas e florestais, apresenta comportamento gregário, apresenta alto número de indivíduos por geração, se desenvolve em diferentes temperaturas e facilidade de dispersão. Tais características favorecem a utilização deste inimigo natural em programas de manejo integrado de pragas.

Estudos de Su et al. (2021) demonstraram que inseticidas sintéticos têm afetado severamente a sobrevivência e parâmetros biológicos desse inimigo natural. O benzoato de emamectina apresentou elevada toxicidade para adultos de *T. howardi*, $CL_{50}$ igual a 0,09 mg/L após 24 horas de exposição. Adicionalmente, Bermúdez et al. (2023) indicaram que bifentrina e tiametoxam afetam severamente a sobrevivência desse parasitoide e seus resíduos continuam atuando sobre o inseto mesmo após 96 horas de exposição. Além disso, estes compostos impediram que as fêmeas conseguissem parasitar seus hospedeiros.

**Figura 11 -** Adultos de *Tetrastichus howardi*, fêmea (F) e macho (M).




Fonte: Barbosa et al. (2015).

### 3.5. Nanomateriais e nanotecnologia

O termo nanotecnologia refere-se ao conjunto de técnicas envolvidas na fabricação, processamento, imagem, medição e aplicação de materiais que se enquadram na faixa de tamanho de até 100 nm. (Ashby; Ferreira; Schodek, 2009a; El-Kady et al., 2023).

Quando a matéria é transformada até atingir a nanoescala apresenta alterações em suas propriedades quando comparada a porções massivas. De modo geral, os nanomateriais demonstram comportamentos físicos alterados que oferecem propriedades mecânicas, termodinâmicas, elétricas, magnéticas e ópticas favoráveis para inúmeras aplicações. No entanto, a implementação de nanomateriais em cada situação requer uma compreensão detalhada das propriedades químicas e físicas dos materiais de base, parâmetros de controle e métodos de produção (Govindaraman et al., 2022; Stander; Theodore, 2011).

A forma utilizada para classificar os nanomateriais é baseada em suas, dimensões. Nanomateriais de dimensão zero, ou 0-D, são aqueles em que todas as dimensões são inferiores a 100 nanômetros, como exemplo pode-se citar os quantum dots. Já os nanomateriais 1-D apresentam uma dimensão que está fora da nanoescala, tal diferença origina materiais em forma de agulha. Como exemplo de nanomateriais 1-D tem-se os nanotubos, nanobastões e nanofibras. Nanomateriais bidimensionais, ou 2-D, são aqueles que apresentam duas dimensões fora da nanoescala, por isso exibem forma semelhante a placas, como exemplos têm-se os filmes de grafeno (Ashby; Ferreira; Schodek, 2009b).

Os nanomateriais podem ser obtidos a partir de diversos materiais. Os materiais metálicos podem ser formados por um único elemento ou por ligas metálicas, os nanomateriais mais comuns são feitos de ouro, prata e cobre. Os materiais poliméricos são macromoléculas formadas por monômeros que normalmente são compostos orgânicos. Materiais compósitos são formados por dois ou mais materiais com propriedades distintas, que agem sinergicamente para criar propriedades que não podem ser alcançadas isoladamente por cada material (Ashby; Ferreira; Schodek, 2009c).

Nanomateriais têm sido amplamente aplicados em diversos setores, como nas indústrias de alimentos, cosméticos, medicina, agricultura e eletrônicos. Essas partículas desempenham um papel importante na solubilização, carreamento, liberação controlada e proteção de princípios ativos contra condições ambientais adversas, como oxidação, variações de pH e hidrólise. Adicionalmente, são utilizadas para alcançar alvos específicos, otimizando os efeitos de permeabilidade e retenção do princípio ativo, que podem ser ajustados conforme necessário (Bissessur, 2020; Gajanan; Tijare, 2018).

### 3.5.1. Nanopartículas poliméricas

Nanopartícula polimérica é um termo que nomeia qualquer tipo de nanopartícula feita a base de polímero, entretanto, os tipos mais comuns são as nanoesferas e nanocápsulas. As nanoesferas são partículas de matriz, ou seja, partículas cuja massa inteira é sólida e outras substâncias podem estar adsorvidas na superfície ou estar dentro da partícula. Em geral, são esféricas, mas podem apresentar formato irregular. As nanocápsulas são sistemas vesiculares, atuando como uma espécie de reservatório, onde as substâncias de interesse são confinadas em uma cavidade constituída por um núcleo líquido, composto por óleo ou água, envolto por uma membrana de material sólido polimérico (Couvreur; Dubernet; Puisieux, 1995; Khan et al., 2023; Vauthier; Couvreur, 2000).

As nanopartículas poliméricas podem ser preparadas a partir de polímeros prontos ou por polimerização direta de monômeros. Métodos como evaporação de solvente, *salting-out*, diálise e tecnologia de fluido supercrítico são utilizados para a preparação das nanopartículas a partir de polímeros prontos. Os métodos para síntese a partir da polimerização de monômeros engloba as técnicas de microemulsão, miniemulsão, emulsão livre de surfactante e polimerização interfacial. A escolha do método de preparação é feita com base em vários fatores, como o tipo de sistema polimérico, área de aplicação, requisitos de tamanho, entre outros (Rao; Geckeler, 2011).

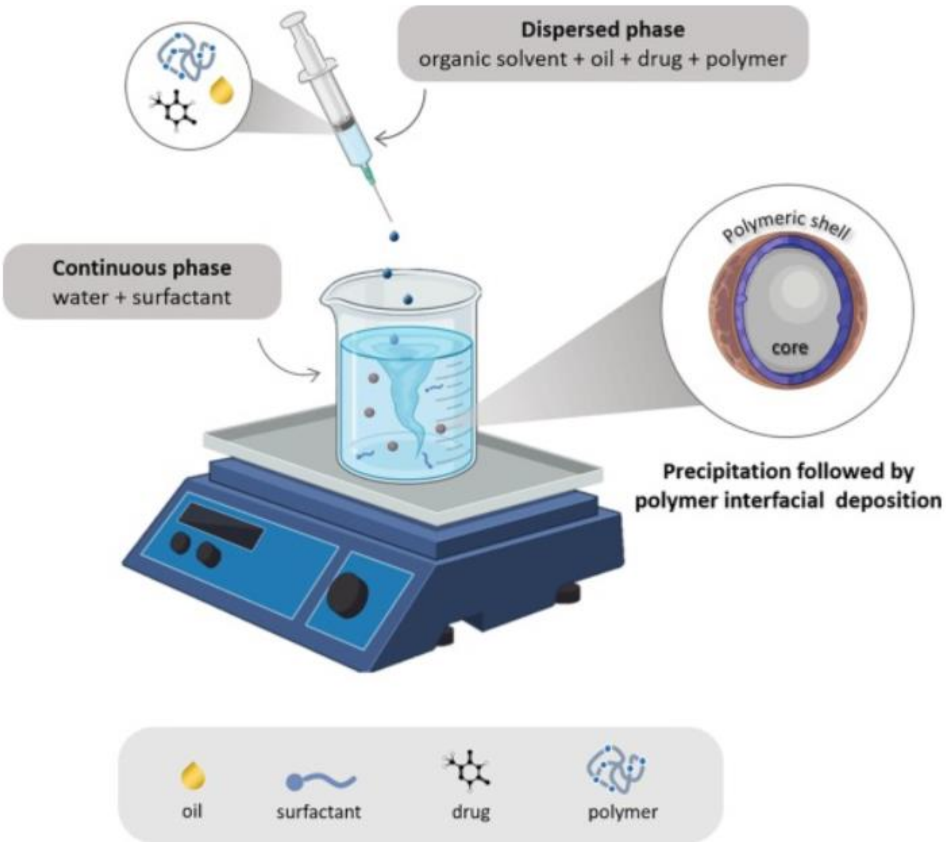
A síntese de novas nanopartículas poliméricas tem recebido atenção crescente devido ao grande número de áreas onde podem ser empregadas, como o desenvolvimento de novos pesticidas, controle de poluição, carreamento de fármacos e desenvolvimento de biosensores (Khan et al., 2023).

#### 3.5.1.1. Síntese de nanopartículas poliméricas pela técnica de emulsão-evaporação de solvente

As nanopartículas poliméricas podem ser preparadas por diferentes métodos, incluindo técnicas físicas, químicas ou biológicas. A escolha do método mais adequado deve considerar as propriedades físico-químicas do polímero e do princípio ativo que se deseja incorporar à matriz polimérica. É importante empregar técnicas que não comprometa ou inative o princípio ativo, pois alguns métodos utilizam solventes orgânicos, ultrassonicação, altas temperaturas e agitação, que podem alterar as propriedades dos compostos químicos de interesse (Castro; Costa; Campos, 2022; Elmowafy et al., 2023).

Entre os métodos disponíveis para a produção de nanopartículas poliméricas, destaca-se o método de nanoprecipitação, (Figura 12), esta técnica é conhecida como emulsão-evaporação do solvente. Neste método há a preparação de duas fases: a fase orgânica, composta pelo polímero e pelo substrato de interesse, e a fase aquosa, formada por água e um agente estabilizante, como compostos surfactantes. A fase orgânica é adicionada gradualmente à fase aquosa, normalmente com o auxílio de uma bomba injetora, resultando na formação de partículas coloidais após a evaporação do solvente orgânico. Diversos parâmetros podem alterar as propriedades físico-químicas das nanopartículas produzidas, como tamanho de partícula, potencial zeta e morfologia. Estes parâmetros incluem a técnica utilizada para evaporar o solvente, a taxa de injeção da fase orgânica na fase aquosa, a natureza e a concentração do agente estabilizante, a concentração do polímero, a velocidade de agitação e o volume da fase aquosa (Iván Martínez-Muñoz; Elizabeth Mora-Huertas, 2022; Lima et al., 2022).

**Figura 12 –** Esquema de síntese de nanopartículas pelo método de nanoprecipitação.



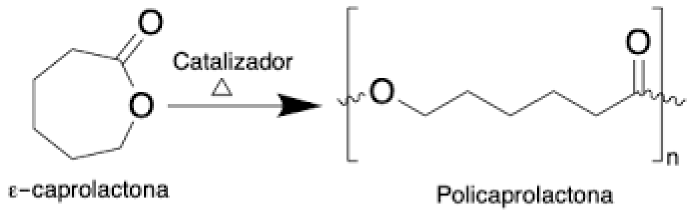

Fonte: Lima et al. (2022).

Esta técnica apresenta diversas vantagens para a preparação de nanopartículas, das quais destacam-se a alta reprodutibilidade em escala nanométrica e a possibilidade de utilização de polímeros biodegradáveis e biocompatíveis, como a poli($\varepsilon$-caprolactona) (PCL) e quitosana. Adicionalmente, este método possibilita o uso de princípios ativos e estabilizantes de baixa toxicidade, como produtos naturais, minimizando os impactos ambientais e toxicológicos causados por agentes nocivos. eficiência do processo, que requer baixos volumes de água, energia e tempo, aliado à simplicidade das etapas experimentais (Barreras-Urbina et al., 2016; Martínez Rivas et al., 2017).

#### 3.5.1.1.1. Nanopartículas de policaprolactona

A policaprolactona (PCL) é um copolímero à base de poliéster alifático que tem potencial para diversas aplicações, como produtos biomédicos e sistemas de liberação controlada de princípios ativos. A polimerização por abertura de anel do monômero de caprolactona com o catalisador é o processo industrial mais comum para síntese de PCL, o esquema dessa reação é apresentado na Figura 13. Este método resulta em polímero com alto peso molecular e polidispersão estreita em comparação à policondensação (Pawar et al., 2023).

**Figura 13 –** Estrutura química da policaprolactona.




Fonte: do autor (2025).

Este polímero e outros poliésteres biodegradáveis são opções muito utilizadas em sistemas de liberação controlada de substâncias, pois a degradação do polímero por meio de processos erosivos permite a liberação dos compostos químicos de interesse (Makadia; Siegel, 2011). Esse material é biodegradável, sendo metabolizado por diversos fungos, bactérias e algas (Leja; Lewandowicz, 2010), e, além disso, apresenta toxicidade irrelevante e baixo custo quando comparado a outros materiais (Cesari et al., 2020).

Estudos demonstram que a incorporação de produtos naturais em nanopartículas de PCL prolongam o efeito inseticida, repelente e tendem a reduzir a concentração de princípio ativo necessário para promover o controle de insetos-praga (Ahsaei; Talebi-Jahromi; Amoabediny, 2022; Caetano et al., 2022; Khoobdel; Ahsaei; Farzaneh, 2017; López et al., 2021; Pardini et al., 2021).

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# SEGUNDA PARTE - ARTIGOS

**ARTIGO I**

**Article published in Industrial Crops and Products: “Terpenes and phenylpropanoids for the control of *Drosophila suzukii* (Diptera: Drosophilidae): Toxicity, oxidative stress, histopathology, and selectivity”.**

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## Highlights

- Five terpenes and four phenylpropanoids were tested on *D. suzukii* adults and larvae.
- Carvone, carvacrol, (*E*)-anethole and (*E*)-cinnamaldehyde were the most toxic.
- Terpenes and phenylpropanoids negatively affected SOD, CAT, and GST activities.
- Terpenes and phenylpropanoids induced histological and histochemical alterations.

## Abstract

Plants' secondary metabolites, in particular terpenes and phenylpropanoids, represent a good source of bioactive molecules for developing biopesticides as an alternative to synthetic pesticides. The great diversity of these classes of biomolecules has not been fully investigated. This work aimed to evaluate the toxicity of five terpenes and four phenylpropanoids to adults, 3rd instar larvae, and pupae of the Spotted wing drosophila, *Drosophila suzukii*; to evaluate the effect of sublethal exposure to $LC_{50}$ concentrations of the four most toxic compounds on oxidative stress enzymes and on histological structures of the adult flies; and to evaluate the potential side effects of these compounds on a non-target organism, the earwig *Doru luteipes*. The results demonstrate that the terpenes L-(-)-carvone and carvacrol and the phenylpropanoids (*E*)-anethole and (*E*)-cinnamaldehyde, with respective $LC_{50s}$ of 3.13, 4.47, 4.22 and 4.60 mM, presented the highest toxicities to *D. suzukii* adults. These compounds promoted considerable mortality of pupae and deformation in adults. The $LC_{50}$s of L-(-)-carvone and carvacrol, (*E*)-anethole, and (*E*)-cinnamaldehyde also affected the activity of the detoxifying enzymes SOD, CAT, and GST mostly 4 hours after exposure. Histological and histochemical analyses revealed that exposure to these compounds promoted changes in the thickness of the exoskeleton, midgut, and hindgut. Furthermore, the area and density of lipid droplets in the fat body and carbohydrate concentration in muscle fibers and the fat body decreased in treated groups. The terpene carvacrol promoted the most severe histological changes. Finally, the survival and feeding capacity of the non-target organism *D. luteipes* were not significantly affected by exposure to the selected terpenes and phenylpropanoids making them promising options for controlling *D. suzukii*.

**Keywords:** Natural products; Spotted wing fly; Oxidative stress; Morphological changes; Eco-friendly.

## 1. Introduction

*Drosophila suzukii* (Diptera: Drosophilidae), commonly called the spotted wing fly (SWD), was initially described in Japan. This fly is dispersed globally, being reported in Europe (Calabria et al., 2012), and North and South America (Deprá et al., 2014). In Brazil, this pest insect was reported for the first time in the southern region in 2013 (Deprá et al., 2014) before spreading to other regions (Andreazza et al., 2016b). The *D. suzukii* adults differ from other species of the same genus by two main characteristics, the presence of dark spots on the edge of the wings of males and serrated ovipositor in females (Cini et al., 2012, Walsh et al., 2011). The serrated ovipositor increases the damage caused by SWD by allowing the infestation of healthy and immature fruits (Hamby et al., 2016, Karageorgi et al., 2017). This species has a great diversity of hosts, including stone fruits (peaches, cherries, and plums), thin-skinned berries (blueberries and strawberries), and a great diversity of wild fruits (Bellamy et al., 2013, Hamby et al., 2016, Lee et al., 2011). In addition to the damage caused by the larvae of this insect, the opening made by the serrated ovipositor allows contamination by a wide range of pathogens (Hamby et al., 2012, Ioriatti et al., 2015).

To reduce the economic damage caused by *D. suzukii*, the main control strategy is based on the use of synthetic insecticides (Beers et al., 2011, Van Timmeren and Isaacs, 2013, Wise et al., 2015). Several chemical compounds from the classes of spinosyns, neonicotinoids, organophosphates, pyrethroids, and diamides are used, which have different results for the different development stages of *D. suzukii* (Bruck et al., 2011, Wiman et al., 2016). In Brazil, only one spinosyn compound (Espinetoram) is registered for use in blackberry, blueberry, raspberry, and grape cultivation (Garcia et al., 2022). The abusive and persistent use of synthetic insecticides can lead to the selection of individuals resistant to such compounds (Karunaratne et al., 2018, Sparks and Nauen, 2015). Many studies have already demonstrated the propensity for resistance to synthetic insecticides of *D. suzukii* (Deans and Hutchison, 2022, Ganjisaffar et al., 2022, Gress and Zalom, 2019).

In addition to the chemical-based form of control, alternative management can be carried out through the use of biological control (Wang et al., 2020), as well as the use of natural products (Keesey et al., 2019) including the use of plants derived pesticides due to their perceived beneficial attributes (Haddi et al., 2020). However, although many studies present promising results for the use of essential oils to control various insect pests, including *D. suzukii* (Caetano et al., 2022, de Souza et al., 2022, Pan et al., 2022, Pineda et al., 2023), the number of plant-based commercial products is still extremely low (Isman, 2020a, Isman, 2015, Isman and Grieneisen, 2014, Pavela and Benelli, 2016). The challenges to the commercial use of these products include some legislation barriers (Isman, 2005) since extensive toxicological studies are normally required before the botanical product's approval (Pavela and Benelli, 2016). Furthermore, the variation in the chemical composition of essential oils from the same plant species is also one of the main factors that hinder the approval of new products (Turek and Stintzing, 2013). Additionally, the instability of some constituents when exposed to environmental conditions also discourages the use of these natural products (Burt, 2004).

Essential oils are made up of terpenes and phenylpropanoids and the evaluation of these isolated constituents is an alternative to overcome some of the limiting factors for obtaining new biopesticides (de Oliveira et al., 2020). Indeed, the known chemical structures of isolated secondary metabolites can help in the development of commercial formulations in the future. Additionally, phenylpropanoids and terpenes in their isolated forms act satisfactorily against insect pests (Almadiy et al., 2022, Kanda et al., 2017, López and Pascual-Villalobos, 2015) and some studies reported good activity against *D. suzukii* of essential oils and their isolated major constituents. Among these, the essential oils of *Satureja montana*, *Cinnamomum cassia*, *Baccharis* spp and *Mentha x piperita* and their major components carvacrol, (*E*)-cinnamaldehyde, limonene, menthone and (-)-mentol showed promising results (Dam et al., 2019, Souza et al., 2021, Wang et al., 2021). However, the great diversity that these classes of biomolecules present needs to be further explored.

Additionally, some studies have demonstrated that these natural products act selectively, sparing non-target organisms (Santos et al., 2023, Toledo et al., 2020). However, there are no reports of the effects on individuals of the generalist predator species *Doru luteipes* (Scudder, 1876) (Dermaptera: Forficulidae). This species presents omnivorous behavior, feeding on different prey during its different stages of development. This characteristic makes this insect a potential agent for use in biological control practices (Haas, 2018, Silva et al., 2023).

The present study aimed to investigate the lethal effects of four phenylpropanoids (eugenol, (*E*)-anethole, (*E*)-cinnamaldehyde and *p*-anisaldehyde) and five terpenes (carvacrol, L-(-)-carvone, 1,8-cineole, *β*-citronellol and *p*-cymene) on *D. suzukii* adults. Subsequently, the two terpenes and the two phenylpropanoids that presented the highest toxicity to SWD were selected to investigate their lethal and sublethal effects. Furthermore, the impact of these compounds on the activity of enzymes related to oxidative stress were assessed. Moreover, the histopathological alterations produced by the selected terpenes and phenylpropanoids in the muscles, midgut, fat body, and exoskeleton of third-instar larvae were also investigated. Finally, the effects on the survival and feeding capacity of the non-target organism *D. luteipes* after exposure to the $LC_{50}$ and $LC_{90}$ of the selected terpenes and phenylpropanoids were also assessed.

## 2. Materials and methods

### 2.1. Phenylpropanoids and terpenes

(*E*)-anethole (99 % purity, CAS 4180–23–8), carvacrol (98 % purity, CAS 499–75–2), 1,8-cineole (98 % purity, CAS 470–82–6), *β*-citronellol (95 % purity, CAS 106–22–9), and eugenol (99 % purity, CAS 97–53–0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-anisaldehyde (99 % purity, CAS 123–11–5), L-(-)-carvone (99 % purity, CAS 6485–40–1), *p*-cymene (99 % purity, CAS 99–87–6) and (*E*)-cinnamaldehyde (99 % purity, CAS 14371–10–9) were purchased from Acros Organics (Geel, Belgium).

## 2.2. Insects

The insects and larvae of *D. suzukii* used in the bioassays were obtained from a stock colony maintained under controlled conditions (temperature: 24 ± 2 °C; relative humidity: 60 ± 5 %; photoperiod: 12 h:12 h) at the Laboratory of Molecular Entomology and Ecotoxicology of the Entomology Department (DEN) at the Universidade Federal de Lavras (UFLA). Rearing was done in plastic cages using an artificial diet prepared in the laboratory and followed previously established methods (Andreazza et al., 2016a, Emiljanowicz et al., 2014, Mendonca et al., 2019). Adults of the earwig *D. luteipes* used in the selectivity bioassay were obtained from a stock colony maintained under controlled conditions (temperature: 25 ± 2 °C; relative humidity: 60 ± 5 % and photoperiods: 12 L:12D) at the Biological Pest Control Laboratory of the DEN. Rearing was carried out on an artificial diet prepared in the laboratory and followed previously established methods (Cruz, 2009).

## 2.3. Assessment of phenylpropanoids and terpenes toxicity to adults of *Drosophila suzukii*

Concentration-mortality bioassays were conducted to determine the contact and ingestion lethal activity of the nine compounds to *D. suzukii* adult flies. The exposure procedure was based on the IRAC (Insecticide Resistance Action Committee) protocol nº 026 (IRAC, 2011), recommended for bioassays with adults of *Musca domestica* L. (Diptera: Muscidae), with modifications. Briefly, seven to nine serial concentrations of each chemical were used with four replications each. The concentrations of the chemicals varied from 0.1 to 100 $\mu L.mL^{-1}$ for the pilot assay. Then, the final concentrations were adjusted according to the results of the previous step. The concentration of the solvent, dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), was kept constant at 2 % (v/v) for all groups, and a 20 % (w/v) sucrose solution was used to complete the volume of the final solution. Previous studies have shown that this concentration of solvent is not harmful to SWD (de Souza et al., 2022, Pineda et al., 2023). For the bioassay, dental cotton (2 cm) treated with 2.2 mL of the final compound solution was deposited in a glass vial (200 mL). For the negative control, only the solvent (2 % v/v DMSO) was used. For each replication, twenty-five unsexed insects of the same age (5–7 days) were introduced into glass vials, which were closed with foam plugs and kept under laboratory conditions at 24 ± 2 °C, 60 ± 5 % RH, and 12-hour photoperiod. Mortality was assessed after 24 hours and flies that did not move even after a gentle brush stimulation were considered dead.

### 2.4. Assessment of phenylpropanoids and terpenes toxicity to *Drosophila suzukii* larvae and pupae

To assess the toxicity of selected phenylpropanoids and terpenes to *D. suzukii* larvae and pupae, an adaptation was made to the methodology of Pan et al. (2022). Newly formed third-instar larvae of *D. suzukii* were exposed to the lethal concentrations $LC_{50}$s and $LC_{90}$s of the two phenylpropanoids, (*E*)-anethol and (*E*)-cinnamaldehyde, and the two terpenes, L-(-)-carvone and carvacrol, found to be the most toxic compounds to adult flies (see results section). Four groups of 15 individuals were placed in Petri dishes with a diameter of 5 cm. Each Petri dish contained a 5 cm diameter filter paper impregnated with 500 µL of solution for each treatment. The insects were exposed to the same impregnated filter paper during the whole duration of the experiment. The tests were randomized, with four repetitions (n = 60). The Petri dishes were maintained in the laboratory at 24 ± 2 °C, 60 ± 5 % RH, and 12 h photoperiod. Larval mortality was assessed every 24 hours until pupae formation. Subsequently, pupal mortality was determined by counting the formed pupae that did not emerge as adults after ten days. Individuals who presented macroscopic morphological changes were considered deformed adults.

### 2.5. Assessment of larval enzymatic responses after exposure to phenylpropanoids and terpenes

For enzymatic analyses, newly formed third-instar larvae of *D. suzukii* were exposed to the $LC_{50}$ of the two phenylpropanoids (*E*)-anethole and (*E*)-cinnamaldehyde, and the two terpenes L-(-)-carvone and carvacrol as well as to the solvent control (2 % v/v DMSO). All enzymatic analyses were performed in five independent replicates containing 40 larvae, totaling 200 individuals for each treatment. Fifteen larvae were collected from each replicate after 4 hours and 24 hours of exposure. Each replicate was homogenized with 400 µL of phosphate buffer solution (PBS), pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged at 10,000 g for ten minutes (Multifuge X1R Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) and supernatants were used in subsequent enzymatic analyses.

**2.6. Superoxide dismutase activity**

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined based on the ability of this enzyme to catalyze the reaction of superoxide radical ($O^-_2$) to hydrogen peroxide, thereby reducing the auto-oxidation rate of pyrogallol following the method described by Madesh and Balasubramanian (1997). In a microplate, 40 μL of the homogenate was diluted in 132 μL of PBS. Subsequently, 8 μL of (3–4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) at 0.6 g $L^{-1}$ and 20 μL of 1.25 mM pyrogallol (Sigma-Aldrich, St. Louis, MO, USA) were added. The plate was incubated at 37°C for 5 minutes. Next, 150 μL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added and the absorbance was read in duplicate for each repetition using a microplate reader at 570 nm (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). Blanks included all components except pyrogallol and homogenate. Total SOD activity was expressed in units per milligram of protein, where one unit of SOD activity is defined as the amount of enzyme required to produce 50 % dismutation of the superoxide radical per minute.

**2.7. Glutathione S-transferase activity**

Glutathione S-transferase (GST, EC 2.5.1.18) activity was assayed according to the formation of glutathione-conjugated 2,4-dinitrochlorobenzene (CDNB) using the method of Habig et al. (1974). A reaction mixture consisting of 380 μL of 0.1 M CDNB in ethanol (Sigma-Aldrich, St. Louis, MO, USA), 2.3 mL of 30.7 mg GSH $mL^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA) and 12.32 mL of PBS (Sigma-Aldrich, St. Louis, MO, USA) was first prepared. Subsequently, 25 μL of the homogenate was added to 125 μL of the reaction mixture in a well of a microplate. Absorbance was measured at 340 nm every 20 seconds, totaling 15 measurements in a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). The GST activity measurements were done in duplicate. As a negative control, PBS only was used. GST activity is expressed in mM per minute per milligram of protein.

## 2.8. Catalase activity

The activity of the catalase (CAT, EC 1.11.1.6) was assessed through the consumption of $H_2O_2$, observed by the decrease in absorbance at 240 nm (Aebi, 1984). For the assay, 10 µL of the supernatant was diluted in 190 µL of PBS to reach a 20-fold dilution. Then, 10 µL of the diluted supernatant and 140 µL of 10 mM $H_2O_2$ (Sigma-Aldrich, St. Louis, MO, USA) were added to a microplate well. Monitoring the reduction in absorbance of the reaction mixture was done at 240 nm every 10 seconds for two minutes in a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA), the measurement was done in triplicate. The specific activity of CAT was estimated in mM $H_2O_2$ consumed per minute per milligram of protein.

## 2.9. Quantification of protein content

The quantification of the protein content in the homogenates was carried out by adapting the Bradford method (Bradford, 1976), which uses bovine serum albumin (BSA) to obtain the analytical curve. Absorbance was measured at 595 nm in duplicate. Protein content was used to normalize the results of the enzymatic tests described above.

## 2.10. Assessment of histopathological alterations induced by phenylpropanoids and terpenes in third-instar larvae of *Drosophila suzukii*

Based on the toxicity results (see results section), L-(-)-carvone, carvacrol, (*E*)-anethole, and (*E*)-cinnamaldehyde were used to assess possible structural changes caused by exposure to low concentrations in the fat body, intestine, exoskeleton and muscle tissues of the third larval stage of *D. suzukii*. Methods for the histopathological analyses are described in detail in de Souza et al., (2022) and are summarized below.

## 2.11. Histopathological and histochemical analyses

For the histopathological analysis, fifty newly formed third-instar larvae of *D. suzukii* were exposed to $LC_{50}$s of L-(-)-carvone, carvacrol, (*E*)-anethol and (*E*)-

cinnamaldehyde, and the solvent control (2 % v/v DMSO). In a 9 cm diameter Petri dish with the bottom covered with filter paper, 1600 µL of each solution was added to the filter paper and then fifty larvae were placed in each Petri dish. After 24 h of exposure, five third-instar larvae were collected for each compound and fixed in a 4 % paraformaldehyde solution for 72 h and transferred to a 70 % ethanol solution. Larvae were then dehydrated using serial concentrations of ethanol (70, 80, 90, and 95 %) and embedded in Leica historesin for 24 hours at 4°C before being transferred to plastic molds for embedding. Subsequently, slides containing 4 µm thick sections were prepared using a microtome (Luptec MRP09). Two slides containing at least six sections were stained with Hematoxylin and Eosin (Junqueira and Junqueira, 1983). To evaluate factors such as the presence, frequency, and distribution of polysaccharides, two slides containing at least six sections were stained using the Periodic Acid-Schiff technique (Junqueira and Junqueira, 1983). Finally, the slides were mounted in Entellan® for subsequent analysis.

### 2.12. Morphometric analyzes

For morphometric analysis, the histological sections were photographed with a trinocular image capture system (Olympus Optical Ltda. Brasil, São Paulo, SP, Brazil) and a camera (SCOS Color for light microscopy). Measurements of abdominal muscle fibers, midgut and hindgut thickness, abdominal exoskeleton thickness, fat body lipid droplet area, lipid droplet density, and the relative glycogen content in the fat body and muscle were performed using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/).

For the abdominal muscle fibers, longitudinal histological sections were selected from the middle portion of the larvae. In these sections, for each insect, ten measurements were made of the thickness of the muscle fibers arranged horizontally. Five insects were used for each compound. To evaluate midgut and hindgut thickness, ten measurements were made of each structure per insect and five insects were used for each treatment.

To determine the average area of fat cells (trophocytes) in the fat body, 50 measurements were taken per insect, five insects for each compound, in histological sections of the abdominal portion. To determine the density of lipid droplets in the fat body, areas in this tissue were delimited and the number of droplets counted; results were expressed as lipid drops per 1000 µm$^2$. To analyze the glycogen present in the muscle and fat body, ten regions of these tissues were selected and the staining intensity was measured using the histogram tool in ImageJ.

## 2.13. Assessment of phenylpropanoids and terpenes selectivity to the non-target predator *Doru luteipes*

To evaluate the selectivity of L-(-)-carvone, carvacrol, (*E*)-anethol, and (*E*)-cinnamaldehyde to the generalist predator *D. luteipes*, toxicity and feeding bioassays were carried out.

In the toxicity bioassay, adults of up to 48 hours of age of *D. luteipes* were exposed to $LC_{50}$ and $LC_{90}$ of the two phenylpropanoids (*E*)-anethole and (*E*)-cinnamaldehyde, and the two terpenes L-(-)-carvone and carvacrol and the solvent control (2 % v/v DMSO), following the methodology of Moreira et al. (2023) with slight modifications. Briefly, groups of 20 individuals were placed in 7 cm Petri dishes containing filter paper (Ø = 7 cm) impregnated with 830 µL of solution for each treatment. Petri dishes with *D. luteipes* individuals were maintained under laboratory conditions at 25 ± 2 °C, 60 ± 5 % RH, and photoperiods: 12 L:12D. The tests were randomized, with four repetitions (n = 80), and mortality was assessed 24 hours after exposure.

In the feeding bioassay, *D. luteipes* adults that survived exposure to terpenes and phenylpropanoids were transferred to a plastic cage (500 mL capacity) and were offered moistened cotton, folding paper for shelter, and an artificial diet (Cruz, 2009) *ad libitum*. The tests were randomized, with four repetitions, 15 earwigs per repetition (n = 60) for each treatment (compound) and control. This experiment was maintained under laboratory conditions as described above. The daily diet mass consumed (food consumption) was measured on an analytical balance (model AUW 220 D, Shimadzu, Kyoto, Japan). Food consumption and survival of the *D. luteipes* adults were assessed every 24 hours for 7 days.

## 2.14. Statistical analyses

Adult *D. suzukii* toxicity data were subjected to probit analysis (SAS Institute, Cary, NC, USA). Toxicity data on *D. suzukii* larvae and *D. luteipes* as well as enzymatic analyses, morphometric, and the *D. luteipes* diet consumption data were subjected to the Shapiro-Wilk normality test. They were then compared by Analysis of Variance (ANOVA) using GraphPad Prism software, version 9.5.0 (GraphPad Software, Boston, MA, USA) followed by Tukey's multiple comparison test ($p < 0.05$), when normal, and Kruskal-Wallis followed by Dunn's post hoc test ($p < 0.05$) when the data normality prerequisites were not fulfilled. The survival of *D. luteipes* was determined by Kaplan-Meier analysis (Log-Rank).

## 3. Results

### 3.1. Toxicity of phenylpropanoids and terpenes to *Drosophila suzukii* adults

The mortality levels obtained in the dose-mortality bioassays (Table 1) were satisfactorily described by the probit model [goodness-of-fit tests exhibiting low $\chi^2$ values (<12) and high p-values (>0.05)]. For the phenylpropanoids, (*E*)-anethole presented the lowest $LC_{50}$ (4.22 mM), followed by (*E*)- cinnamaldehyde ($LC_{50}$ = 4.60 mM), eugenol ($LC_{50}$ = 4.68 mM) and *p*-anisaldehyde ($LC_{50}$ = 5.33 mM). For the terpenes, L-(-)-carvone had the lowest $LC_{50}$ (3.13 mM), followed by carvacrol ($LC_{50}$ = 4.47 mM), *β*-citronellol ($LC_{50}$ = 5.14 mM), *p*-cymene ($LC_{50}$ = 41.81 mM) and 1,8-cineole ($LC_{50}$ = 49.68 mM). Both the two terpenes *p*-cymene and 1,8-cineole were the least toxic to the adults of SWD compared to the other compounds.

### 3.2. Toxicity of selected phenylpropanoids and terpenes to *Drosophila suzukii* larvae and pupae

The $LC_{90}$ of the phenylpropanoid (*E*)-anethole caused the highest average larval mortality compared to the other treatments ($F_{(8,27)} = 4.23$; $p = 0.0022$) (Fig. 1A). The average pupal mortalities caused by the $LC_{90}$ of (*E*)-anethole and carvacrol were similar (Fig. 1B), and significantly higher ($F_{(8,27)} = 12.25$; $p < 0.0001$) than the pupal mortalities caused by other treatments. All treatments significantly increased ($F_{(8,27)} = 16.86$; $p < 0.0001$) body deformation in emerged adults. The main observed morphological changes included the presence of twisted legs, a reduced and wrinkled abdomen, wings not completely expanded, and pronotum with an irregular surface. Additionally, some of the insects were unable to completely emerge from the pupa or died shortly after this process without completing metamorphosis. These alterations were more evident in groups treated with carvacrol ($LC_{50}$ and $LC_{90}$) and (*E*)-anethole ($LC_{90}$) (Fig. 1C).

### 3.3. Effect of exposure to $LC_{50}$s of selected phenylpropanoids and terpenes in the larval SOD, GST, and CAT enzymatic activities

The effect of exposure to the selected terpenes and phenylpropanoids depended largely on the time after exposure and the enzyme type. Most of the change in the activity was observed 4 hours after exposure for all the assessed enzymes (SOD, GST, and CAT) (Fig. 2).

The enzymatic activity of the Superoxide Dismutase (SOD) was significantly ($F_{(4,20)}$ = 9.60; p = 0.0002) reduced compared to the control after 4 hours due to exposure to carvacrol ($LC_{50}$) but not to the other tested compounds (Fig. 2A). Furthermore, a similar decrease in the SOD activity was observed also in individuals exposed to $LC_{50}$s of (*E*)-cinnamaldehyde and L-(-)-carvone but only after 24 hours (Fig. 2D). For Glutathione-S-transferase and Catalase, significant increases in enzymatic activity were induced by exposure to $LC_{50}$s of (*E*)-anethole and (*E*)-cinnamaldehyde (GST: $F_{(4,20)}$ = 15.55; p < 0.0001; Fig. 2B) and to $LC_{50}$ of (*E*)-anethole (CAT: $F_{(4,20)}$ = 8.59; p = 0.0003; Fig. 2C) after 4 hours of exposure. However, no statistical differences were found in the enzymatic activity of GST ($F_{(4,20)}$ = 0.60; p = 0.6649) and CAT ($F_{(4,20)}$ = 2.07; p = 0.1228) after 24 hours of exposure to the $LC_{50}$s of any of the test compound compared to the control (Fig. 2E and F).

### 3.4. Histopathological changes in third-instar larvae of *Drosophila suzukii*

The exposure of the third instar larvae to the $LC_{50}$s of L-(-)-carvone, carvacrol, (*E*)-anethol, and (*E*)-cinnamaldehyde, resulted in structure-dependent histopathological alterations (Table 2 and Fig. 3). Overall, although no morphometric changes were revealed between the control and treatments for the muscle fibers' thickness, ($F_{(4,245)}$ = 0.13; p = 0.9711), significant reductions in the thickness of the midgut ($F_{(4,245)}$ = 49.00; p < 0.0001) and hindgut epitheliums ($F_{(4,245)}$ = 25.09; p < 0.0001), as well as the area ($F_{(4,1245)}$ = 29.89; p < 0.0001), and the density ($F_{(4,1245)}$ = 29.89; p < 0.0001) of lipid droplets in the fat body ($F_{(4,1245)}$ = 29.89; p < 0.0001), were observed after exposure to $LC_{50}$s of the selected terpenes and phenylpropanoids. Exposure to $LC_{50}$ of carvacrol caused the greatest reduction in the thickness of the midgut epithelium and the area of lipid droplets in the fat body while the (*E*)-anethole significantly reduced the hindgut epithelium thickness and the (*E*)-cinnamaldehyde reduced the density lipid droplets in the fat body.

Interestingly, the exposure to $LC_{50}$s the selected terpenes, and phenylpropanoids promoted a significant ($F_{(4,245)} = 106.50$; $p < 0.0001$) thickening of the exoskeleton compared to the control. The compounds (*E*)-cinnamaldehyde and carvacrol promoted the greatest alterations in this structure.

The analysis of muscle fibers through the PAS technique showed that there was a reduction in the carbohydrates content in this tissue in treatments with the $LC_{50}$ of (*E*)-anethole, L-(-)-carvone, and carvacrol, these treatments did not differ from each other but significantly differed ($F_{(4,1245)} = 21.88$; $p < 0.0001$) from the control and $LC_{50}$ of (*E*)-cinnamaldehyde. Regarding the fat body, carvacrol was the compound that promoted the greatest reduction ($F_{(4,1245)} = 19.60$; $p < 0.0001$) in the carbohydrate content in this tissue. The (*E*)-anethole, (*E*)-cinnamaldehyde, and L-(-)-carvone also promoted a reduction in carbohydrate content compared to the control but did not differ from each other.

### 3.5. Toxicity to adults of *Doru luteipes*

The exposure of *D. luteipes* adults to $LC_{50}$ and $LC_{90}$ of (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone, and carvacrol did not induce a significant difference ($H = 14.64$, $df = 8$, $p = 0.07$) in the predator mortality after 24 hours compared to the control (Fig. 4). Carvacrol was the most toxic of all the treatments but exposure to the $LC_{90}$ caused less than 15 % of mortality in *D. luteipes* adults (Fig. 4). Furthermore, the survival of *D. luteipes* was not significantly affected at the end of seven days after exposure to $LC_{50}$s ($\chi^2 = 2.36$, $df = 4$, $p = 0.63$) and $LC_{90}$s ($\chi^2 = 0.5360$, $df = 4$, $p = 0.97$) of the selected terpenes and phenylpropanoids (Table 3). Similarly, no difference was observed in the total amount of artificial diet consumed, over seven days period, by the control *D. luteipes* adults and adults that were exposed to either $LC_{50}$s ($F_{(4,15)} = 0.2452$; $p = 0.91$) (Fig. 5A) or $LC_{90}$s ($F_{(4,15)} = 0.3824$; $p = 0.82$) (Fig. 5B) of the selected terpenes and phenylpropanoids.

## 4. Discussion

*Drosophila suzukii* is a species that has the potential to cause great economic losses. Several studies investigated alternative ways to control this species, especially through essential oils. In this sense, our work stands out by evaluating the effects of nine secondary metabolites commonly found as major compounds in essential oils. We evaluated a series of parameters related to the survival, oxidative stress, and internal morphology of *D. suzukii* as well as the effects on the survival and food consumption of the non-target organism *D. luteipes*. Our results demonstrate that the compounds L-(-)-carvone, carvacrol, (*E*)-anethol, and (*E*)-cinnamaldehyde are promising for controlling *D. suzukii* while being selective to the generalist predator *D. luteipes*.

In the present study, L-(-)-carvone, carvacrol, and *β*-citronellol stood out as the most lethal terpenes to *D. suzukii* adults while all phenylpropanoids presented similarly good efficacy. In third-instar larvae, the phenylpropanoid (*E*)-anethole and the terpene carvacrol induced the highest larval lethality and sub-lethal effects expressed as adult deformation at emergence. These compounds, alone or as major constituents of essential oils, were reported to exhibit insecticidal potential against adults and young stages of other insect species, including dipterans including *D. suzukii* (Baker et al., 2023, da Silva et al., 2020, Pascual-Villalobos et al., 2020, Xie et al., 2019). Indeed, for (*E*)-anethole, $LC_{50}$ values of 1.75 $mg.L^{-1}$ in males and 3.00 $mg.L^{-1}$ in females were reported in a test that evaluated fumigant activity (Kim et al., 2016) while carvacrol presented $LD_{50\,s}$ equal to 1.63 µg per fly for males and 2.60 µg per fly for females following topical application (Park et al., 2016). Additionally, the (*E*)-cinnamaldehyde caused the mortality of approximately 80 % of flies when topically applied at a dose of 0.50 µL per fly (Jabeen et al., 2021). The slight differences between these studies and our findings might be inherent to the differences in the exposure routes (fumigation, contact, and ingestion exposure). Moreover, it is important to note that males and females present different susceptibility to insecticides, in which greater mortality is observed in males subjected to the same concentration (Kim et al., 2016, Park et al., 2016). We have used unsexed insects in our study to simulate natural conditions. However, the fact that we did not determine the $LC_{50}$ values for both males and females is a limitation of our study. Thus, further studies should assess insecticidal activity of chemicals for both sexes.

The insecticidal activity of terpenes and phenylpropanoids could be linked to several mechanisms. Firstly, these compounds may cause genotoxicity and mutagenicity (Nesterkina et al., 2023). Additionally, these natural chemicals may inhibit cholinergic enzymes, neural receptors, and ion transport channels (Isman, 2020b, Liu et al., 2022). Finally, they can induce oxidative stress (Chaudhari et al., 2021, Magierowicz et al., 2019) ultimately leading to morphological changes in organs particularly important for their survival (Chaaban et al., 2019, de Souza et al., 2022, Souza et al., 2021).

The differentiated toxic activity of phenylpropanoids and the terpene carvacrol against the *D. suzukii* larvae and adults might be due to their chemical structure. Phenylpropanoids and carvacrol have in their structure an aromatic ring conjugated with organic oxygenated functions. This factor may indicate greater reactivity of these compounds with insect structures, membranes, organelles, enzymes, and other biomolecules, justifying their good insecticidal performance (Liu et al., 2022). Additionally, although the terpene L-(-)-carvone is not an aromatic compound, it has a carbonyl in its structure that also represents a highly reactive site (Carson and Hammer, 2011).

Redox reactions are fundamental in living organisms, acting on bioenergetics, metabolism, and other vital functions. Under normal conditions, there is a balance between oxidizing and antioxidant compounds, called redox balance. When the balance is disturbed and there is an excess of oxidants, redox signaling can be interrupted and molecular damage can occur in the body. This phenomenon is called oxidative stress (Sies, 2020, Sies, 2018, Sies et al., 2017). The inactivation of oxidizing agents can occur through the action of antioxidant enzymes produced by the organism itself or by xenobiotic compounds. Among the antioxidant enzymes, catalases (CAT), superoxide dismutases (SOD), and glutathione S-transferases (GST), among others (Sies, 1997, Zhao et al., 2017), stand out. SODs are metalloenzymes that act intracellularly in aerobic organisms, promoting defense against reactive oxygen species (Perry et al., 2010). CATs are tetrameric heme that contain enzymes that dismutate hydrogen peroxide into water and oxygen gas, acting mainly in peroxisomes (Nicholls, 2012). GSTs catalyze the conjugation of xenobiotic compounds with the glutathione peptide, therefore, they have the potential to remove cytotoxic or genotoxic compounds (Strange et al., 2000).

Our results suggest that the detoxifying enzymes CAT and GST acted intensively in the initial hours of exposure of third-instar larvae to terpenes and phenylpropanoids, however, SOD had its activity suppressed. Exposure to terpenes and phenylpropanoids may promote the action of these enzymes as a defense mechanism. Alternatively, these chemicals may interact with these detoxifying enzymes and inhibit their activity (Achiri et al., 2022), as observed for SOD in our study. Indeed, antioxidant enzymes can interact with some xenobiotics resulting in their depletion and subsequent neurotoxicity as previously reported for *Drosophila melanogaster* (Shilpa et al., 2021). Sub-lethal concentrations of (*E*)-anethol were shown to increase the activity of SOD and CAT enzymes in *Hyphantria cunea* (Pour et al., 2022), and the activity of GST in *Ephestia kuehniella* (Shahriari et al., 2018). Carvacrol elevated CAT activity in *Acrobasis advenella* (Magierowicz et al., 2019) and elevated CAT and SOD activity, and reduced GST activity in *Lymantria dispar* (Chen et al., 2021). The suppression of SOD activity is directly related to the reduction in lifespan due to the action of reactive oxygen species (Landis and Tower, 2005). The inability to promote the neutralization of reactive chemical species can lead to the occurrence of histopathological changes (El-Ashram et al., 2021).

Morphological changes can compromise the perfect performance of physiological functions essential to maintaining life in insects. Organs may not function properly, such as loss of ability to absorb nutrients through the intestine or damage to the ovary that reduces reproductive efficiency. Muscle changes can compromise insect locomotion or flight. Changes in *D. suzukii* larvae were described after exposure to *Litsea cubeba* essential oil (Pan et al., 2022) and species of the genus Baccharis and the terpene limonene (Souza et al., 2021). Finally, de Souza et al. (2022) reported histopathological changes in adults of this species after exposure to *Illicium verum* essential oil.

The exoskeleton is usually the first insect structure that comes into contact with pesticides. The exoskeleton has the functions of protecting against physical and chemical damages, preventing infection by microorganisms and water loss, in addition to supporting muscles during locomotion (Gunderson and Schiavone, 1989, Zhu et al., 2016). We observed that the selected terpenes and phenylpropanoids promoted the thickening of the exoskeleton of the 3rd instar larvae of *D. suzukii*, especially after exposure to the $LC_{50}$ of (*E*)-cinnamaldehyde, which almost doubled the thickness of this structure. Although such thickening can be considered as a defense response to prevent the chemical compounds tested from reaching the interior of the insects' bodies, the reason why only (*E*)-cinnamaldehyde caused the thickening is still unclear. Previous studies reported changes in the exoskeleton of *D. suzukii* larvae, including variation in pigmentation, deformation of exoskeleton cuticles, and peeling (Pan et al., 2022, Souza et al., 2021, Trombin de Souza et al., 2022), however, none of these studies evaluated variations in exoskeleton thickness.

Besides the changes in the insect's external structure (exoskeleton), the histopathological analyses also revealed the occurrence of internal morphological changes. We observed a reduction in the thickness of the midgut and hindgut caused by all treatments, especially carvacrol ($LC_{50}$). These alterations in intestinal thickness can be a result of a necrosis process in the digestive tract of this pest species as was shown for different essential oils and their isolated constituents (Pan et al., 2022) including essential oils rich in (*E*)-anethole (de Souza et al., 2022).

The above-mentioned histopathological alterations in the intestines suggest that the larvae had difficulty absorbing nutrients from the food that was present in their digestive tract thus negatively impacting the energy reserves of glycogen and lipids. We observed that the area of lipid droplets in the fat body was reduced, especially in larvae exposed to the $LC_{50}$ of carvacrol. This reduction implies that the insect had to metabolize this reserve to maintain its vital functions and that exposure to essential oils or their isolated components promotes metabolic disorders that force the insect to consume its lipid reserves. In our previous work (de Souza et al., 2022), when evaluating energy reserves in adult females of *D. suzukii,* we observed that the glycogen reserves were well distributed throughout the fat body and muscles of the control group but these reserves were consumed in insects treated with essential oil rich in (*E*)-anethole. Furthermore, a similar decrease in the lipid droplets area was observed in the fat body of exposed adult females and larvae of *D. suzukii*. Conversely, in the present study, we did not observe considerable amounts of glycogen either in the control or any of the treatments. This may be due to the insects remaining fasting for 24 hours during exposure to terpenes and phenylpropanoids. Yamada et al. (2018) demonstrated that after 4 hours of fasting, glycogen reserves in the fat body of Drosophila flies are almost completely consumed.

The search for alternative pesticides must consider not only the target insect pests but also non-target arthropods like the natural enemy *D. luteipes* (Bacci et al., 2001, Campos et al., 2011, Moreira et al., 2023). This species is important for integrated pest management, as it is a generalist predator with known potential to control several insect pest species (Pacheco et al., 2021, Silva et al., 2023). Our results demonstrated that none of the selected compounds affected the survival or feeding capacity of *D. luteipes*. The amount of artificial diet consumed daily by *D. luteipes* (2–3 mg/insect/day) agrees with a previous study that investigated the susceptibility of this insect to different pesticides, in which the consumption ranged from approximately 2 to 4 mg/insect/day in the control group (Moreira et al., 2023). Interestingly, the group exposed to the pesticide metaflumizone significantly decreased feed consumption in nymphs, but not in adults. These findings indicate that these terpenes and phenylpropanoids are not harmful to the natural enemy while presenting good toxicity to *D. suzukii*.

In this sense, the terpenes, L-(-)-carvone and carvacrol, and the phenylpropanoids, (*E*)-anethole, and (*E*)-cinnamaldehyde, are promising for *D. suzukii* control as they negatively affected its survival, through oxidative stress, and by causing alterations in the internal and external structures of this species. Furthermore, these compounds did not affect the natural predator *D. luteipes*. Studies involving formulations with these compounds are necessary to verify their effectiveness in the field, possibly with encapsulation techniques to ensure greater stability and controlled release.

## CRediT authorship contribution statement

**Luciano de Souza:** Writing – original draft, Investigation, Formal analysis. **Maria das Graças Cardoso:** Writing – original draft, Validation, Supervision, Resources, Methodology, Conceptualization. **Isaac Konig:** Writing – original draft, Methodology, Investigation. **Stefânia Priscilla de Souza:** Investigation, Data curation. **Ana Luísa Rodrigues Silva:** Methodology, Investigation. Naiara Melo: Methodology, Investigation. **Rosangela Cristina Marucci:** Writing – original draft, Validation, Supervision, Methodology. **Khalid Haddi:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Data Availability**

Data will be made available on request.

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**Table 1 -** Toxicological activity of four phenylpropanoids and five terpenes on adults of *Drosophila suzukii* following exposure for 24 hours.

| Compounds | Chemical Class | Nº. Insects | $LC_{50}$ (95% F.I) (mM) | $LC_{90}$ (95% F.I) (mM) | $LC_{95}$ (95% F.I) (mM) | $X^2$ | *P* | TR $LC_{50}$ (95% F.I) (mM) |
|---|---|---|---|---|---|---|---|---|
| **(*E*)-anethole** | phenylpropanoids | 900 | 4.22(3.95-4.49) | 7.84(7.14-8.88) | 9.37(8.35-10.88) | 7.9216 | 0.2439 | 1.35(1.33-1.37) |
| ***p*-anisaldehyde** | phenylpropanoids | 700 | 5.33(5.00-6.26) | 9.37(8.53-10.62) | 11.00(9.82-12.85) | 6.4488 | 0.1680 | 1.71(1.68-1.92) |
| **(*E*)-cinnamaldehyde** | phenylpropanoids | 900 | 4.60(4.39-4.80) | 7.15(6.73-7.71) | 8.10(7.53-8.89) | 4.8830 | 0.5589 | 1.47(1.47-1.47) |
| **eugenol** | phenylpropanoids | 800 | 4.68(4.42-4.94) | 7.70(7.07-8.61) | 8.85(7.99-10.19) | 7.1113 | 0.2125 | 1.51(1.49-1.52) |
| **carvacrol** | Terpenes | 800 | 4.47(4.19-4.75) | 8.70(8.00-10.37) | 10.87(9.49-13.10) | 5.2693 | 0.3839 | 1.44(1.41-1.46) |
| **L-(-)-carvone** | Terpenes | 700 | 3.13(2.97-3.26) | 4.71(4.44-5.08) | 5.29(4.92-5.83) | 1.4755 | 0.6879 | * |
| **1,8-cineole** | Terpenes | 800 | 49.68(48.90-50.39) | 57.98(56.78-59.47) | 60.54(59.05-62.51) | 8.0307 | 0.1546 | 15.87(15.46-16.46) |
| ***β*-citronellol** | Terpenes | 700 | 5.14(4.70-5.59) | 11.73(10.45-13.51) | 14.82(12.92-17.60) | 6.1479 | 0.1884 | 1.65(1.58-1.71) |
| ***p*- cymene** | Terpenes | 700 | 41.81(39.04-44.83) | 57.97(52.70-67.77) | 63.60(56.85-76.89) | 8.7825 | 0.0668 | 13.48(13.14-13.75) |

$LC_{50}$, $LC_{90}$, and $LC_{95}$ are the lethal concentrations that kill 50 %, 90 %, and 95 % of tested individuals; F.I: 95 % Fiducial intervals; $\chi^2$: Chi-square test; P: test probability; TR: toxicity ratio calculated by dividing the $LC_{50}$ of each compound by the minor value between all $LC_{50}$s. (*): the compound that presented the lowest $LC_{50}$ (L-(-)-carvone) and used to compute TR.

**Table 2 -** Morphometric analysis of structures of third-instar *Drosophila suzukii* larvae untreated (2 % v/v DMSO), and exposed for 24 hours to $LC_{50}$ de of the two phenylpropanoids (*E*)-anethole and (*E*)- cinnamaldehyde and the two terpenes L-(-)-carvone, and carvacrol.

| Structures | Water + DMSO | $LC_{50}$ (*E*)-anethole | $LC_{50}$ (*E*)-cinnamaldehyde | $LC_{50}$ L-(-)-carvone | $LC_{50}$ carvacrol |
|---|---|---|---|---|---|
| **Thickness of muscle fibers (µm)** | $15.09 \pm 2.20^{a}$ | $15.11 \pm 2.19^{a}$ | $15.15 \pm 1.91^{a}$ | $15.13 \pm 1.93^{a}$ | $15.56 \pm 2.31^{a}$ |
| **Thickness of the midgut epithelium (µm)** | $43.37 \pm 6.37^{a}$ | $36.51 \pm 4.63^{b}$ | $40.00 \pm 4.72^{c}$ | $42.05 \pm 7.08^{a,c}$ | $29.78 \pm 4.07^{d}$ |
| **Thickness of the hindgut epithelium (µm)** | $26.96 \pm 5.25^{a}$ | $18.45 \pm 4.41^{b}$ | $22.60 \pm 4.34^{c}$ | $21.63 \pm 3.51^{c}$ | $22.33 \pm 3.75^{c}$ |
| **Thickness of exoskeleton (µm)** | $12.04 \pm 2.05^{a}$ | $18.45 \pm 1.89^{b}$ | $20.23 \pm 2.73^{c}$ | $15.55 \pm 2.09^{d}$ | $19.03 \pm 2.42^{b,c}$ |
| **Average area of lipid droplets of fat body (µm²)** | $67.46 \pm 20.41^{a}$ | $47.96 \pm 17.16^{b}$ | $49.37 \pm 18.55^{b}$ | $36.08 \pm 15.19^{c}$ | $18.97 \pm 8.04^{d}$ |
| **Density of lipid droplets (lipid droplets in 1000 µm²)** | $7.51 \pm 1.52^{a}$ | $5.99 \pm 1.31^{b}$ | $5.63 \pm 1.02^{b}$ | $5.88 \pm 0.75^{b}$ | $7.48 \pm 1.21$a |
| **Intensity of PAS staining in muscle fibers (a. u.)** | $197.71 \pm 10.55^{a}$ | $177.71 \pm 19.46^{b}$ | $196.95 \pm 16.46^{a}$ | $184.60 \pm 13.61^{b}$ | $178.32 \pm 13.10^{b}$ |
| **Intensity of PAS staining in the fat body (a. u.)** | $194.40 \pm 13.94^{a}$ | $180.20 \pm 14.02^{b}$ | $180.13 \pm 18.15^{b}$ | $186.17 \pm 11.88^{b}$ | $169.56 \pm 14.10^{c}$ |

Data normality was checked using the Shapiro-Wilk test. Means in the same line followed by the same letters do not differ from each other by Analysis of Variance (ANOVA) followed by Tukey's post hoc test ($p > 0.05$). CL = lethal concentration. µm = micrometer and a.u. = arbitrary units.

**Table 3 -** Survival of *Doru luteipes* adults exposed to $LC_{50}$ and $LC_{90}$ de of the two phenylpropanoids (*E*)-anethole and (*E*)-cinnamaldehyde and the two terpenes L-(-)-carvone and carvacrol.

| **Treatment** | **Survival $LC_{50}$ (days)** | **Survival $LC_{90}$ (days)** |
|---|---|---|
| Control | 6.817[a] (6.531 - 7.102) | 6.850[a] (6.640 - 7.060) |
| (*E*)-anethole | 6.667[a] (6.305 - 7.029) | 6.967[a] (6.875 - 7.058) |
| (*E*)-cinnamaldehyde | 6.667[a] (6.298 - 7.035) | 6.833[a] (6.512 - 7.155) |
| L-(-)-carvone | 6.750[a] (6.437 - 7.063) | 6.867[a] (6.624 - 7.110) |
| carvacrol | 6.917[a] (6.718 - 7.115) | 6.983[a] (6.938- 7.029) |

Survival was analyzed using the Kaplan-Meier test. Means in the same column followed by the same letters do not differ from each other ($p > 0.05$). LC = lethal concentration. The insects were exposed to the chemicals for 24 hours and mortality was monitored daily for seven days.

**Fig. 1 -** Larval (A) and pupal (B) mortality and adult deformation (C) of *Drosophila suzukii* individuals after exposure of the third instar larvae to $LC_{50}$ and $LC_{90}$ of (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol. The insects were exposed once at the beginning of the experiment and monitored for ten days. Bars represent mean ± SE. Different letters indicate statistical differences following the Tukey Test.

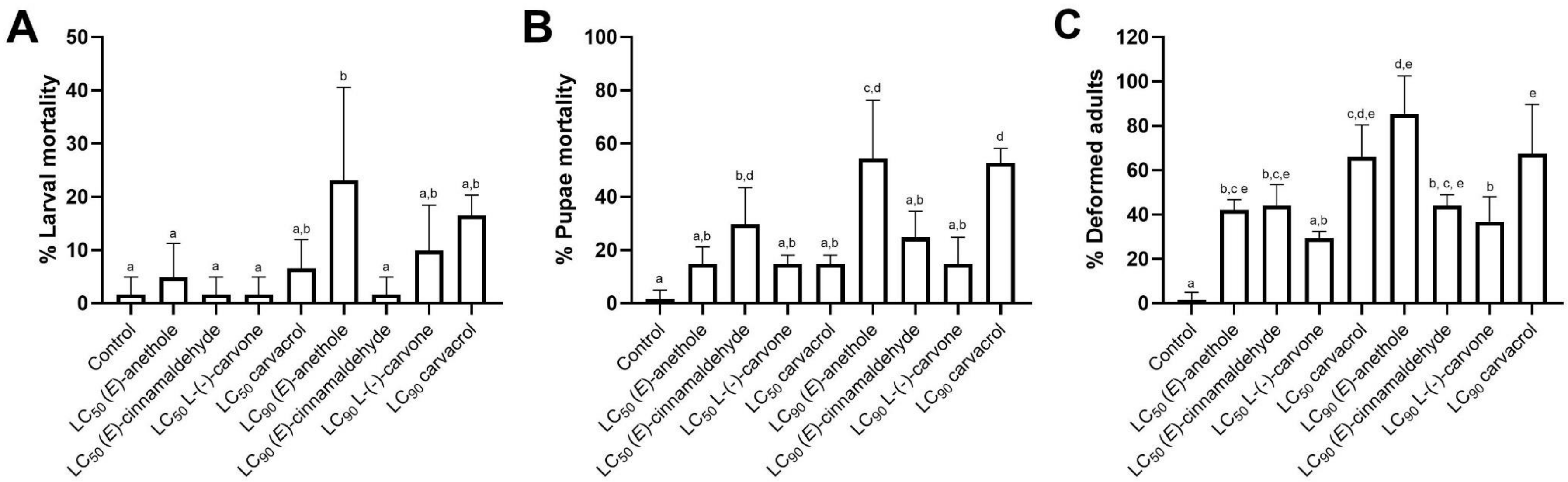


**Fig. 2 -** Enzymatic activity of Superoxide dismutase (A and D), Glutathione-S-transferase (B and E) and Catalase (C and F) in 3rd instar larvae of *Drosophila suzukii* exposed to $LC_{50}$ of (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol after 4 (A, B, C) and 24 hours (D, E, F). Bars represent mean ± SE. Different letters indicate statistical differences following the Tukey Test.

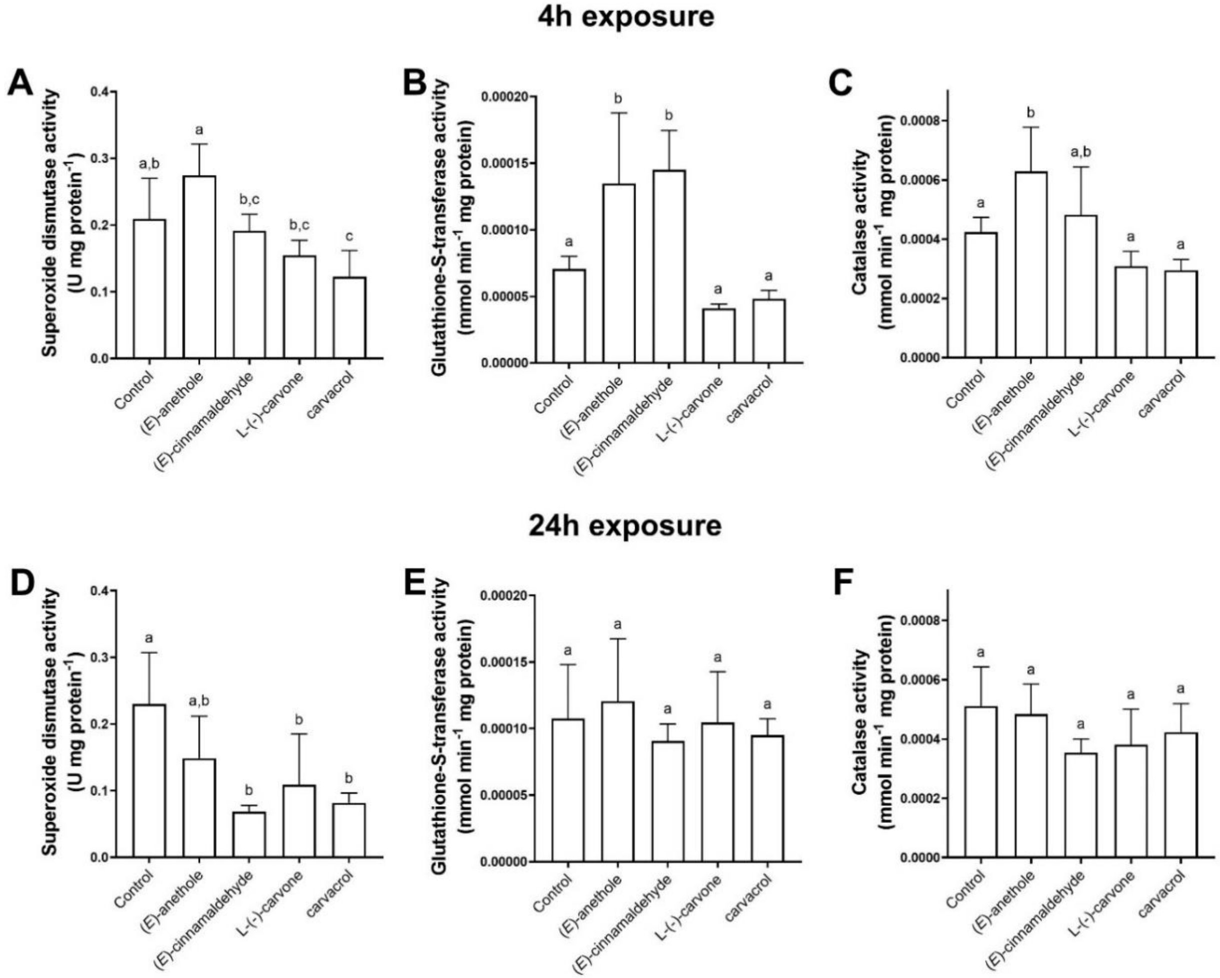


**Fig. 3 -** Representation of the main histological changes in 3rd instar larvae of *Drosophila suzukii* untreated (A and B) and exposed to $LC_{50}$ of (*E*)-anethole (C and D), (*E*)-cinnamaldehyde (E and F), L-(-)-carvone (G and H) and carvacrol group (I and J). Histological sections stained with hematoxylin and eosin (HE) (A, C, E, G, and I) and periodic acid-Schiff technique (PAS) (B, D, F, H, and J). Scale bars: 50 µm. Captions: AD – adipocyte; DA – adipocyte density; IF – intensity of PAS staining in the fat body; IM – intensity of PAS staining in muscle fibers; PAS+ - positive PAS region; ExT – exoskeleton thickness; IL – Intestinal lumen; IET – thickness of the intestinal epithelium; RIET – reduction in the thickness of the intestinal epithelium; RAD – reduced adipocyte density; IExT – increased exoskeleton thickness; RIF – reduction in the intensity of PAS staining in the fat body; RIM – reduction in the intensity of PAS staining in muscle fibers; RA – reduction in adipocyte area.

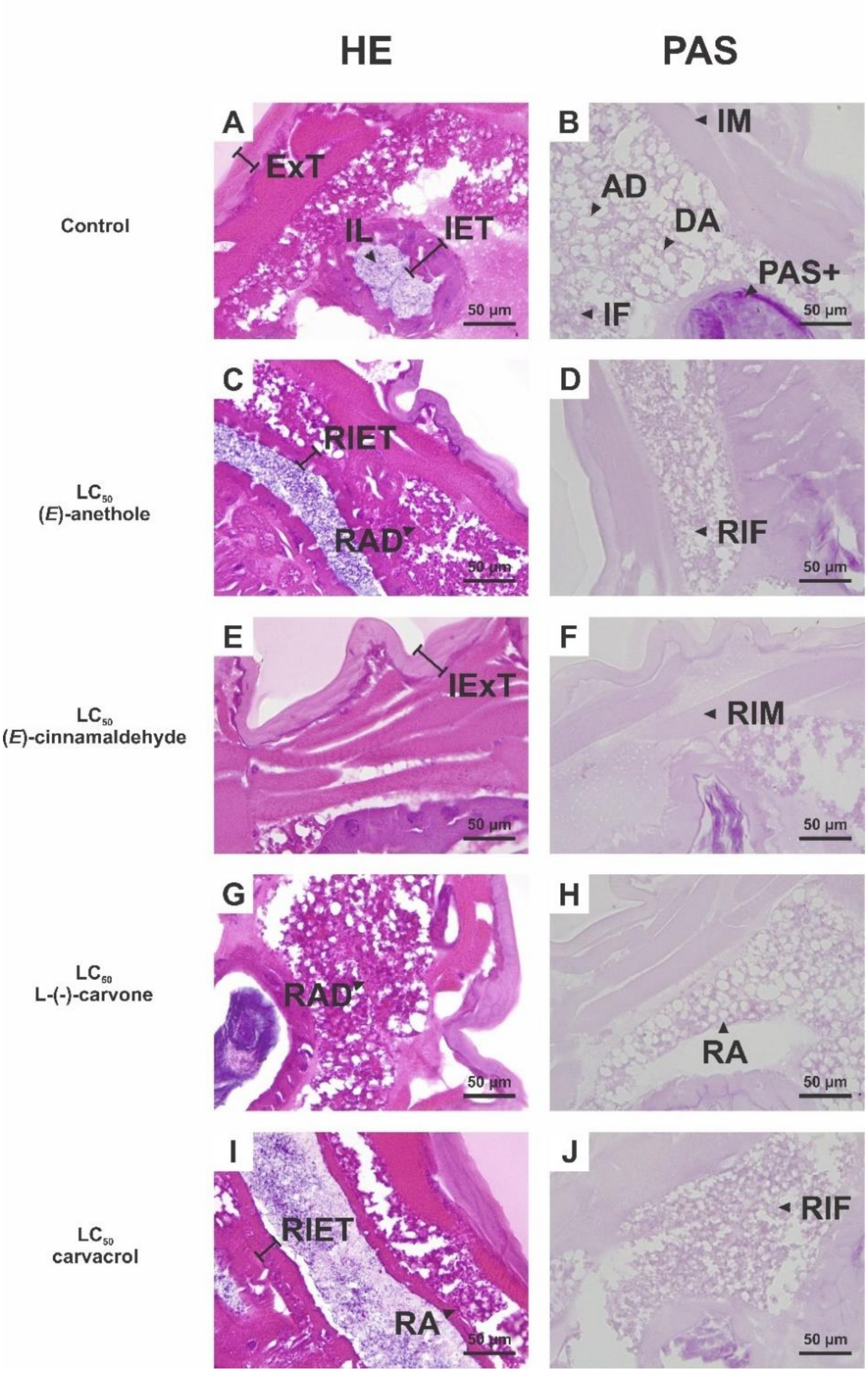


**Fig. 4 -** Mortality of *Doru luteipes* adults 24 hours after exposure to the $LC_{50}$ and $LC_{90}$ of (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol. Boxes indicate the median and its range of dispersion, lower and upper quartiles, and outliers. Similar letters indicate no statistical differences following the Kruskal-Wallis test.

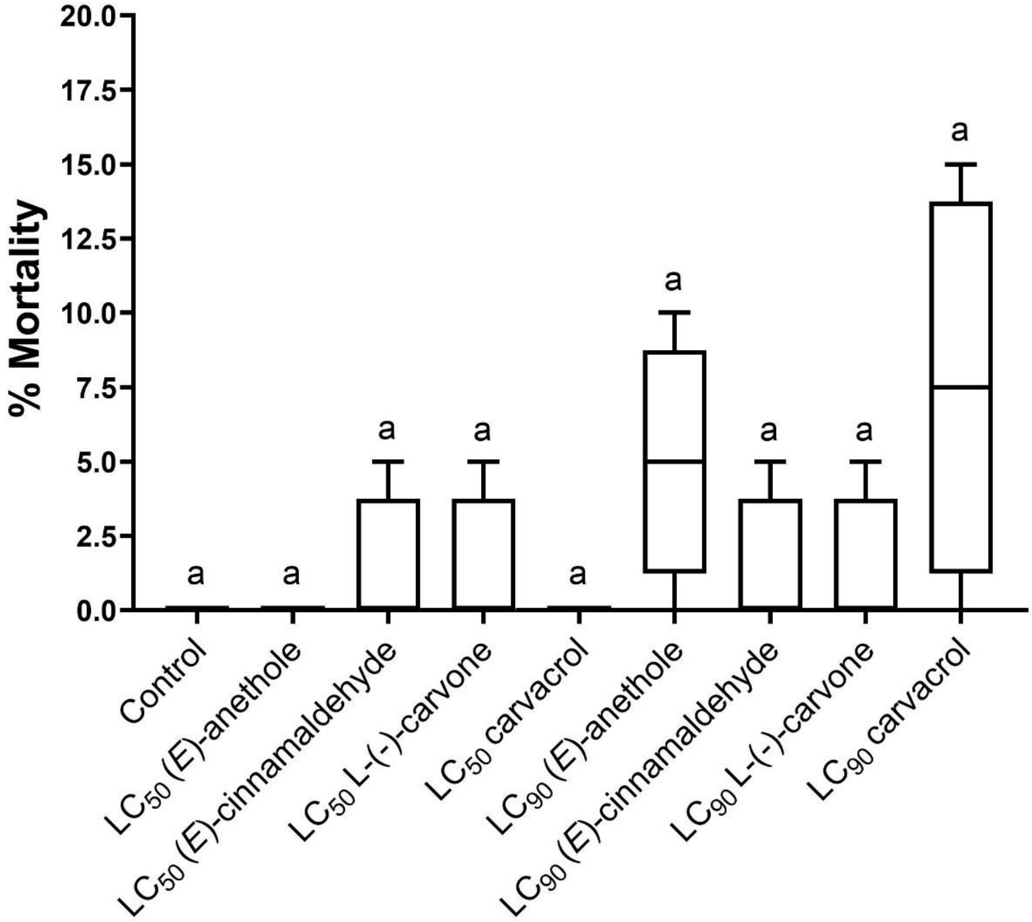
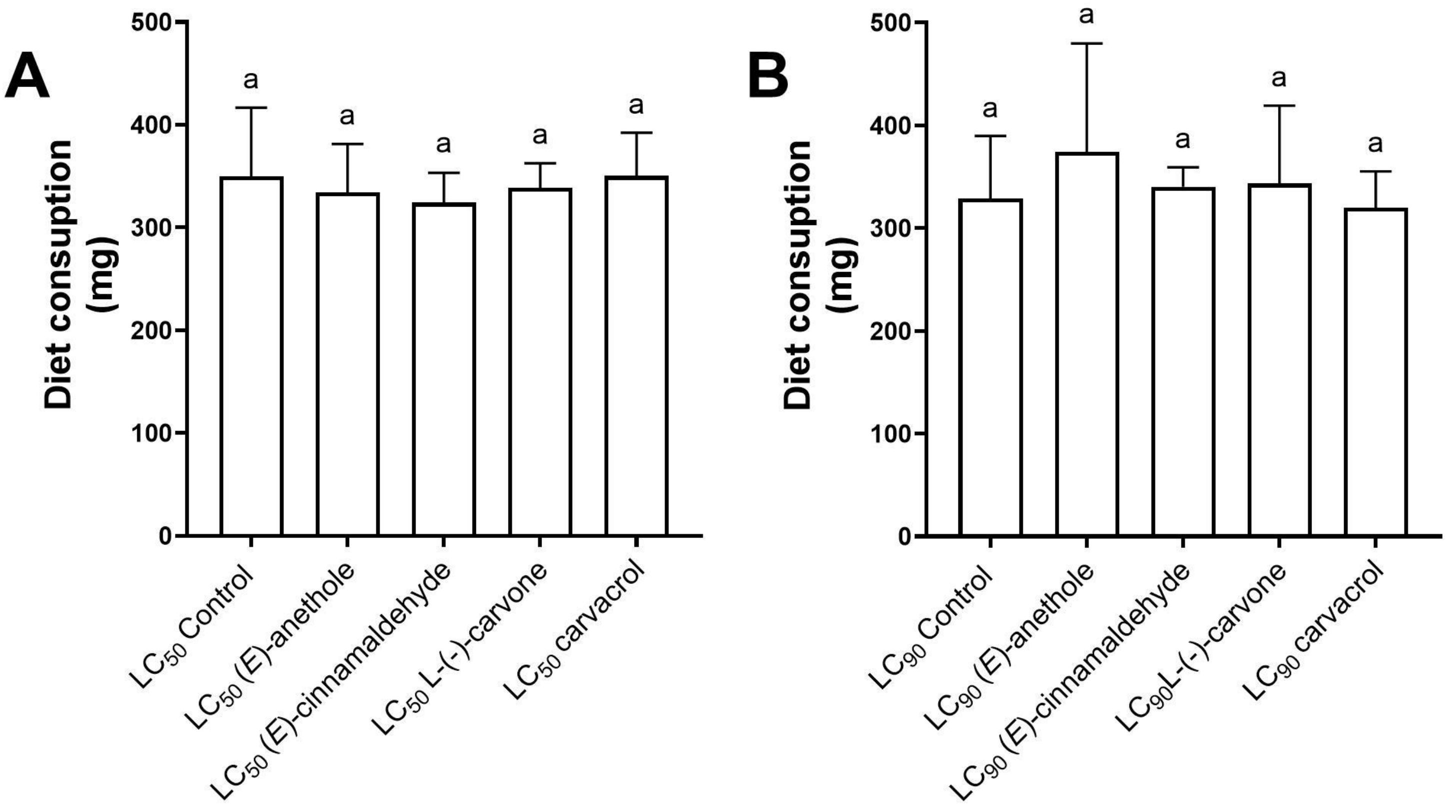


**Fig. 5 -** Total diet consumption over seven days period of *Doru luteipes* adults groups (n = 4 replicates each with one group of 15 insects/treatment) exposed to the $LC_{50}$ and $LC_{90}$ of (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol. Bars represent mean ± SE. Similar letters indicate no statistical differences following the Tukey Test.



**ARTIGO II**

**"Poly(ε-caprolactone) nanoparticles of terpenes and phenylpropanoids promote prolonged insecticidal activity and induce morphophysiological disturbances in *Drosophila suzukii*"**

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## Highligths

- Isolated L-(-)-carvone had the best insecticidal activity against *Drosophila suzukii*.
- Nanoparticles of poly(*ε*-caprolactone) (PCL) prolonged their insecticidal activities.
- PCL-(*E*)-cinnamaldehyde presented the lowest $LC_{50}$ value, $LC_{50}$ = 17.12 µL.mL$^{-1}$.
- PCL-carvacrol was the most promising in terms of inducing oxidative stress.
- PCL-treated flies exhibited evident internal morphological alterations.


## Abstract

The use of plant-derived products in crop protection is still hampered by their high volatility and susceptibility to degradation. New technologies based on nanoemulsions and nanoencapsulation are currently under scrutiny to improve plant-derived biopesticides' efficacy and stability. This study aimed to investigate the insecticidal activity of carvacrol, L-(-)-carvone, (*E*)-anethole, and (*E*)-cinnamaldehyde in their isolated form and as nanoparticles of poly(ε-caprolactone) (PCL) against *Drosophila suzukii* adults. Additionally, we evaluated the effects of these compounds on biomarkers of oxidative stress and histopathology of $LC_{50}$-treated *D. suzukii* adult females. Finally, the effects of these compounds on the non-target organism *Palmistichus elaeisis* were also assessed. Isolated L-(-)-carvone exhibited the greatest insectidal activity ($LC_{50}$ = 0.49 µL.mL$^{-1}$), whereas for the nanoparticles PCL-(*E*)-cinnamaldehyde presented the lowest $LC_{50}$ value ($LC_{50}$ = 17.12 µL.mL$^{-1}$). The nanoparticles of all compounds exhibited prolonged insecticidal activities compared to their isolated form, particularly PCL-carvacrol. This same pattern was observed for oxidative stress in which strong activation of catalase, glutathione-S-transferase, and glutathione peroxidase (GPx) was observed for PCL-carvacrol. For lipid peroxidation, PCL-control and isolated compounds increased MDA content compared to the solvent control (2% DMSO). Furthermore, PCL-treated flies exhibited decreased muscle fiber, increased midgut epithelium thickness, and reduced lipid droplet area in the fat body. Thoracic exoskeleton thickness increased in flies treated with isolated compounds. In their isolated or nanoparticle forms, these terpenes and phenylpropanoids were not harmful to the parasitoid *P. elaeisis*, suggesting that nanoparticles of these natural compounds should be further studied as new alternatives to the control of *D. suzukii*.

**Keywords:** Nanoinsecticide; Spotted-wing fly; Oxidative stress; Morphological changes; Eco-friendly.

## 1. Introduction

The spotted-wing fly *Drosophila suzukii* (Diptera: Drosophilidae) is a worldwide distributed pest that has been reported in Europe (Calabria et al. 2012), North America (Lee et al. 2011), Africa (Boughdad et al. 2021) and South America (Deprá et al. 2014), including Brazil (Andreazza et al. 2016b). Controlling this species is an ongoing challenge, primarily carried out by chemical methods (Van Timmeren and Isaacs 2013). However, few alternatives, such as biological control (Wang et al. 2020), control of growing conditions (Tochen et al. 2016), use of genetically improved plants (Murphy et al. 2016), and the use of natural products (Keesey et al. 2019) are being frequently advocated and further investigated.

Plant-derived compounds, like essential oils and their major constituents, are promising alternatives for controlling pest insects including *D. suzukii* (Souza et al. 2021; Pan et al. 2022; Pineda et al. 2023). More than sixty-five oils extracted from plants were previously tested through different exposure routes against *D. suzukii*, and their insecticidal efficiency, as well as their major compounds, was reported (Xavier et al. 2024). For example, the essential oil of *Illicium verum*, with (*E*)-anethole as a major component, showed good insecticidal activity in adults of *D. suzukii* with an $LC_{50}$ equal to 1.9 $\mu L.mL^{-1}$ and caused morphological damage in the intestine, muscles, and fat body of the flies (de Souza et al. 2022). The essential oil of *Anethum graveolens*, rich in carvone (30.11%), performed well as a fumigant at a concentration of 5% on *D. suzukii* and promoted insect repellency (Bošković et al. 2023) while the carvacrol found in the *Thymus zygis* essential oil was more toxic to both males and females of *D. suzukii* after contact exposure (Park et al. 2016). Similarly, *Cinnamomum cassia* oil, rich in (*E*)-cinnamaldehyde (81.6%), promoted high mortality of *D. suzukii* in contact exposure at a dose of 5 µg (Kim et al. 2016). Furthermore, a previous study that assessed nine terpenes and phenylpropanoids demonstrated that the compounds (*E*)-anethole, (*E*)-cinnamaldehyde, carvacrol, and L-(-)-carvone presented low $LC_{50}$s, induced oxidative stress, and caused morphological damage in immature stages of the contact fly (de Souza et al. 2024).

However, although promising, these plant-derived compounds generally present low stability against oxidation, low water solubility, and high volatility, making field applications difficult (Pavela and Benelli 2016; Mossa 2016). These limitations imposed by the plant-derived compounds' physical-chemical properties could be overcome through nanotechnology processes (Giunti et al. 2023) and the use of nanoparticles, which are nanometric-sized structures with large surface area compared to materials on a macroscopic scale (Lee et al. 2015). Different studies investigated the optimization of insecticidal activity with the use of nanoparticles containing essential oils (Ahmed et al. 2023; Yeguerman et al. 2023). Even on *D. suzukii*, *Rosmarinus officinalis* oil, composed mainly of camphor (35.38%), 1,8-cineole (17.05%), and *α*-pinene (12.90%), when incorporated into poly(ε-caprolactone) nanoparticles, prolonged the insecticidal activity when compared to the pure essential oil (Caetano et al. 2022). Nevertheless, although the previous results are promising, this area of knowledge remains little explored for the control of *D. suzukii*, and studies assessing the efficacy of terpenes and phenylpropanoids nanoparticles, important candidates for the development of botanical pesticides, are still very scarce. Furthermore, the effects of terpenes and phenylpropanoids in general and specifically of L-(-)-carvone, carvacrol, (*E*)-anethole, and (*E*)-cinnamaldehyde when applied in their pure or nano-formulated forms on different non-target organism like natural enemies is generally overlooked.

In this sense, the present work evaluated the acute toxicity and insecticidal activity over time of L-(-)-carvone, carvacrol, (*E*)-anethole, and (*E*)-cinnamaldehyde in their pure and poly-ε-caprolactone nanoparticles forms on adults of *D. suzukii*. In addition, the physiological alterations resulting from exposure to these compounds on the activity of enzymes related to oxidative stress, and lipid peroxidation levels as well as histopathological analyses of muscle, intestine, fat body, and exoskeleton were assessed. We also evaluated the effects of these compounds in their pure and nanoparticle forms on the survival and reproductive parameters of the non-target organism *Palmistichus elaeisis*, a pupal endoparasitoid frequently used in the integrated management of different agricultural and forestry pest species. We expect that the data from this study will contribute to the ongoing research on effective and environmentally friendly alternative pesticides for the control of *D. suzukii*.

## 2. Material and methods

### 2.1. Chemical compounds and insects

The chemical compounds (*E*)-anethole (99% purity, CAS 4180-23-8) and carvacrol (98% purity, CAS 499-75-2) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and L-(-)-carvone (99% purity, CAS 6485-40-1) and (*E*)-cinnamaldehyde (99% purity, CAS 14371-10-9) were purchased from Acros Organics (Geel, Belgium).

The insects of the species *D. suzukii* used in the bioassays were obtained from a stock colony maintained in the Laboratory of Molecular Entomology and Ecotoxicology of the Department of Entomology (DEN) of UFLA. The rearing was done on an artificial diet (Emiljanowicz et al. 2014; Andreazza et al. 2016a; Mendonca et al. 2019) and under controlled conditions (24 ± 2 °C; 60 ± 5% and photoperiod: 12:12 h L/D).

The adults of the pupae-parasitoid *Palmistichus elaeisis* used in the selectivity bioassays were obtained from a colony kept at the Biological Pest Control Laboratory (DEN, UFLA). The rearing was carried out in acrylic cages (40×40×60 cm) using pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) as host (Rolim et al. 2020). Newly emerged *P. elaeisis* individuals were fed with pure honey *ad libitum*. Larvae and adults of *T. molitor* were kept in plastic boxes (40×30×12 cm) and fed *ad libitum* with wheat bran and pieces of chayote (*Sechium edule* Swartz) until pupation. The parasitoid and its host were reared under a controlled environment (25 ± 2 °C, 70 ± 10% RH, and a photoperiod of 12:12 h L/D) (Rolim et al. 2020). Pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were obtained from the Biological Pest Control Laboratory of the DEN of UFLA. They were used in the selectivity experiment and for mass rearing of *Palmistichus elaeisis*. These insects were kept under controlled conditions (25 ± 2 °C; 70 ± 10% RH and photoperiod of 12:12 h L/D).

## 2.2. Synthesis and characterization of Poly-ε-caprolactone nanoparticles

### 2.2.1. Synthesis of the nanoparticles

Nanoparticles of poly-ε-caprolactone (PCL) (MW = 50,000 g/mol) (Perstorp, Warrington, Cheshire, United Kingdom) were obtained by the emulsification-solvent evaporation technique, as proposed by Caetano et al., (2022). Briefly, a PCL-acetone solution was prepared by adding 130 mg of PCL polymer to 27 mL of acetone and subjected to magnetic stirring at 37 °C until complete dissolution of the polymer. Then, 4 mL of Tween 80 surfactant (Sigma-Aldrich, St. Louis, MO, USA) was added, and the solution was stirred at room temperature until complete homogenization. Using an injection pump (NE-300 Just Infusion™, New Era Pump Systems, Inc., Farmingdale, NY, USA), the suspension obtained was added at a rate of 300 $\mu L.min^{-1}$ to 53 mL of deionized water under magnetic stirring, and the mixture was kept stirring at room temperature until the complete evaporation of the acetone solvent. The evaporated acetone volume was replaced by distilled water. The preparation of terpenes and phenylpropanoids nanoparticles followed the same procedure using a 5% (v/v) emulsion of each compound instead of the Tween 80 surfactant alone.

### 2.2.2. Characterization of the synthesized nanoparticles

All obtained nanoparticles were characterized as previously described (Fraj et al. 2019).

The zeta potential, the mean diameter of the nanoparticles, and the polydispersity index were determined using dynamic light scattering equipment (Zetasizer Nano ZS, Malvern Panalytical Inc., Malvern, Worcestershire, United Kingdom). Distilled water was used as a dispersant to avoid multiple dispersion effects and interactions between nanoparticles. The cumulative mean diameter (z-mean) and the polydispersity index (PdI) were used to describe the size and distribution of the nanoparticles, respectively.

Furthermore, infrared spectroscopy using a Fourier transform infrared spectrophotometer (Frontier, Perkin Elmer, São Paulo, SP, Brazil) was carried out. The nanoparticle samples were deposited on the equipment crystal for a sufficient time for the solvent to evaporate before the spectra were obtained by the attenuated reflectance technique

(ATR). Sixty-four scans were performed in the range between 400 and 4000 $cm^{-1}$ with a resolution of 1 $cm^{-1}$.

### 2.3. Toxicity of pure terpenes and phenylpropanoids and their nanoparticles to *Drosophila suzukii* adults

Concentration-mortality bioassays were performed to evaluate the lethal activity of contact and ingestion of the selected compounds and their nanoparticles on adult *D. suzukii* flies. The exposure procedure followed the IRAC protocol no. 026 (Insecticide Resistance Action Committee), recommended for bioassays with *Musca domestica* L. adults, with adaptations (IRAC 2011). Five to nine increasing concentrations of each compound were tested, with four replicates each. The concentrations ranged from 0.1 to 100 $\mu L.mL^{-1}$ in the pilot test and were adjusted based on the preliminary results. The dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was used as the solvent and was maintained at 2% (v/v) in the solutions, while a 20% (w/v) sucrose solution completed the final volume. Previous studies confirmed the non-toxicity of this solvent concentration for *D. suzukii* (de Souza et al. 2022, 2024). For the bioassays, dental cotton treated with 2.2 mL of the prepared solution was placed in 200 mL glass vials. The negative control consisted only of the solvent and sucrose solution. Twenty-five randomly collected adult flies of the same age range (5-7 days) were introduced into each vial, which was closed and kept under controlled conditions of temperature (24 ± 2 °C), relative humidity (60 ± 5% RH), and 12-h photoperiod. Mortality was assessed after 24 hours, considering flies that did not move after a slight stimulus as dead.

### 2.4. Residual effect of pure terpenes and phenylpropanoids and their nanoparticles on adults of *Drosophila suzukii*

The residual insecticidal activity of isolated terpenes and phenylpropanoids nanoparticles was evaluated over 120 hours using the previously obtained $LC_{50s}$ and following the same exposure methodology. DMSO-sucrose solutions at 2% v/v and PCL nanoparticle emulsion at 2.5% v/v were used as controls. For each treatment, 100 insects were divided into four replicates. The treatments were applied only once, and at each 24-hour interval, all individuals were removed from the containers, mortality was assessed, and new insects were introduced to the vial.

### 2.5. Effects of exposure to isolated terpenes and phenylpropanoids and their nanoparticles on enzymatic activity and oxidative stress of *D. suzukii* females

Adult females of *D. suzukii* were previously exposed to $LC_{50}$s of the (*E*)-anethole and (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol, and their PCL nanoparticles before carrying the enzymatic analyses. All analyses were performed in five independent replicates containing 40 insects, totaling 200 individuals for each treatment. Ten females were collected from each replicate after 4 hours of exposure. Each replicate was homogenized with 400 µL of phosphate buffer solution (PBS), pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged at 10,000 g for ten minutes (Multifuge X1R Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). The supernatants were used in subsequent analyses.

#### 2.5.1. Superoxide dismutase enzyme activity

Superoxide dismutase (SOD, EC 1.15.1.1) enzyme activity was assessed following an adaptation of the method described by Madesh and Balasubramanian, (1997). For each treatment, 40 µL of insects' supernatant was diluted in 132 µL of PBS (Sigma-Aldrich, St. Louis, MO, USA) in a well of a microplate. Then, 8 µL of 0.6 g $L^{-1}$ (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) and 20 µL of 1.25 mM pyrogallol (Sigma-Aldrich, St. Louis, MO, USA) were added. The plate was incubated at 37 °C for 5 minutes before adding 150 µL of DMSO (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured in duplicate for each replicate using a microplate reader at 570 nm (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). Negative controls consisted of all components except pyrogallol and the sample. Total SOD activity was expressed in units per milligram of protein, where one unit of SOD activity is defined as the amount required to trigger 50% dismutation of the superoxide radical per minute.

### 2.5.2. Glutathione S-transferase enzyme activity

The activity of the glutathione S-transferase (GST, EC 2.5.1.18) enzyme was evaluated using 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) as substrates as described by Habig et al., (1974). In a microplate, 25 μL of the supernatant and 125 μL of the reaction medium, composed of 380 μL of 0.1 M CDNB in ethanol (Sigma-Aldrich, St. Louis, MO, USA), 2.3 mL of GSH 30.7 mg $mL^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA) and 12.32 mL of PBS (Sigma-Aldrich, St. Louis, MO, USA) were combined. Absorbance was measured every 20 seconds, totaling 15 measurements, in a microplate reader at 340 nm, with duplicates in each reading. The negative control consisted of PBS only. The GST activity was expressed in units of enzyme activity per milligram of protein.

### 2.5.3. Glutathione peroxidase enzyme activity

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined according to the method described by Günzler et al. (1984). The reaction mixture used in the assay consisted of 10 mL of PBS, 2.6 mL of 10 mM GSH solution, 2.6 mL of 1.6 mM NADPH solution, 2.6 mL of 10 mM $NaN_3$ solution, and 1 μL of GR enzyme (0.5 $U.mL^{-1}$). Then, 40 μL of the supernatant, 200 μL of the reaction mixture, and 25 μL of 10 mM hydrogen peroxide solution were added to each microplate well. The reaction was monitored at 340 nm, and the GPx value was expressed as a unit of enzyme activity per milligram of protein.

### 2.5.4. Catalase enzyme activity

The activity of the catalase enzyme (CAT, EC 1.11.1.6) was measured based on the consumption of $H_2O_2$, evidenced by the reduction of absorbance at 240 nm (Aebi 1984). For the assay, 10 μL of the supernatant was diluted in 190 μL of PBS to achieve a 20-fold dilution. Then, 10 μL of the diluted supernatant and 140 μL of 10 mM $H_2O_2$ (Sigma-Aldrich, St. Louis, MO, USA) were added to a well of a microplate. The reduction in absorbance of the reaction mixture was monitored at 240 nm every 10 seconds for two minutes, with measurements made in triplicate. The specific activity of CAT was estimated in mM of $H_2O_2$ consumed per minute per milligram of protein.

### 2.5.5. Quantification of protein content

The protein content quantification in the homogenates was performed based on an adaptation of the method described by Bradford, (1976). For the assay, 10 µL of the supernatant was added to 200 µL of Bradford's reagent. An analytical curve was determined using albumin as a standard. Absorbance was measured at 595 nm in duplicate. Protein content was used to normalize the results of the enzymatic tests described above.

## 2.6. Evaluation of lipid peroxidation in adult female *Drosophila suzukii*

Lipid peroxidation was evaluated by quantifying the production of thiobarbituric acid reactive substances (TBARS), using malondialdehyde (the main product of lipid peroxidation) as a standard, as proposed by Buege and Aust, (1978). For the assay, 150 µL of the supernatant was added to 200 µL of the TBARS solution before being vortexed and incubated in a water bath at 37 °C for 15 minutes. After cooling to room temperature, 420 µL of butyl alcohol was added to the solution, which was vortexed and centrifuged (Multifuge X1R Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes at 5000 g. The supernatant of all samples was read in 96-well flat-bottom microplates, and the readings were performed in a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA) with absorbance at 532 nm. The readings were performed in duplicate with five samples per treatment. The results were expressed as malondialdehyde concentration in $mg.L^{-1}$.

## 2.7. Histopathological evaluation in exposed adult females of *Drosophila suzukii*

Adult females of *D. suzukii* were exposed to the $LC_{50}$s of L-(-)-carvone, carvacrol, (*E*)-anethole, and (*E*)-cinnamaldehyde in their isolated and nanoparticle forms. Structural changes in the fat body, intestine, exoskeleton, and muscle tissues of the insects after exposure to these compounds were evaluated.

### 2.7.1. Histopathological and histochemical analyses

For histopathological analysis, fifty adult females of *D. suzukii* were exposed to each treatment, and to the control solutions of 2% v/v DMSO and 2.5% v/v PCL nanoparticle emulsion. After 48 h of exposure, five females were collected from each treatment, fixed in 4% paraformaldehyde solution for 72 h, and transferred to 70% ethanol solution. The insects were then dehydrated using serial ethanol concentrations (70, 80, 90, and 95%) and embedded in historesin (Leica Biosystems, Nussloch, Germany) for 24 h at 4 °C before being transferred to plastic molds for embedding. Subsequently, slides containing 4 µm-thick sections were prepared using a microtome (Luptec MRP09). Two slides containing at least six sections were stained with Hematoxylin and Eosin (Junqueira and Junqueira 1983). Finally, the slides were mounted in Entellan® (Merck KGaA, Darmstadt, Germany) for further analysis.

### 2.7.2. Morphometric analyses

Morphometric analyses were performed as described by de Souza et al., (2024). The histological sections were photographed using a DM750 trinocular image capture system (Leica Microsystems, São Paulo, SP, Brazil) and a Flexacam C1 camera (Leica Microsystems, São Paulo, SP, Brazil). Measurements of the thickness of the thoracic muscle fibers, midgut, and thoracic exoskeleton, as well as the area and density of lipid droplets in the fat body, were performed using Image J software (National Institutes of Health, Bethesda, Maryland, USA). For the thoracic muscle fibers, longitudinal histological sections of the midportion of the larvae were selected, where ten measurements of the thickness of the horizontal fibers were made per insect. Five insects were used in each treatment. For the thickness of the midgut, ten measurements were made per structure in each insect, with five insects used per treatment. The mean area of the fat body's adipose cells (trophocytes) was calculated with 50 measurements per insect, totaling five insects per treatment in histological sections of the abdominal portion. To determine the density of lipid droplets, specific areas of the fat body were delimited, and the number of droplets was counted, with the results expressed in lipid droplets per 1.000 µm².

## 2.8. Selectivity to the pupae parasitoid *Palmistichus elaeisis*

To evaluate the effect of isolated and nanoparticle chemical compounds on *P. elaeisis*, a methodology adapted from Pereira Costa et al. (2020) and Vieira Caldeira et al. (2022) was used. Newly formed (less than 24 h) pupae of *T. molitor*, with an average weight of 82.4 ± 3.9 mg, were immersed for ten seconds in 10 mL of $LC_{50}$ solutions of the compounds L-(-)-carvone, carvacrol, (*E*)-anethole and (*E*)-cinnamaldehyde in their isolated and nanoparticle forms, and control solutions of 2% v/v DMSO and 2.5% v/v PCL nanoparticle emulsion. The pupae were placed on paper towels to remove excess solution. Next, one pupa was placed in a flat-bottomed glass tube (25×85 mm) containing six *P. elaeisis* mated females that had emerged no more than 48 h earlier. The tube contained a drop of honey and was sealed with voile fabric, thus constituting a sampling unit. Each treatment had 20 replicates. The *P. elaeisis* females were allowed to parasitize the treated pupae for 48 h. After this period, they were transferred to plastic tubes (8×60 mm). The parasitism and emergence percentages, the number of emerged parasitoids, the sex ratio (RS = number of emerged females/total emerged parasitoids), and the egg-to-adult period (days) were evaluated. A change in color of the pupae to brownish indicated parasitism. Emergence was recorded daily for 10 days after the first emergence. The sex ratio was obtained by counting the total number of females and males emerged per *T. molitor* pupa. The egg-to-adult period was obtained by the number of days between the moment when females begin parasitism and the emergence of the first insects of the new generation of the parasitoid. In addition, the survival of *P. elaeisis* females after contact with treated *T. molitor* pupae was assessed by daily mortality counts.

## 2.9. Statistical analyses

The toxicity data on *D. suzukii* adults were subjected to probit analysis (SAS Institute, Cary, NC, USA). The toxicity data over time were subjected to regression analyses, with models chosen based on parsimony, lowest standard errors, and steep increases in $R^2$ with model complexity. Regression analysis was performed using the curve-fitting procedure of SigmaPlot 15.0 (Systat Software Inc., Palo Alto, CA, USA). Data from enzymatic analyses, malondialdehyde content, and histomorphometry of *D. suzukii*, as well as emergence, egg-to-adult period, progeny, and sex ratio of *P. elaeisis* were subjected to the Shapiro-Wilk normality test using GraphPad Prism software, version 10.2.0 (GraphPad Software, Boston, MA, USA). Then, the data was subjected to Analysis of Variance (ANOVA), followed by Dunnett's multiple comparisons test to compare pure compounds with 2% DMSO control and PCL-encapsulated compounds with the PCL control. A pairwise comparison between the pure and PCL-encapsulated compounds was also done using Welch’s t-test. The *P. elaeisis* survival was determined by Kaplan-Meier (Log-Rank) analysis, using SigmaPlot software (Systat Software Inc., Palo Alto, CA, USA). *P. elaeisis* parasitism was analyzed using a generalized linear model (GLM), using R studio software, version 3.6.1 (Posit, Boston, MA, USA). For all endpoints, the significance level was set at $p < 0.05$.

## 3. Results

### 3.1. Characterization of the synthesized particles

The FTIR spectra obtained for the nanoparticles containing the active ingredients did not show any significant shift in relation to the bands of the main vibrational modes when compared to the isolated substances (Supplementary Figure 1). A decrease in the intensity of the bands was observed, mainly in PCL-(*E*)-cinnamaldehyde and PCL-carvacrol. However, characteristic signals of terpenes and phenylpropanoids, such as the bands related to the unsaturated bonds between carbons (around 900 and 1.600 $cm^{-1}$) and stretching of single bonds between carbon and oxygen (around 1.300 $cm^{-1}$) could be observed in the spectra of the prepared nanoparticles. Such evidence confirms the encapsulation of the active ingredients in the PCL nanoparticles. The characterization of the nanoparticle size indicated that the isolated PCL particles had the smallest size, 175 nm (96.5%). The particles with the encapsulated compounds showed an increase in size, with PCL-(*E*)-anethole having the largest average diameter, 314 nm (85.4%) (Table 1). Regarding the polydispersity index, the values ranged from 0.181 ± 0.003 for PCL nanoparticles to 0.663 ± 0.048 for PCL-carvacrol. Finally, the zeta potential ranged from -5.67 mV for PCL-(*E*)-anethole to -24.6 mV for PCL-carvacrol.

**3.2. Toxicity of terpenes, phenylpropanoids, and their nanoparticles to *Drosophila suzukii* adults**

The probit model satisfactorily described (goodness-of-fit tests showing $\chi^2$ values < 8 and p values >0.05) mortality levels obtained in the dose-mortality bioassays (Table 2). All compounds in their isolated form were similarly toxic to adults of *D.suzukii*. The $LC_{50}$ ranged from L-(-)-carvone was the most toxic presenting the lowest $LC_{50}$s ranged from 0.49 (0.47-0.51) $\mu L.mL^{-1}$ for L-(-)-carvone to 0.71 (0.65-0.77) $\mu L.mL^{-1}$ for carvacrol.

Regarding nanoparticles, the (*E*)-cinnamaldehyde nanoparticles were the most toxic with $LC_{50}$ equal to 17.12 $\mu L.mL^{-1}$ while the (*E*)-anethole nanoparticles were the least toxic ($LC_{50}$ = 25.30 $\mu L.mL^{-1}$). It is noteworthy that the amount of active ingredient present in the nanoparticles and correspondent to the $LC_{50}$ value of PCL-encapsulated compounds is similar or slightly smaller than the $LC_{50}$s of their respective isolated forms (Table 2).

Furthermore, the nanoparticles containing terpenes and phenylpropanoids optimized the toxic effect over time of these compounds when compared to their isolated forms (Figure 1). While initially, no differences ($p > 0.05$) in terms of mortality were observed 24 h after exposure for any of the isolated compounds when compared to the nanoencapsulated ones, after 48 h, the nanoencapsulated compounds consistently showed superior activity for all the tested compounds (from the summary of statistical analyses presented in Supplementary Table 1). The adequacy of the results obtained experimentally for mortality over time to different nonlinear regression models confirms that the process of incorporating the compounds into PCL polymeric nanoparticles enhanced their effectiveness. The models used and parameters obtained for the nonlinear regression are presented in Supplementary Table 2.

**3.3. Effect of exposure to terpenes and phenylpropanoids and their nanoparticles on the enzymatic activity of *Drosophila suzukii* adults**

Catalase activity was not affected by treatment with the compounds in their pure form when compared to the 2% DMSO control ($F_{(4.18)} = 1.51$, $p = 0.241$) (Figure 2A). However, PCL-carvacrol increased CAT activity compared to PCL control ($F_{(4.16)} = 4.23$, $p = 0.016$). Pairwise comparisons showed reduced CAT activity of PCL control ($t = 4.41$, $p = 0.003$), whereas an increase was detected in PCL carvacrol ($t = 3.06$, $p = 0.020$). Regarding SOD activity, there were no differences in pure compounds compared to 2% DMSO control ($F_{(4.20)}$ = 0.27, p = 0.890) and nanoencapsulated forms compared to PCL control ($F_{(4.20)}$ = 1.64, p = 0.201). On the other hand, PCL-(*E*)-cinnamaldehyde increased SOD activity compared to its pure form (t = 2.41, p = 0.041) (Figure 2B). The GST enzyme's activity increased by treatment with (*E*)-anethole compared to the 2% DMSO control ($F_{(4.17)}$ = 4.81, p = 0.008). Similarly, the PCL-carvacrol group had GST activity increased compared to the PCL control ($F_{(4.16)}$ = 11.09, p < 0.001). Pairwise comparisons showed reduced activity following PCL-(*E*)-anethole exposure (t = 3.60, p = 0.011) and increased activity with PCL-L-(-)-carvone (t = 4.99, p = 0.001) and PCL-carvacrol (t = 3.39, p = 0.014) treatments (Figure 2C). Finally, treatment with (*E*)-cinnamaldehyde, L-(-)-carvone, and carvacrol increased GPx activity compared to the 2% DMSO control ($F_{(4.19)}$ = 25.32, p < 0.001). Similarly, exposure to the nanoparticles L-(-)-carvone and PCL-carvacrol caused an increase in GPx activity compared to the PCL control ($F_{(4.15)}$ = 5.25, p = 0.007). Exposure to all of the nanoparticles increased GPx activity compared to their respective pure compound and control (t = 17.16, p < 0.001), (*E*)-anethole (t = 6.12, p = 0.0005), (*E*)-cinnamaldehyde (t = 4.50, p = 0.002), L-(-)-carvone (t = 4.11, p = 0.004), and carvacrol (t = 2.56, p = 0.043) (Figure 2D).

Pure compounds promoted an increase in lipid peroxidation in insects compared to the 2% DMSO control ($F_{(4.20)}$ = 27.93, p < 0.001). The PCL control presents higher MDA content than the nanoparticles ($F_{(4.20)}$ = 6.88, p = 0.001). Pairwise comparisons showed that PCL control exhibits higher MDA content than 2% DMSO control (t = 10.00, p < 0.001). (*E*)-cinnamaldehyde (t = 3.45, p = 0.0086) and carvacrol (t = 6.54, p = 0.0002) reduced lipid peroxidation in its nanoparticle form (Figure 3).

### 3.4. Histopathological Changes in Adult Females of *Drosophila suzukii*

The thickness of thoracic muscle fibers was not affected by treatment with the compounds in their pure form when compared to the 2% DMSO control ($F_{(4,245)}$ = 1.21, p = 0.282) (Table 3). However, treatment with PCL-(*E*)-anethole, PCL-L-(-)-carvone, and PCL-carvacrol reduced the thickness of this structure compared to the PCL control ($F_{(4,245)}$ = 33.42, p < 0.001). Pairwise comparisons showed a reduction in thickness in the treatment with PCL-L-(-)-carvone (t = 4.67, p < 0.001) and PCL-carvacrol (t = 8.93, p < 0.001). Regarding midgut thickness, there were no differences in the pure compounds compared to the 2% DMSO control

($F_{(4,245)}$ = 1.24, p = 0.23). In the nanoencapsulated forms compared to the PCL control, there was an increase in all treatments ($F_{(4,245)}$ = 6.56, p < 0.001). Pairwise comparisons demonstrated an increase in midgut thickness in the treatments with PCL-(*E*)-anethole (t = 3.79, p = 0.0003), PCL-(*E*)-cinnamaldehyde (t = 3.72, p = 0.0003), and PCL-carvacrol (t = 2.71, p = 0.008). Thoracic exoskeleton thickness increased for all compound treatments alone compared to the 2% DMSO control ($F_{(4,245)}$ = 16.81, p < 0.001), and for all encapsulated compounds compared to the PCL control ($F_{(4,245)}$ = 12.76, p < 0.001). Pairwise comparisons showed increased thickness following exposure to PCL-(*E*)-cinnamaldehyde (t = 3.70, p = 0.0004) and PCL-L-(-)-carvone (t = 2.42, p = 0.017). Additionally, the lipid droplet area of adipose tissue was unchanged for pure compounds compared to the 2% DMSO control ($F_{(4,620)}$ = 1.07, p = 0.37). The encapsulated compounds promoted a reduction in the area compared to the PCL control ($F_{(4,620)}$ = 18.94, p < 0.001). Pairwise comparisons showed a reduction in the area after exposure to PCL-(*E*)-anethole (t = 6.47, p < 0.0001), PCL-(*E*)-cinnamaldehyde (t = 5.76, p < 0.0001), PCL-L-(-)-carvone (t = 6.42, p < 0.0001) and PCL-carvacrol (t = 4.75, p < 0.0001). Finally, there was no change in lipid droplet density in the fat body in treatments with pure compounds compared to the 2% DMSO control ($F_{(4,120)}$ = 1.60, p = 0.18), nor in treatments with nanoparticle compounds compared to the PCL control ($F_{(4,120)}$ = 1.68, p = 0.16).

### 3.5. Toxicity on *Palmistichus elaeisis*

Exposure to $LC_{50}$ of terpenes and phenylpropanoids, pure or in nanoparticles, did not affect the parasitism of *P. elaeisis* females, being 100% in all treatments. Similarly, there were no differences among treatments in progeny emergence: pure compounds compared to the 2% DMSO control ($F_{(4.95)}$ = 1.52, p = 0.202) and PCL nanoparticles compared to the PCL control ($F_{(4.95)}$ = 1.38, p = 0.246) (Figure 5A). For the egg-adult development period, there were no differences for the pure compounds compared to the 2% DMSO control ($F_{(4.89)}$ = 0.95, p = 0.44) and for the PCL nanoparticles and the PCL control ($F_{(4.92)}$ = 0.26,p = 0.90). However, a reduction in the development period was observed in the PCL control compared to 2% DMSO control (t = 3.02, p = 0.005) and PCL-(*E*)-anethole compared to the pure compound (t = 2.04, p = 0.04) (Figure 5B). Regarding the number of offspring, there was no significant difference for the pure compounds compared to the 2% DMSO control ($F_{(4.89)}$ = 1.13, p = 0.34). In contrast, an increase in progeny was observed for the PCL-(*E*)-anethole group ($F_{(4.95)}$ = 2.54 p = 0.017).

However, there was an increase in the number of insects in the PCL-carvacrol treatment compared to pure carvacrol ($t = 2.22$, $p = 0.03$) (Figure 5C). In the sex ratio, there were no differences for the pure compounds compared to the 2% DMSO control ($F_{(4.89)} = 1.47$, $p = 0.21$) and for the PCL nanoparticles and the PCL control ($F_{(4.92)} = 0.69$, $p = 0.60$). However, a reduction in the sex ratio was observed in the treatment with PCL-L-(-)-carvone compared to its pure form ($t = 2.63$, $p = 0.012$) (Figure 5D).

The mean survival time was significantly smaller for the insects exposed to the control PCL ($\chi^2 = 10.091$, df = 1, and $p < 0.001$), (*E*)-anethole alone ($\chi^2 = 6.793$, df = 1, and $p = 0.009$) and PCL-(*E*)-anethole ($\chi^2 = 5.682$, df = 1, and $p = 0.017$) compared with the insects exposed to the 2% DMSO control group and other treatments (Table 4 and Supplementary Figure 2).

## 4. Discussion

Bioactive compounds present in essential oils stand out as promising alternatives for the control of *D. suzukii*. However, these compounds have the disadvantage of being usually volatile, insoluble in water, and presenting low stability against oxidation (Giunti et al. 2023). These disadvantages can be overcome by using nanotechnology to increase the bioavailability of the active ingredients (Singh and Pulikkal 2022; Xavier et al. 2024). In the present study, we evaluated the insecticidal activity of four plant secondary metabolites in their isolated and poly(*ε*-caprolactone) nanoparticle forms. Our results demonstrated that the poly(*ε*-caprolactone) nanoencapsulation prolonged the insecticidal activity of the active ingredients and altered the morphophysiology of *D. suzukii*, mainly through increased activity of enzymes related to oxidative stress and morphological damage caused after exposure to these compounds. Furthermore, these compounds did not affect many parameters, such as the egg-to-adult development period, progeny, and sex ratio of a non-target organism, the pupa parasitoid *P. elaeisis*.

The characterization of the nanoparticles obtained in this study demonstrated that there was an increase in the average diameter of the particles containing terpenes and phenylpropanoids when compared to the PCL nanoparticle control, especially the PCL-(*E*)-anethole nanoparticles that increased by approximately 140 nm. The increase in particle size is an indication that the active ingredients of interest were encapsulated in the polymer matrix (Rabha et al. 2021; Dadashpour et al. 2023). Regarding the zeta potential, all nanoparticles containing terpenes and phenylpropanoids presented values similar to the control, except for those containing carvacrol, which presented a value of -24.6 mV. The zeta potential indicates the electrochemical equilibrium between particles and liquids that form a system, as is the case of colloidal solutions of nanoparticles (Lunardi et al. 2021). The increase in this parameter indicates an increase in charges on the surface of the nanoparticles, probably due to the lower affinity between the carvacrol molecules and the polymer. Consequently, the hydroxyls of carvacrol may become more exposed and undergo deprotonation, resulting in a variation in the zeta potential (Seremeta et al. 2013). Finally, the infrared spectra indicate the presence of an intense band near 1720 $cm^{-1}$ that refers to the stretching vibrational mode of the carbonyl group, and a band in the region of 1290 $cm^{-1}$ characteristic of the stretching of the main chain related to the single bonds between carbon atoms and between carbon and oxygen of the PCL polymer. Additionally, intense broadband was observed in the region of 1100 $cm^{-1}$, which is characteristic of the ester bonds of the surfactant used in this synthesis (Zanetti et al. 2019; Caetano et al. 2022). In the spectra of nanoparticles containing terpenes and phenylpropanoids, signals related to the stretching of characteristic bonds are observed, such as the stretching of single bonds between carbon and oxygen (1280 $cm^{-1}$), stretching of double bonds between carbon and oxygen (1670 $cm^{-1}$), twisting and stretching of double bonds between carbons (970 and 1600 $cm^{-1}$, respectively) (Li et al. 2013; Sinha et al. 2014; Ramamoorthy and Rajiv 2014; Rajkumar et al. 2018).

In our study, all four isolated compounds tested (L-(-)-carvone; (*E*)-cinnamaldehyde; (*E*)-anethole; and carvacrol) demonstrated toxicity to the adults of *D. suzukii* presenting low $LC_{50}$s. Other studies have already demonstrated the insecticidal potential of (*E*)-anethole, (*E*)-cinnamaldehyde, carvacrol, and L-(-)-carvone isomers; however, different exposure methodologies were used. (*E*)-anethole presented $LC_{50}$ equal to 1.75 and 3.0 $mg.L^{-1}$ in males and females of *D. suzukii*, respectively, in a toxicity test via fumigation (Kim et al. 2016). In a topical toxicity test, (*E*)-cinnamaldehyde caused 80% mortality of individuals at a concentration of 0.50 µL per fly (Jabeen et al. 2021). *Anethum graveolens* essential oil, which contains carvone (30.11%) as its major constituent, showed 97.5% and 100% mortality in *D. suzukii* adults by fumigation and topical contact methods, respectively, at a concentration of 5% (Bošković et al. 2023). Carvacrol, in a contact toxicity test, showed $LD_{50}$ equal to 1.63 and 2.60 µg per fly for males and females, respectively (Park et al. 2016). Moreover, the $LC_{50}$ value obtained here for the (*E*)-anethole was even considerably lower than that obtained for the essential oil of *I.verum*, with a major component (*E*)-anethole (99.6%), which presented an $LC_{50}$ equal to 1.9 µL.mL$^{-1}$ at the same time and form of exposure (de Souza et al. 2022). These findings reinforce the insecticidal activity and potential use in pest management of such plant secondary metabolites as previously described in the literature.

The process of incorporating the four compounds into PCL polymeric nanoparticles maintained their insecticidal activity. It is important to emphasize that although the prepared nanoparticles presented higher $LC_{50}$s than their respective isolated compounds, the concentration of active ingredients in the nanoparticles was very similar or even lower than that of the isolated compounds. PCL-(*E*)-cinnamaldehyde stands out, in which the concentration of (*E*)-cinnamaldehyde was 0.43 µL.mL$^{-1}$, a reduction of approximately 25% compared to the isolated compound. The appreciable performance of the compounds under study, isolated or in nanoparticles, can be explained by their structural characteristics. All compounds have oxygenated organic functions in their structures and have aromatic rings, except for L-(-)-carvone, which is a cyclic but non-aromatic monoterpene (Carson and Hammer 2011). These factors allow the interaction of these molecules with several enzymes, receptors, membranes, as well as other macromolecules and cellular structures of insects, which justifies the insecticidal potential of these compounds (Liu et al. 2022).

Furthermore, the nanoencapsulation with the PCL prolonged the insecticidal activity of the isolated compounds over time. Our results showed that the nanomaterials obtained in this study, PCL-(*E*)-cinnamaldehyde and PCL-carvacrol, promoted significant insecticidal activity over 96 and 120 hours, respectively, on *D. suzukii* adults. Previous studies also reported prolonged insecticidal activity of essential oils through nanoencapsulation on different species of insect pests and vectors (Ferreira et al. 2019; Caetano et al. 2022; Ahsaei et al. 2022). It was previously demonstrated that PCL nanoparticles containing *Rosmarinus officinalis* essential oil promoted the mortality of *D. suzukii* adults for up to 72 hours only (Caetano et al. 2022) and that PCL and chitosan nanoparticles containing *R. officinalis* and *Zataria multiflora* essential oil increased insecticidal activity over time on *Tribolium confusum* (Ahsaei et al. 2022).

The use of polymeric nanomaterials is a viable alternative to overcome the low persistence of biological activity that some chemical compounds present due to their physicochemical properties (Wang et al. 2017). Among the available polymers, poly(ε-caprolactone) (PCL) stands out (Sachan et al. 2023). This polymer and other biodegradable polyesters are widely used options in controlled release systems of different substances since the degradation of the polymer through erosive processes allows the release of the chemical compounds of interest (Makadia and Siegel 2011). This material is biodegradable, being metabolized by several fungi, bacteria, and algae (Leja and Lewandowicz 2010), and, in addition, it presents irrelevant toxicity and low cost compared to other materials (Cesari et al. 2020). These factors qualify pesticides formulated with natural products and PCL as environmentally friendly alternatives to the use of conventional pesticides (Mondéjar-López et al. 2024).

In a recent review on the control of *D. suzukii* through natural products, Xavier et al. (2024) highlighted that the sublethal effects and possible molecular targets of these compounds on this pest remain little explored. In our study, we evaluated the effects of isolated and nanoparticle compounds on enzymes related to oxidative stress and detoxification, SOD, CAT, GPx, and GST. When there is an accumulation of reactive oxygen species (ROS) in the organism due to oxidative stress, there is activation of defense mechanisms via hormonal signaling, which includes an increase in the activity of these detoxifying enzymes (Velki et al. 2011; Kodrík et al. 2015). Our results demonstrate that, in general, PCL-carvacrol caused the most significant response due to the greatest increase in the activity of the enzymes CAT, GST, and GPx when compared to the other treatments. This may indicate that this treatment was more aggressive to insects, promoting greater production of ROS, which triggered a more intense defense response represented by the high activity of the highlighted enzymes. Carvacrol also promoted an increase in the activity of SOD and CAT enzymes in *Lymantria dispar* larvae (Chen et al. 2021), increased the activity of SOD and GST in *Spodoptera littoralis* larvae (Agliassa and Maffei 2018), and increased the activity of CAT in *Acrobasis advenella* larvae (Magierowicz et al. 2019). When defense mechanisms do not act efficiently, the homeostasis balance is not maintained in cells, and excessive accumulation of ROS occurs in tissues, which can cause physiological and morphological damage, such as protein oxidation, damage to nucleic acids, and membrane lipid peroxidation (Li-Byarlay and Cleare 2020; Lennicke and Cochemé 2021).

Some molecules are indicators of damage caused by oxidative stress, notably malondialdehyde (MDA). ROS, such as superoxide radical anion and non-radical peroxide anion, are highly reactive, abstracting electrons from several classes of biomolecules, including proteins, nucleic acids, and lipids, especially polyunsaturated fatty acids (Tsikas 2017). Radical peroxidation of fatty acids, such as arachidonic or linoleic acid, leads to the formation of MDA

(Nam 2011). In this study, a greater increase in MDA concentration was observed in treatments in which the chemical compounds were in their isolated form. This observation suggests that the low defensive response promoted by the non-increase in the activity of detoxifying enzymes may have caused the accumulation of ROS in insects, promoting greater lipid peroxidation. Treatments with compounds in PCL nanoparticles promoted a slight reduction in MDA content when compared to the PCL control. In these treatments, there was a more effective defense response, with increased activity of detoxifying enzymes, especially in PCL-carvacrol. This factor probably reduced the availability of ROS, decreasing MDA production.

In addition to promoting lipid peroxidation, the accumulation of ROS can promote histopathological changes in organs that are particularly important for insect survival (El-Ashram et al. 2021). Histopathological analyses showed that the terpenes and phenylpropanoids compounds, either isolated or in nanoparticle form, increased the thickness of the thoracic exoskeleton of female *D. suzukii*. The exoskeleton is normally the first barrier of protection of insects against damage caused by chemical, physical, and biological agents (Zhu et al. 2016). In a previous study, an increase in the thickness of the exoskeleton was also observed in *D. suzukii* larvae (de Souza et al. 2024), suggesting a defense response to prevent toxic compounds from reaching the interior of the organism. Regarding the midgut epithelium, treatments with nanoparticle compounds increased the thickness of this structure. The insect midgut is a tube formed by a cell monolayer (Hakim et al. 2010). This is the region of the digestive tract of insects most susceptible to the action of ingested pesticides, and cell elongation can be observed in the presence of xenobiotic compounds (Lavarías et al. 2017). This fact may justify the increase in thickness observed in this study, indicating a probable difficulty in absorbing nutrients. As for thoracic muscle fibers, treatments with nanocompounds, especially PCL-carvacrol, reduced fiber thickness. In a previous study, star anise oil, composed basically of (*E*)-anethole, also reduced the thickness of thoracic muscle fibers in female *D. suzukii* (de Souza et al. 2022). Muscle damage can compromise the insects' ability to move and fly, reducing their action potential. Finally, the analysis of lipid droplets in the fat body showed a reduction in the area of these structures in treatments with PCL nanoparticle compounds. No difference was observed in the density of these structures in the fat body. The reduction in lipid reserves in the fat body may indicate that the insect needed to use this energy source due to the impossibility of absorbing nutrients from the food present in the digestive tract or due to the high energy demand resulting from the detoxification process (Li-Byarlay and Cleare 2020; Lennicke and Cochemé 2021).

The search for environmentally safer pesticides must be aligned with the search for alternatives that cause less harm to non-target organisms (Konig et al. 2023). The search for alternative pesticides is also necessary to reduce the damage caused by synthetic pesticides to non-target organisms. In this sense, the evaluation of lethal and sub-lethal effects of plant-derived compounds on insect pests and non-target organisms is becoming more frequent. In our study, terpenes and phenylpropanoids in their isolated or nanoparticle form did not affect the parasitism, emergence, and sex ratio of *P. elaeisis*. The PCL nanoparticles and nanoparticles containing terpenes and phenylpropanoids promoted slightly faster development of parasitoid progeny. Additionally, PCL nanoparticles containing terpenes and phenylpropanoids induced a slight increase in offspring generation. This parasitoid is used in integrated pest management (IPM) programs to control different pest insects (Rolim et al. 2020). A recent study that evaluated the effect of terpenes and phenylpropanoids (*E*)-anethole, (*E*)-cinnamaldehyde L-(-)-carvone and carvacrol on the predator *Doru luteipes*, demonstrated that these compounds did not affect the survival and feeding capacity of this insect (de Souza et al. 2024). Because they do not drastically affect the reproductive parameters of natural enemies such *P. elaeisis* and *D. luteipes* like some synthetic commercial products, the compounds under study can be considered new alternatives to be implemented in IPM programs.

In conclusion, the incorporation of terpenes and phenylpropanoids into PCL nanoparticles prolonged the insecticidal activity of these compounds over time, particularly the compounds PCL-carvacrol and PCL-(*E*)-cinnamaldehyde. The compounds in nanoparticles demonstrated greater potential to activate enzymatic defenses against oxidative stress, especially PCL-carvacrol, indicating greater potential to cause damage to the organism of insect pests. The compounds in their isolated form promoted more significant lipid peroxidation, indicated by the higher concentration of malondialdehyde, possibly because they did not generate high activity of detoxifying enzymes. Despite promoting increased activity of enzymes that combat oxidative stress, the nanoparticle compounds demonstrated increased potential to cause histopathological damage in several structures of adult females of *D. suzukii*. Finally, the compounds under study did not affect the non-target organism *P. elaeisis*. These results are promising and accredit the compounds under study as possible candidates for use in IPM. However, further studies are needed to evaluate the performance of these nanomaterials under realistic field environmental conditions as well as their toxicity to other organisms and ecosystems.

**CRediT authorship contribution statement**

Luciano Souza: Investigation, Formal analysis, Writing – original draft; Maria das Graças Cardoso: Resources, Conceptualization, Methodology, Validation, Supervision, Writing – original draft; Isaac Filipe Moreira Konig: Methodology, Investigation, Writing – original draft; Stefânia Priscilla de Souza: Investigation, Data curation; Cassia Duarte Oliveira: Methodology, Investigation; Ezequiel Gracia de Souza: Methodology, Investigation; Juliano Elvis de Oliveira: Resources, Methodology, Validation, Supervision; Hallen Daniel Rezende Calado: Methodology, Validation; Rosangela Cristina Marucci: Resources, Methodology, Validation, Supervision, Writing – original draft; Khalid Haddi: Resources, Conceptualization, Methodology, Validation, Supervision, Formal analysis, Writing – review & editing.

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**Declaration of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Table 1 -** Average size, polydispersity index and zeta potential of isolated polycaprolactone (PCL) particles containing the terpenes carvacrol and L-(-)-carvone and the phenylpropanoids (*E*)-anethole and (*E*)-cinnamaldehyde.

| Sample | Average particle size (nm) | Polydispersity index | Zeta potential(mV) |
|---|---|---|---|
| PCL | 175 (96.5%) | 0.181 | -9.19 |
| PCL-(*E*)-anethole | 314 (85.4%) | 0.558 | -5.67 |
| PCL-(*E*)-cinnamaldehyde | 235 (96.6%) | 0.277 | -7.19 |
| PCL-L-(-)-carvone | 247 (97.8%) | 0.248 | -9.94 |
| PCL-carvacrol | 282 (88.2%) | 0.663 | -24.6 |

**Table 2 -** Toxicological performance of the terpenes carvacrol and L-(-)-carvone and the phenylpropanoids (*E*)-anethole and (*E*)-cinnamaldehyde isolated or in poly(ε-caprolactone) (PCL) nanoparticles on *Drosophila suzukii* adults.

| **Chemical compound** | **Form** | **Number of insects** | **$LC_{50}$ (95% FI) (µL.mL$^{-1}$)** | **Active ingredient concentration (95% FI) (µL.mL$^{-1}$)** | $\chi^2$ | ***P*** | **TR $LC_{50}$ (95% IF)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $TR_1$ | $TR_2$ | $TR_3$ |
| (*E*)-anethole | Isolated | 600 | 0.63 (0.52-0.77) | 0.63 (0.52-0.77) | 7.2009 | 0.0658 | 1.29 | - | 40.15 |
| | Nanoparticle | 500 | 25.30 (23.85-26.88) | 0.63 (0.60-0.67) | 0.7123 | 0.7004 | - | 1.48 | |
| (*E*)-cinnamaldehyde | Isolated | 500 | 0.58 (0.55-0.61) | 0.58 (0.55-0.61) | 0.3157 | 0.8540 | 1.18 | - | 29.52 |
| | Nanoparticle | 600 | 17.12 (16.04-18.17) | 0.43 (0.40-0.45) | 5.4277 | 0.1430 | - | * | |
| carvacrol | Isolated | 500 | 0.71 (0.65-0.77) | 0.71 (0.65-0.77) | 4.1367 | 0.1264 | 1.45 | - | 34.82 |
| | Nanoparticle | 600 | 24.72 (21.83-27.65) | 0.61 (0.55-0.69) | 1.2112 | 0.7503 | - | 1.44 | |
| L-(-)-carvone | Isolated | 500 | 0.49 (0.47-0.51) | 0.49 (0.47-0.51) | 1.4755 | 0.6879 | * | - | 37.59 |
| | Nanoparticle | 600 | 18.42 (17.41-19.47) | 0.46 (0.44-0.49) | 3.4554 | 0.3266 | - | 1.08 | |

Where: $LC_{50}$ is the lethal concentration for 50% of individuals; (95% IF) represents the 95% fiducial interval; $\chi^2$ is the chi-square for lack of fit to the probit model; P is the probability associated with the chi-square statistic. TR: toxicity ratio calculated by dividing the $LC_{50}$ of each compound by the lowest value between all $LC_{50}$s. $TR_1$: toxicity ratio calculated for isolated compouds.$TR_2$: toxicity ratio calculated for Nanoparticles.$TR_3$: toxicity ratio calculated for the isolated compounds versus their nanoparticles. (*): the compound that presented the lowest $LC_{50}$ (L-(-)-carvone for the isolated and PCL-(*E*)-cinnamaldehyde for the nanoparticles) and was used to compute TR. The $TR_3$ for the isolated compounds versus their nanoparticles form was calculated using the lowest $LC_{50}$ values as a reference, which was always the isolated form.

**Table 3 -** Morphometric analysis of structures of adult female *Drosophila suzukii* exposed for 24 hours to the controls 2% DMSO and poly(ε-caprolactone) (PCL) nanoparticles and to the $LC_{50}$ of (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol in their isolated forms or in nanoparticles.

| | **Thoracic Muscle fiber thickness (µm)** | **Midgut epithelium thickness (µm)** | **Thoracic Exoskeleton thickness (µm)** | **Area of the fat body lipid droplets (µm²)** | **Density of lipid droplets of the fat body (lipid droplets/ 1000 µm²)** |
|---|---|---|---|---|---|
| 2% DMSO control | 91.20 ± 18.25 a | 132.60 ± 23.98 a | 4.58 ± 0.52 a | 181.10 ± 50.72 a | 3.05 ± 0.66 a |
| (*E*)-anethole | 92.30 ± 15.5 a | 132.00 ± 24.31 a | 6.06 ± 1.17 ****, a | 179.70 ± 50.73 a | 2.87 ± 0.31 a |
| (*E*)-cinnamaldehyde | 90.72 ± 17.28 a | 133.20 ± 24.79 a | 5.24 ± 0.91 **, a | 179.20 ± 49.41 a | 2.84 ± 0.33 a |
| L-(-)-carvone | 93.92 ± 17.81 a | 142.20 ± 21.07 a | 5.44 ± 0.91 ****, a | 169.80 ± 44.38 a | 2.84 ± 0.40 a |
| carvacrol | 93.04 ± 18.25 a | 135.20 ± 29.83 a | 5.58 ± 1.03 ****, a | 180.60 ± 57.41 a | 2.76 ± 0.32 a |
| PCL control | 97.34 ± 16.21 a | 127.50 ± 24.95 a | 4.66 ± 0.83 a | 180.60 ± 50.89 a | 2.76 ± 0.45 a |
| PCL-(*E*)-anethole | 86.51 ± 15.76 **, a | 153.30 ± 31.30 ***, b | 5.71 ± 1.36 ****, a | 137.80 ± 51.67 ****, b | 2.98 ± 0.31 a |
| PCL-(*E*)-cinnamaldehyde | 89.96 ± 20.86 a | 152.70 ± 27.6 ***, b | 6.00 ± 1.13 ****, b | 144.90 ± 44.69 ****, b | 2.87 ± 0.49 a |
| PCL-L-(-)-carvone | 78.66 ± 14.73 ****, b | 150.00 ± 33.79 **, a | 5.91 ± 1.03 ****, b | 133.30 ± 45.52 ****, b | 2.89 ± 0.37 a |
| PCL-carvacrol | 60.14 ± 18.59 ****, b | 152.50 ± 36.10 ***, b | 5.93 ± 1.11 ****, a | 149.40 ± 45.92 ****, b | 2.73 ± 0.28 a |

Isolated compounds were compared with 2% DMSO control, and PCL-encapsulated compounds were compared with PCL control using one-way ANOVA followed by Tukey's post hoc test, and significant results are represented by an asterisk (**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Pairwise comparisons between the isolated and its respective PCL-encapsulated compound were conducted using Welch's t-test, indicated by different lowercase letters for significant results, $p < 0.05$.

**Table 4 -** The mean survival time (days) of *Palmistichus elaeisis* females exposed to *Tenebrio molitor* pupae treated with the $LC_{50}$ of the compounds (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol isolated or in poly($\varepsilon$-caprolactone) (PCL) nanoparticles.

| Treatment | Survival time (days) | SE |
|---|---|---|
| Control DMSO 2% v/v | 14.542 (13.246-15.839) [a] | 0.662 |
| Control PCL | 12.958 (12.021-13.895) [b] | 0.478 |
| (*E*)-anethole | 12.112 (10.832-13.392) [b] | 0.653 |
| PCL-(*E*)-anethole | 12.737 (11.586-13.888) [b] | 0.587 |
| (*E*)-cinnamaldehyde | 13.822 (12.669-14.975) [a] | 0.588 |
| PCL-(*E*)-cinnamaldehyde | 15.714 (14.762-16.667) [a] | 0.486 |
| L-(-)-carvone | 14.278 (12.842-15.715) [a] | 0.733 |
| PCL-L-(-)-carvone | 13.025 (11.835-14.216) [a] | 0.607 |
| carvacrol | 15.009 (13.550-16.467) [a] | 0.744 |
| PCL-carvacrol | 14.017 (12.922-15.112) [a] | 0.558 |

Survival was analyzed using the Kaplan-Meier test. Means in the same column followed by the same letters do not differ compared to the 2% DMSO control group ($p > 0.05$). LC = lethal concentration.

**Figure 1 -** Toxicity over time of the phenylpropanoids (*E*)-anethole (A) and (*E*)-cinnamaldehyde (B) and the terpenes carvacrol (C) and L-(-)-carvone (D) isolated or in poly(ε-caprolactone) (PCL) nanoparticles on *Drosophila suzukii* adults. The mortality of each isolated compound and its nanoparticle was compared at each time interval and different letters indicate statistical differences at that given interval of time (α = 0.05).

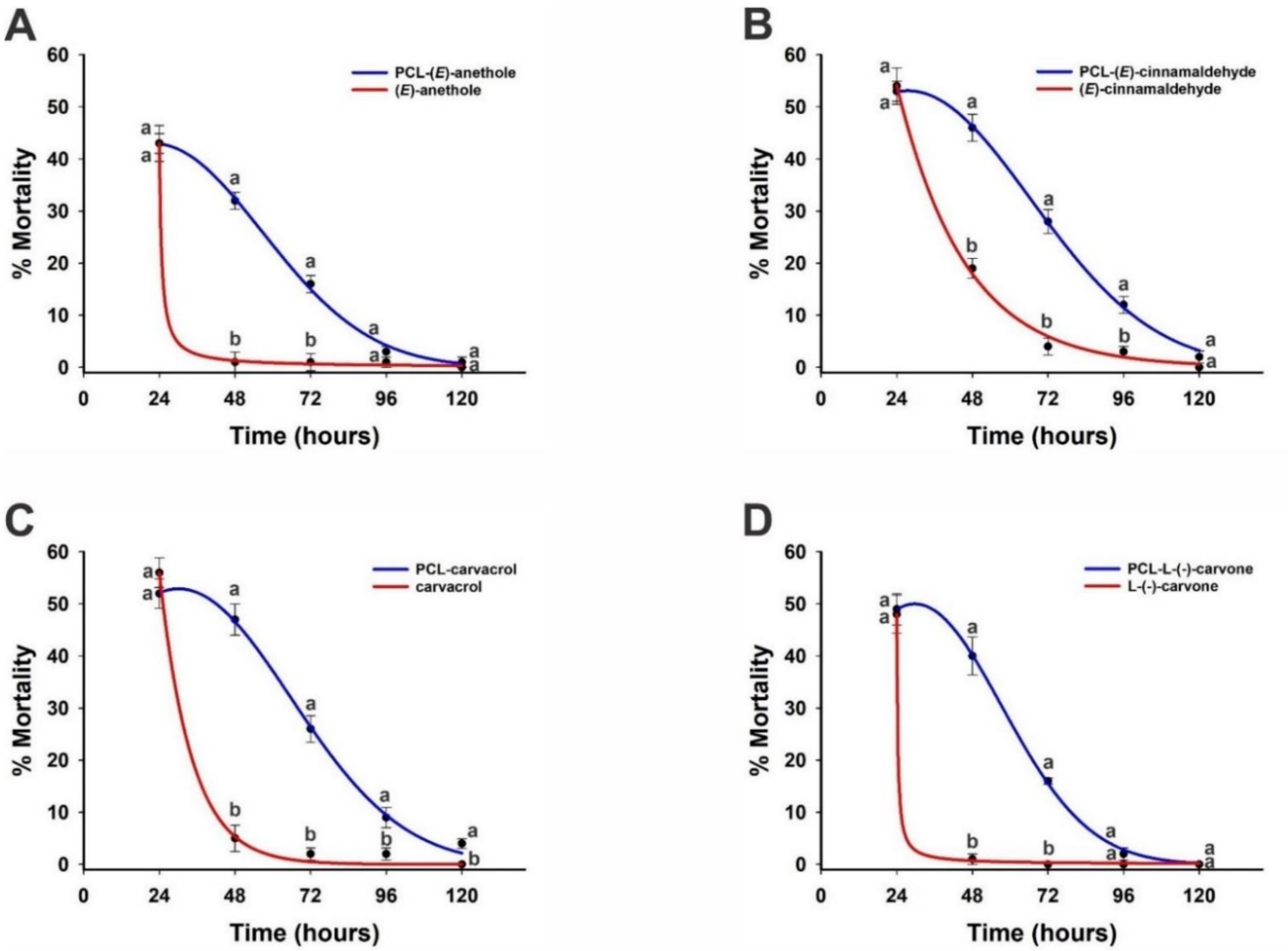


**Figure 2 -** Activity of Catalase (A), Superoxide dismutase (B), Glutathione S-transferase (C), and Glutathione peroxidase (D) enzymes in adult female *Drosophila suzukii* exposed for 4 hours to the control groups and to the $LC_{50}$ of the compounds (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol isolated or in poly(ε-caprolactone) (PCL) nanoparticles. One-Way ANOVA followed by Dunnett's post hoc test for comparisons of isolated compounds with 2% DMSO control and PCL-encapsulated compounds with PCL control, indicated by asterisk $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. Pairwise comparisons between the isolated and its respective PCL-encapsulated compound were conducted using Welch's t-test, indicated by different capital letters for significant results, $p < 0.05$, n=5/treatment.

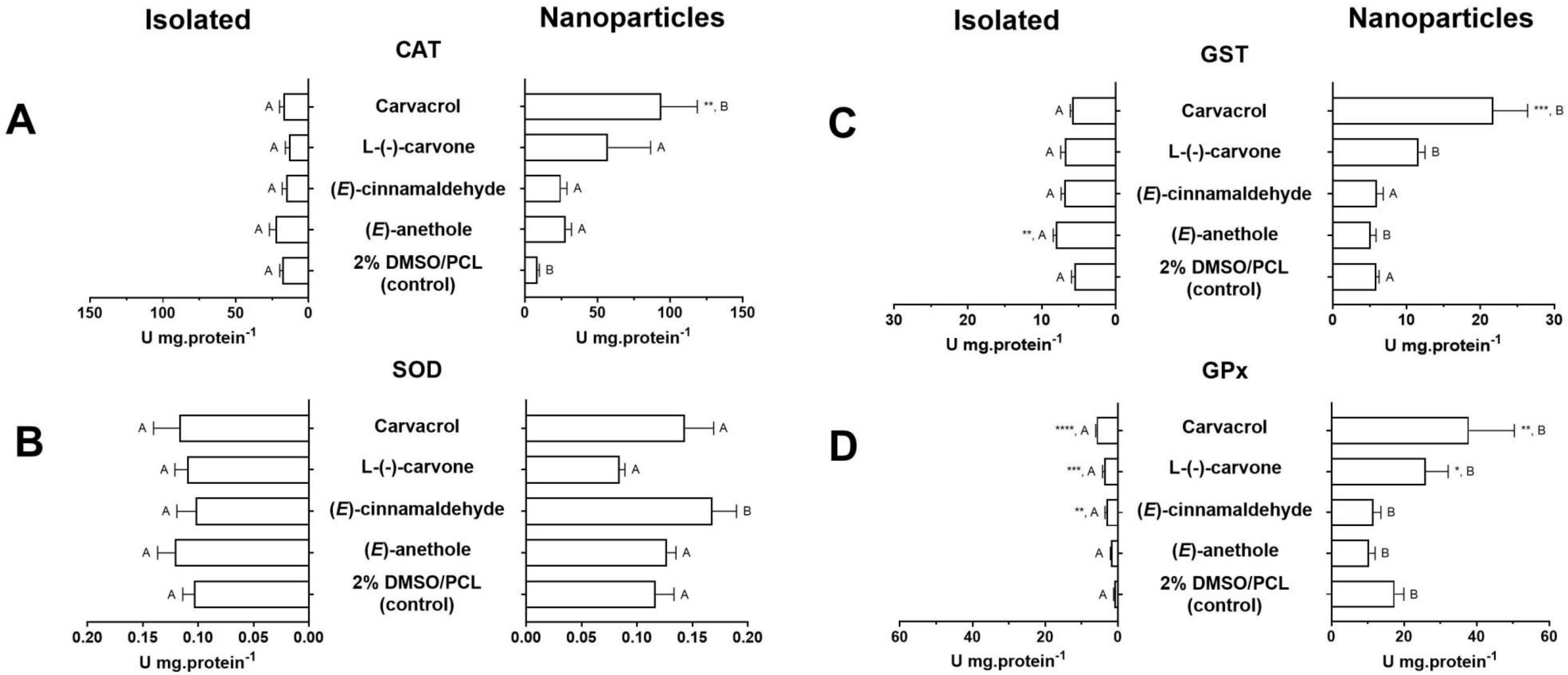


**Figure 3 -** Lipid peroxidation determined by the concentration of malondialdehyde (MDA) in adult females of *Drosophila suzukii* exposed for 4 hours to the control groups and to the $LC_{50}$ of the compounds (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol alone or in poly(ε-caprolactone) (PCL) nanoparticles. One-Way ANOVA followed by Dunnett's post hoc test for comparisons of isolated compounds with 2% DMSO control and PCL-encapsulated compounds with PCL control, indicated by asterisk *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Pairwise comparisons between the isolated and its respective PCL-encapsulated compound were conducted using Welch's t-test, indicated by different capital letters for significant results, $p < 0.05$, n=5/treatment.

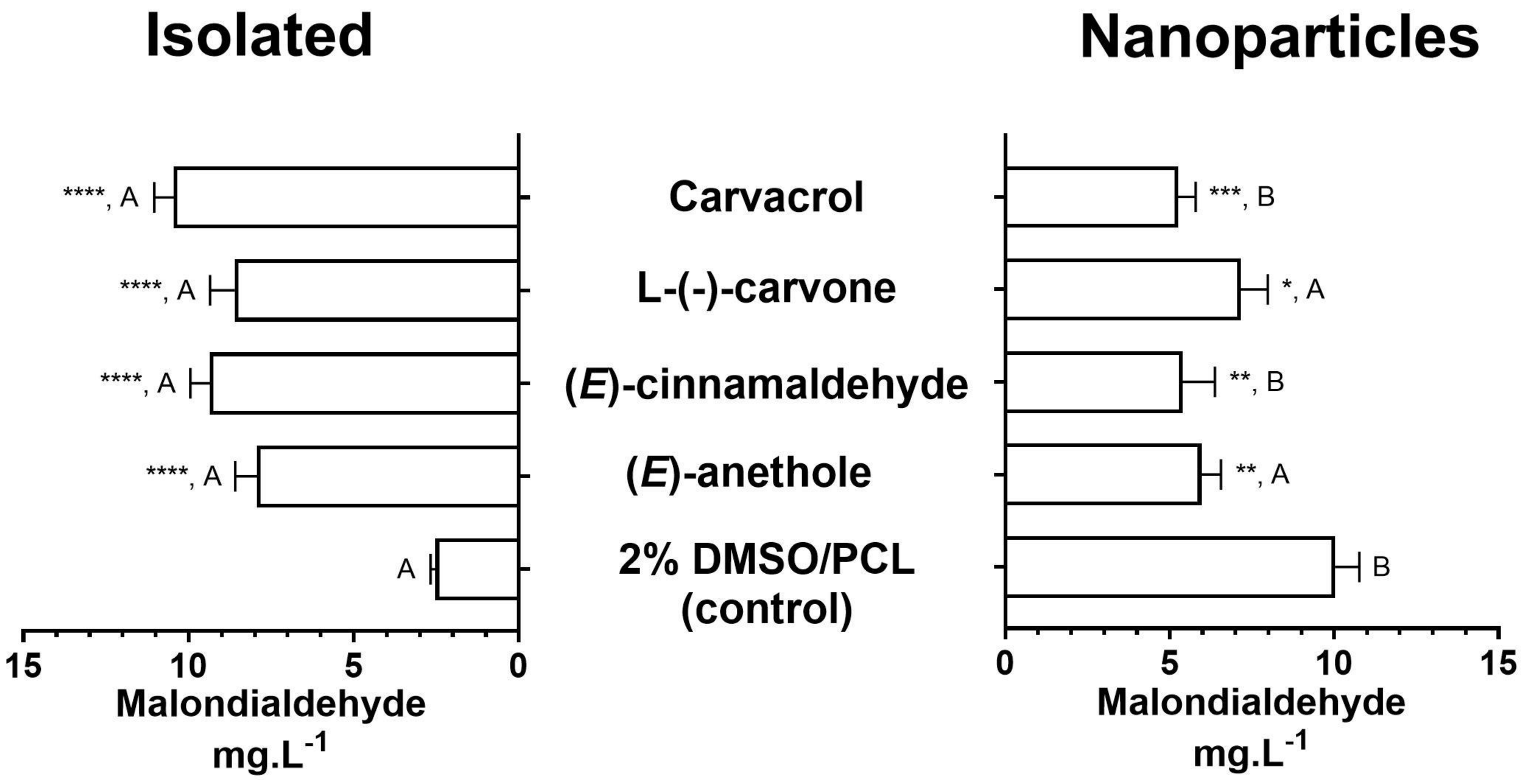


**Figure 4 -** Representation of the main histological alterations in adult females of *Drosophila suzukii*. All histological sections were stained with hematoxylin and eosin. Solvent control group (A); PCL control group (B); $LC_{50}$ PCL-L-(-)-carvone group (C); $LC_{50}$ PCL-carvacrol group (D); Group $CL_{50}$ PCL-(*E*)-anethole (E) and Group $CL_{50}$ (*E*)-anethole (F). Scale bars: 50 µm. Captions: AD – adipocyte; DMF – reduction in thoracic muscle fiber thickness; E – exoskeleton; IET – intestinal epithelial thickness; RAD – reduction in adipocyte area; RMF – muscle fiber rupture; TE – exoskeleton thickening; TIE – intestinal epithelial thickening; TMF – thoracic muscle fiber thickness.

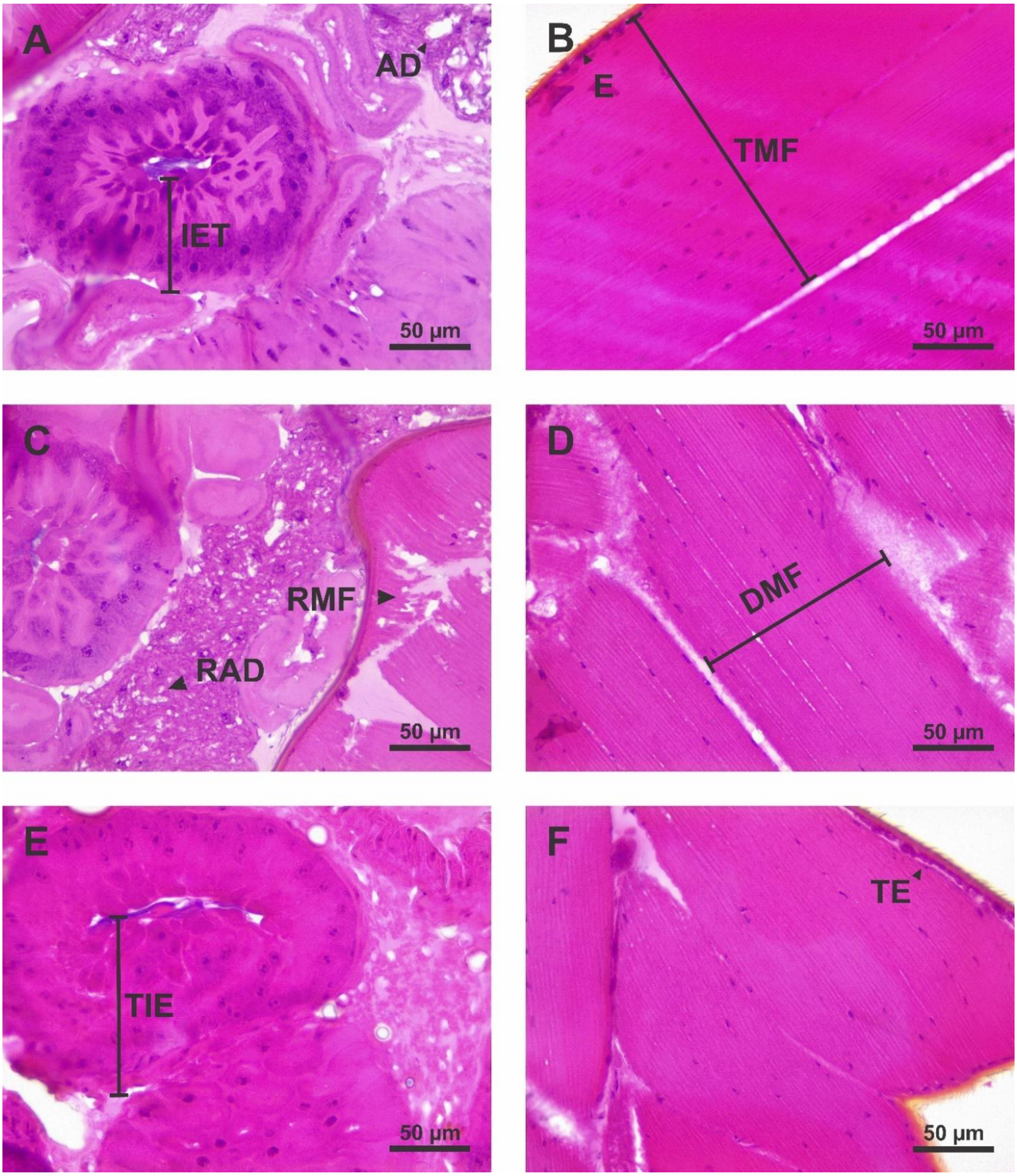
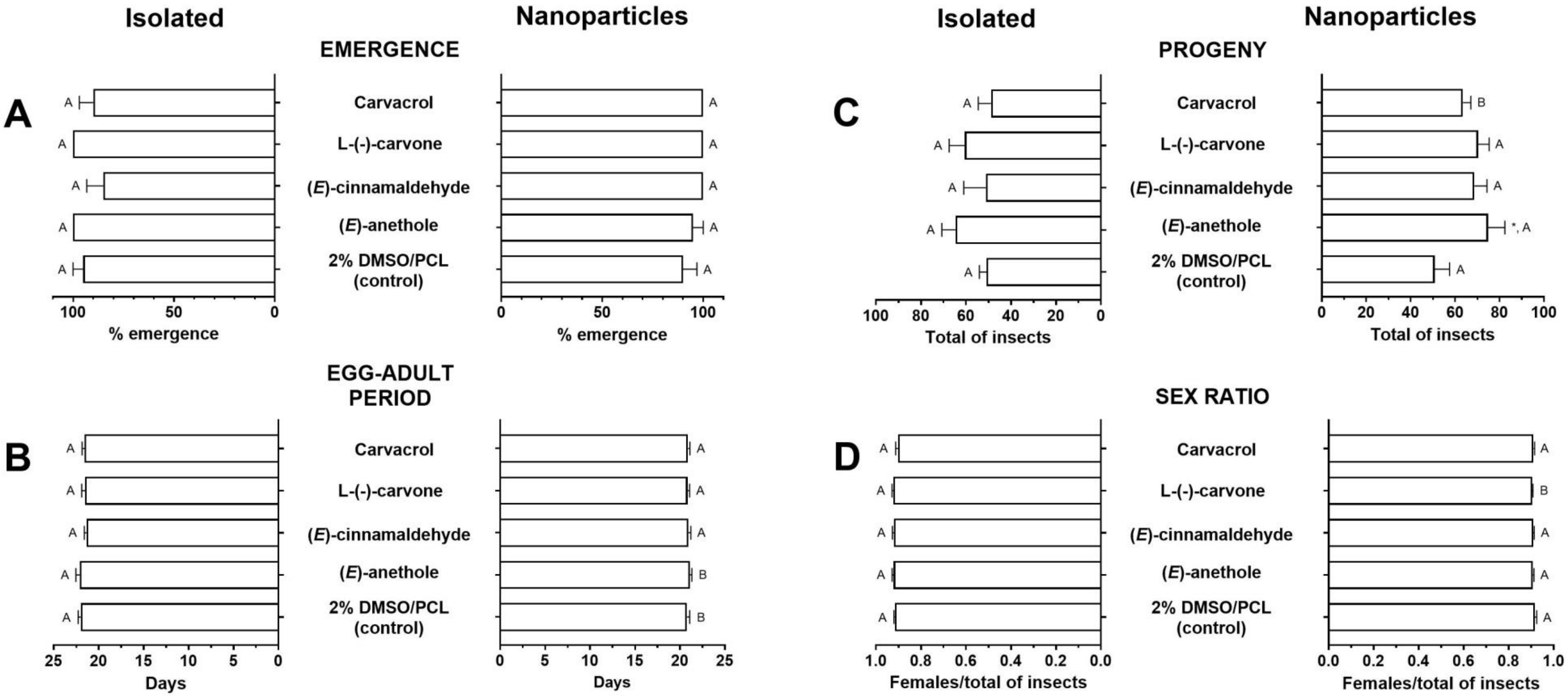


**Figure 5 -** Emergence (A), egg-to-adult period (B), progeny (C), and sex ratio (D) of *Palmistichus elaeisis* obtained from parasitized *Tenebrio molitor* pupae treated with the $CL_{50}$ of the compounds (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol alone or in poly(ε-caprolactone) (PCL) nanoparticles. (One-Way ANOVA followed by Dunnett's post hoc test for comparisons of isolated compounds with 2% DMSO control and PCL-encapsulated compounds with PCL control, indicated by asterisk $*p \leq 0.05$. Pairwise comparisons between the isolated and its respective PCL-encapsulated compound were conducted using Welch's t-test, indicated by different capital letters for significant results, $p < 0.05$, n=20/treatment).



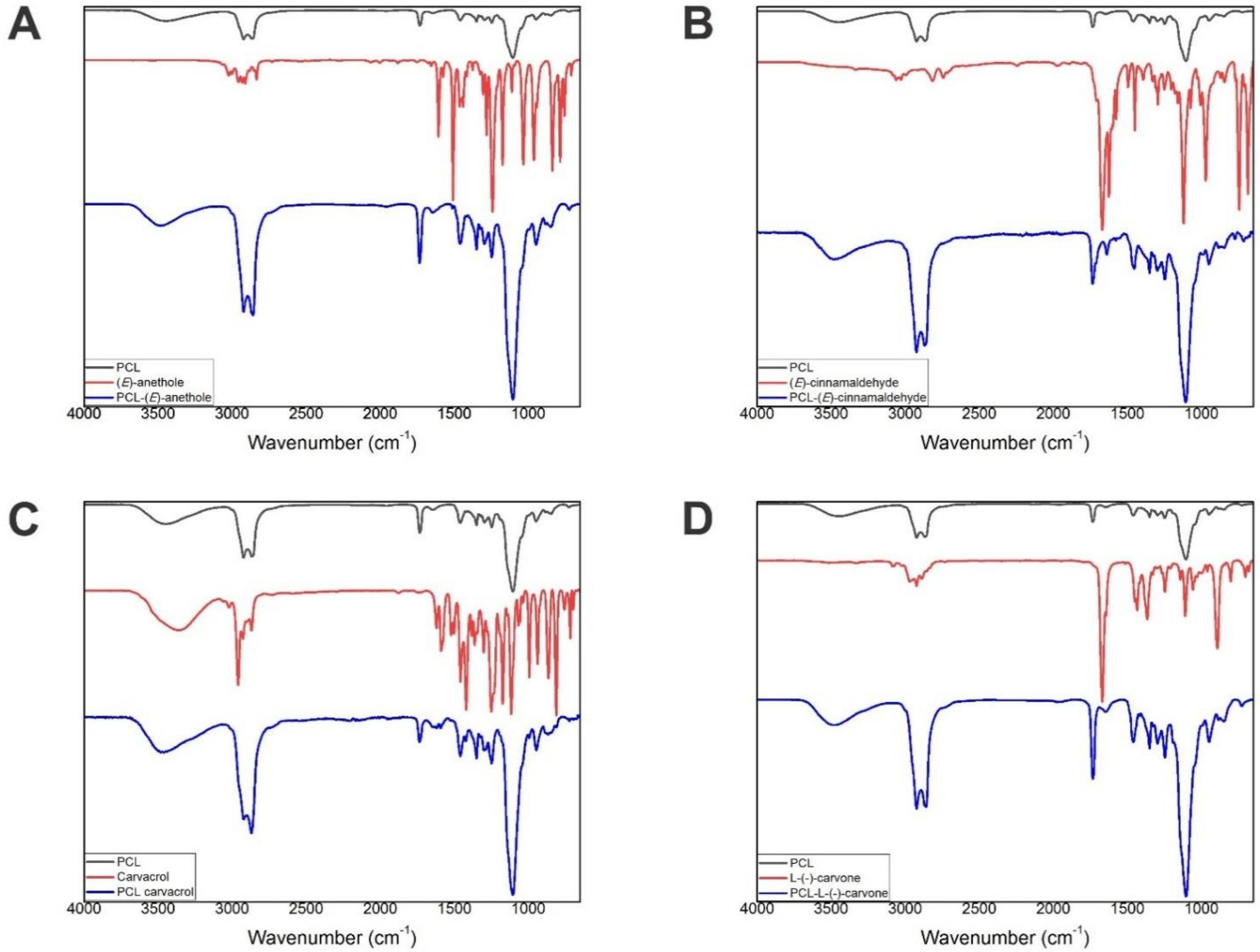
**Supplementary Table 1 -** Statistical outcomes regarding pairwise comparisons between carvacrol, L-(-)-carvone, (*E*)-anethole, and (*E*)-cinnamaldehyde in their isolated form with their respective poly(ε-caprolactone) (PCL) nanoparticles against *Drosophila suzukii* adults, t-test, $p < 0.05$.

| **Time point** | (*E*)-anethole | (*E*)-cinnamaldehyde | carvacrol | L-(-)-carvone |
|---|---|---|---|---|
| **24 h** | t = 0.000, $df$ = 6.02, $p$ = 1.000 | t = 0.253, $df$ = 4.67, $p$ = 0.811 | t = 1.000, $df$ = 6.00, $p$ = 0.356 | t = 0.212, $df$ = 5.78, $p$ = 0.840 |
| **48 h** | t = 16.189, $df$ = 4.97, $p$ < 0.001 | t = 8.399, $df$ = 5.53, $p$ < 0.001 | t = 10.726, $df$ = 5.80, $p$ < 0.001 | t = 10.301, $df$ = 3.45, $p$ = 0.001 |
| **72 h** | t = 7.833, $df$ = 4.97, $p$ < 0.001 | t = 8.480, $df$ = 5.40, $p$ < 0.001 | t = 8.485, $df$ = 4.15, $p$ < 0.001 | t = 27.713, $df$ = 3.00, $p$ < 0.001 |
| **96 h** | t = 1.414, $df$ = 6.00, $p$ = 0.207 | t = 4.700, $df$ = 4.97, $p$ = 0.005 | t = 3.130, $df$ = 4.93, $p$ = 0.026 | t = 1.732, $df$ = 3.00, $p$ = 0.182 |
| **120 h** | t = 1.000, $df$ = 3.00, $p$ = 0.391 | t = 1.732, $df$ = 3.00, $p$ = 0.182 | t = 4.382, $df$ = 3.00, $p$ = 0.022 | t = 8.000, $df$ = 4.00, $p$ = 1.000 |

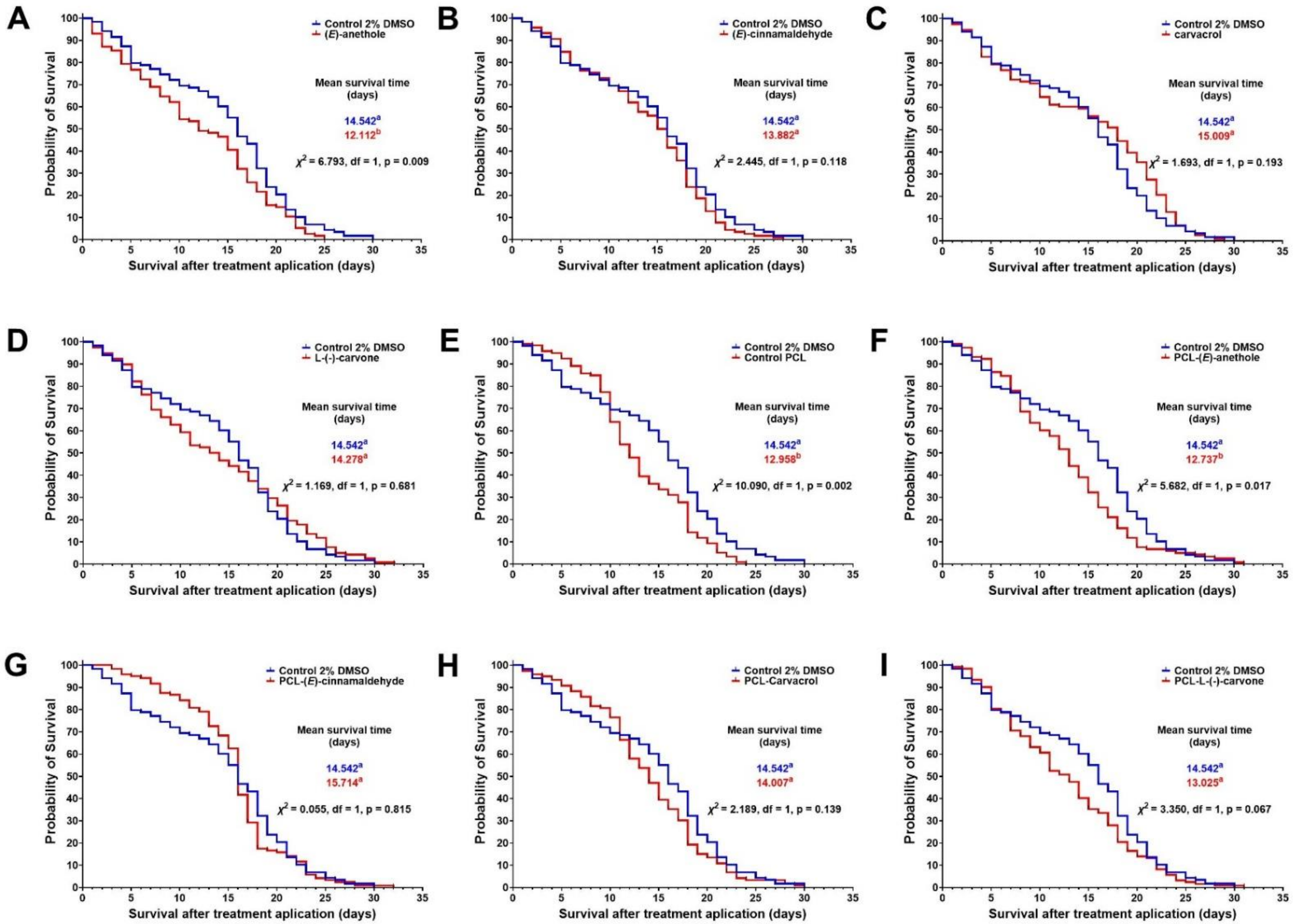
**Supplementary Table 2 -** Summary of nonlinear regression analyses of toxicity over time on adult female *Drosophila suzukii* exposed to $LC_{50}$ of (*E*)-anethole, (*E*)-cinnamaldehyde, L-(-)-carvone and carvacrol in their isolated or nanoparticle forms.

| Treatments | Models | Estimated parameters | | | $df_{error}$ | F | *P* | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| | | ***a*** | ***b*** | $x_0$ | | | | |
| (*E*)- cinnamaldehyde | $y = a^{(-bx)}$ | 163 (121 – 206) | 0.05 (0.04 – 0.06) | - | 4 | 943 | < 0.0001 | 0.9968 |
| carvacrol | | 587 (86 – 1088) | 0.10 (0.06 – 0.13) | - | 4 | 1,131 | < 0.0001 | 0.9974 |
| (*E*)-anethole | $y = {}^{a}/_{(1+bx)}$ | -1.37 (-2.73 – -0.01) | -0.043 (-0.044 – -0.041) | - | 4 | 6,833 | < 0.0001 | 0.9996 |
| L-(-)-carvone | | -0.71 (-1.61 – -0.17) | -0.042 (-0.043 – -0.041) | - | 4 | 11,481 | < 0.0001 | 0.9998 |
| PCL-(*E*)- cinnamaldehyde | $y = a^{\left[-0.5\left(\frac{x-x_0}{b}\right)^2\right]}$ | 53 (49 – 57) | 39 (32 – 47) | 28 (18 – 37) | 4 | 955 | 0.0010 | 0.9990 |
| PCL-carvacrol | | 53 (48 – 58) | 36 (26 – 45) | 30 (19 – 41) | 4 | 4,723 | 0.0021 | 0.9979 |
| PCL-(*E*)-anethole | | 43 (37 – 49) | 34 (22 – 47) | 22 (5 – 40) | 4 | 473 | 0.0021 | 0.9979 |
| PCL-L-(-)-carvone | | 50 (47 – 53) | 28 (24 – 32) | 30 (25 – 34) | 4 | 1,975 | 0.0005 | 0.9995 |

**Supplementary Figure 1 -** Infrared spectra obtained for the phenylpropanoids (*E*)-anethole (A) and (*E*)-cinnamaldehyde (B) and the terpenes carvacrol (C) and L-(-)-carvone (D) isolated and in poly(ε-caprolactone) (PCL) nanoparticles.



**Supplementary Figure 2 -** Survival analysis curves of *Palmistichus elaeisis* females exposed to *Tenebrio molitor* pupae treated with the $LC_{50}$ of the compounds (*E*)-anethole (A), (*E*)-cinnamaldehyde (B), carvacrol (C), L-(-)-carvone (D), control PCL (E), PCL-(*E*)-anethole (F), PCL-(*E*)-cinnamaldehyde (G), PCL-carvacrol (H), and PCL-L-(-)-carvone (I). (Log-rank analysis followed by the Holm-Sidak test, n=120/treatment).



# ARTIGO III

## "Synergism, biochemical changes and morphophysiological impacts induced by terpenes and phenylpropanoids in *Sitophilus zeamais*".

Artigo redigido conforme a norma do periódico Pesticide Biochemistry and Physiology a qual foi submetido.

### Highlights

- Carvacrol had the best insecticidal activity against *Sitophilus zeamais*.
- A mixture of (*E*)-anethole and *p*-anisaldehyde showed the strongest synergistic effect.
- *p*-anisaldehyde induced the activation of oxidative stress-related enzymes.
- *p*-anisaldehyde inhibited *α*-amylase and increased lipase activity.
- (*E*)-cinnamaldehyde and *p*-anisaldehyde induced the most histopathological alterations.


### Abstract

This study aimed to investigate the insecticidal properties of pure and binary mixtures L-(-)-carvone, carvacrol, β-citronellol, (*E*)-anethole, (*E*)-cinnamaldehyde, p-anisaldehyde, and eugenol on adults of *Sitophilus zeamais*, an important pest of stored grains. Subsequently, the effects of selected compounds on the activity of oxidative stress-related detoxifying enzymes and digestive enzymes, as well as on the histopathology of *S. zeamais* adults were evaluated. Finally, the selectivity of these compounds to the non-target pupal parasitoid *Tetrastichus howardi* was assessed. Carvacrol exhibited the highest insecticidal activity ($LC_{50}$ = 3.31 $\mu L.mL^{-1}$), followed by (*E*)-cinnamaldehyde ($LC_{50}$ = 4.15 $\mu L.mL^{-1}$) and *p*-anisaldehyde ($LC_{50}$ = 4.48 $\mu L.mL^{-1}$) meanwhile (*E*)-anethole showed the greatest synergistic activity in binary mixtures. p-anisaldehyde promoted strong activation of the enzymes catalase, glutathione-S-transferase, and glutathione peroxidase while (*E*)-cinnamaldehyde and *p*-anisaldehyde increased the malondialdehyde content and lipid peroxidation compared to the control. Additionally, *p*-anisaldehyde strongly inhibited *α*-amylase activity and increased lipase activity. Moreover, insects exposed to carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde exhibited decreased abdominal muscle fiber thickness, midgut epithelial thickness, midgut regenerative crypt diameter, as well as reduced lipid droplet area and carbohydrate concentration in the fat body. (*E*)-cinnamaldehyde was the compound that showed the greatest compatibility with the non-target organism *T. howardi*. Taken together, our findings suggest that these natural compounds promote appreciable insecticidal activity and are alternatives for the control of *S. zeamais*.

**Keywords**: Bioinsecticide. Corn weevil. Oxidative stress. Morphological changes. $\alpha$-amylase inhibition. Eco-friendly.

## 1. Introduction

Terpenes and phenylpropanoids are structurally diverse families of organic plant secondary metabolites that play an important role in plant-insect and plant-pathogen interactions and provide an array of biological activities of interest for agricultural systems (Cheng et al., 2007). These compounds, alone or as major constituents of essential oils (EOs), are frequently reported to exhibit insecticidal activity against adults and young stages of different insect species (Baker et al., 2023; da Silva et al., 2020; Pascual-Villalobos et al., 2020; Xie et al., 2019) and may offer to the pesticides industry promising sources of new insecticidal molecules and insecticide synergists (Baker et al., 2023). The assessment of the potential of synthesized pure plant secondary metabolites like terpenes and phenylpropanoids can be a good alternative to the use of naturally occurring mixtures like plants' essential oils. Especially since most of the studies with essential oils are not directly converted into commercial products due to the variation in the EOs chemical composition within the same plant species (Isman, 2005; Pavela and Benelli, 2016; Turek and Stintzing, 2013), low stability against oxidation, low water solubility, and high volatility, complicating practical field applications (Mossa, 2016; Pavela and Benelli, 2016).

Although the subjacent mechanisms of EOs toxicity have not been fully elucidated, it is known that as part of the protection mechanisms against herbivory, plant metabolites can affect several vital physiological functions in insects.  EOs generally present mixtures of different compounds in variable proportions, resulting in a variety of biological activities, unspecific targets, and complementary mechanisms of action (Shaaya and Rafaeli, 2007). However, the hyperactivity, convulsions, tremors, and paralysis observed in insects after exposure to EOs or their major compounds point toward the insect´s nervous system as a potential target site. Such neurotoxic action could be the result of interference in the activity of neural enzymes and receptors like acetylcholinesterase, GABA ionotropic, and octopamine receptors (Jankowska et al., 2017). Furthermore, mechanisms involved in the insecticidal action of EOs could derive from intrinsic properties of EOs as the bioactive plants' secondary metabolites can impact key metabolic, biochemical, and physiological in the insect body as well as induce histopathological alterations in insect internal structures (Campolo et al., 2018; Chaaban et al., 2019; Dutra et al., 2019; Osman et al., 2016).

The search for plant-derived alternatives of pest control is essential to reduce the shortcomings of synthetic insecticides. In this sense, in addition to the evaluation of lethal and sub-lethal effects, understanding the potential biochemical, physiological, and histological modes of action, both in insect pests and non-target organisms, is extremely important to ensure the compatibility of the new products with other frequently used biocontrol agents (Haddi et al., 2020; Horowitz and Ishaaya, 2004; Konig et al., 2023).

The present study used the maize weevil *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)  one of the main insect pest species that develop in stored products (Napoleão et al., 2015), and *Tetrastichus howardi* (Hymenoptera: Eulophidae), a frequently used parasitoid in biological control programs, to evaluate the insecticidal activity of L-(-)-carvone, carvacrol, *β*-citronellol, (*E*)-anethole, (*E*)-cinnamaldehyde, *p*-anisaldehyde, and eugenol, alone and in binary combinations. Furthermore, the changes induced by the compounds that presented the best toxicological performance on the activity of enzymes related to oxidative stress, digestion processes and lipid peroxidation levels, in addition to the histopathological alterations in internal structures (abdominal muscles, midgut, fat body, and exoskeleton) were assessed in both the insect pest and non-target organisms.

The control of this insect pest is mainly done through the use of synthetic chemical insecticides (Haddi et al., 2015a). However, few compounds approved for the management of this species, combined with indiscriminate use, contributed to the development of resistance in *S. zeamais* (Correa et al., 2014; Haddi et al., 2018, 2015a) to pyrethroids, indoxacarb, malathion, pirimiphos-methyl, fenitrothion, and phosphine (Corrêa et al., 2011; Haddi et al., 2015a; Machuca-Mesa et al., 2024; Pereira et al., 2009).

To circumvent the obstacle of resistance to synthetic insecticides and the environmental impacts they cause, natural products derived from plants are promising alternatives (Isman, 2020; Souto et al., 2021; Sparks and Bryant, 2022). Among the classes of natural products that have the potential to promote the control of insect pests, essential oils stand out (Haddi et al., 2015b; Karabörklü and Ayvaz, 2023), which are formed by mixtures of terpenes and phenylpropanoids (Rehman et al., 2016).

Studies have already demonstrated promising results for the use of essential oils in the control of *S. zeamais* (Bernardi et al., 2024; Moutassem et al., 2024; Pimentel et al., 2023). In fact, the essential oils of *Syzygium aromaticum* L., rich in eugenol, and *Cinnamomum zeylanicum* L., rich in (*E*)-cinnamaldehyde, showed similar insecticidal toxicity through exposure by contact with dry residues, with $LC_{95}$ = 3.96 and 3.47 µL/cm$^2$, respectively. Sublethal doses promoted a stimulating effect on the mean survival time, altered their mobility capacity, and reduced the respiration rate of the insects. Furthermore, the oil of *S. aromaticum* at a sublethal dose increased the number of *S. zeamais* larvae (Haddi et al., 2015b). Additionally, a study evaluated the activity of phenolic compounds on *S. zeamais*, including the terpenes carvacrol and thymol, which are isomers. The authors concluded that the highlighted compounds showed the best performance in the dry residue contact toxicity test, carvacrol ($LC_{50}$ = 221 µmol/cm$^2$) and thymol ($LC_{50}$ = 196 µmol/cm$^2$). Both compounds inhibited the acetylcholinesterase enzyme, with $IC_{50}$ values of 0.019 and 0.96 mM, for carvacrol and thymol, respectively. In addition, thymol caused the greatest weevil repellency, 63% at 40 µM (Rodríguez et al., 2022).

Studies have shown that synthetic insecticides have affected the survival of the parasitoid used in biological control programs, *Tetrastichus howardi* (Hymenoptera: Eulophidae) (Souza Sarmento Moraes et al., 2024; Su et al., 2021). *T. howardi* is a pupal parasitoid that naturally parasitizes a wide variety of lepidopteran pests (Pereira et al., 2021; Silva-Torres et al., 2010), and insects from other orders, such as *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Tiago et al., 2019). Given the importance of this parasitoid, the search for compounds that are less aggressive to non-target organisms, such as *T. howardi*, is necessary.

The objectives of the present study were to evaluate the insecticidal activity of L-(-)-carvone, carvacrol, *β*-citronellol, (*E*)-anethole, (*E*)-cinnamaldehyde, *p*-anisaldehyde and eugenol, alone and in combination, to demonstrate possible synergistic effects on adults of *S. zeamais*. We also evaluated the effects of these selected compounds on the survival and reproductive parameters of the non-target organism *T. howardi*. In this sense, we hope that the data from this study will contribute to the search for effective and environmentally friendly alternative pesticides for the control of *S. zeamais*.

Based on their good performance in the toxicity test (see results section), the compounds carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde were selected for the analysis of the effects of exposure (to $LC_{50}$s) of *S. zeamais* adults on the digestive enzyme inhibition.

## 2. Material and methods

### 2.1. Chemical compounds

The analytical standards of the (*E*)-anethole (99% purity, CAS 4180–23–8), carvacrol (98% purity, CAS 499–75–2), *β*-citronellol (95% purity, CAS 106–22–9), and eugenol (99% purity, CAS 97–53–0) were purchased from Sigma-Aldrich (St. Louis, MO, USA) while *p*-anisaldehyde (99% purity, CAS 123–11–5), L-(-)-carvone (99% purity, CAS 6485–40–1), and (*E*)-cinnamaldehyde (99% purity, CAS 14371–10–9) were purchased from Acros Organics (Geel, Belgium).

### 2.2. Insects

The insects of the species *S. zeamais* used in the bioassays were obtained from a stock colony maintained under controlled conditions (28 ± 2 °C; 70 ± 5% and photoperiod: 12:12 h L/D) at the Laboratory of Molecular Entomology and Ecotoxicology of the Department of Entomology (DEN) of UFLA. The rearing was maintained in glass containers (1 L) and the rearing diet consisted of non-transgenic and pesticide-free corn grains (Haddi et al., 2018). The insects of the species *T. howardi* used in the bioassays were obtained from a colony kept at the Biological Pest Control Laboratory (DEN; UFLA). The adults of this parasitoid were reared in acrylic cages (40×40×60 cm) containing *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) pupae as a host. Newly emerged *T. howardi* individuals were fed pure honey *ad libitum*. The parasitoid rearing was maintained in a room with a controlled environment (25 ± 2 °C, 70 ± 10% RH, and photoperiod of 12:12 h L/D) (Rolim et al., 2020).

## 2.3. Toxicity Bioassays

### 2.3.1. Toxicity of pure phenylpropanoids and terpenes to adults of *Sitophilus zeamais*

All compounds were dissolved in acetone (Sigma-Aldrich, St. Louis, MO, USA) to prepare 6 to 10 serial concentrations ranging from 1 to 10 $\mu L.mL^{-1}$. 200 µL of solution were applied to 5 cm diameter filter paper discs. After complete evaporation of the solvent, the discs were placed on the bottom of 5 cm diameter Petri dishes, before introducing ten unsexed adults of *S. zeamais* per dish (replicate) and sealing them with PVC plastic film. Each concentration had four replicates (n = 40), and acetone alone was used as a control. The Petri dishes were maintained under controlled conditions (25 ± 2 °C, 70 ± 10% RH, and photoperiod of 12:12 h L/D) and mortality was assessed after 72 h. The insects were considered dead when no movement was recorded after a gentle prodding.

### 2.3.2. Toxicity of phenylpropanoids and terpenes binary mixtures to adults of *Sitophilus zeamais*

The testing of the effect of binary mixtures was carried out for (*E*)-anethole, carvacrol, eugenol, *p*-anisaldehyde, L-(-)-carvone, and (*E*)-cinnamaldehyde. The *S. zeamais* adult insects were exposed, as previously described, to the $LC_{50}$ of each compound alone and all possible pairwise combinations for the substances at the $LC_{50}$ and 1:1 ratio (Hummelbrunner and Isman, 2001; Trisyono and Whalon, 1999). The type of interaction was determined by comparing the actual mortalities to the theoretical mortalities based on the equation:

$$E = O_a + O_b\ (1 - O_a)$$

where E is the theoretical mortality for the mixture, and $O_a$ and $O_b$ are the observed mortalities for the isolated compounds at the concentration under study. By definition, $O_a$ will be the highest observed mortality between the pair and, consequently, $O_b$ the lowest mortality.

The effects of the mixtures are designated as antagonistic, additive, or synergistic by analysis using $\chi^2$ comparisons determined by the following equation:

$$\chi^2 = [(O_m - E)]^2 / E$$

where $O_m$ is the observed mortality of the binary mixture and E is the theoretical mortality.

For this experimental arrangement with degrees of freedom equal to 1 and a statistical probability of 95%, the reference value of $\chi^2$ is 3.84. If the experimental value of $\chi^2$ is greater than 3.84, the effect of the interaction can be antagonistic or synergistic. It is concluded that the interaction is antagonistic if the experimental mortality caused by the mixture is less than the theoretical mortality, and the interaction is synergistic if the experimental mortality promoted by the mixture is greater than the theoretical mortality. If the calculated $\chi^2$ value is less than 3.84, it is concluded that the interaction was additive.

### 2.4. Effects of exposure to carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde on oxidative stress biomarkers in adults of *Sitophilus zeamais*

For the analyses of enzymatic activities and lipid peroxidation, adults of *S. zeamais* were exposed to $LC_{50}$ of the compounds carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde as described in section 2.3. All analyses were performed in five independent replicates containing 20 insects, totaling 100 individuals for each treatment. Ten individuals were collected from each replicate after 24 hours of exposure as proposed by Liao et al. (2016). Each replicate was homogenized with 400 µL of phosphate buffer solution (PBS), pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged at 10,000 g for ten minutes (Multifuge X1R Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). The supernatants were used for enzymatic analyses. The activity of the superoxide dismutase enzyme (SOD, EC 1.15.1.1) was evaluated following an adaptation of the method described by Madesh & Balasubramanian (1997), with reading at 570 nm (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). The activity of the glutathione-S-transferase enzyme (GST, EC 2.5.1.18) was evaluated following the method described by Habig et al. (1974), with a reading at 340 nm. The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined according to the method described by Günzler et al. (1984), with a reading at 340 nm. Catalase enzyme activity (CAT, EC 1.11.1.6) was measured based on the method of Aebi (1984) and the reading was performed at 240 nm. Total protein content was determined according to Bradford (1976)Bradford, (1976).

All enzymatic analyses were performed in duplicate and the results were expressed in U/mg protein.

Lipid peroxidation was evaluated by quantifying the production of thiobarbituric acid reactive substances (TBARS), using malondialdehyde (MDA) as a standard, as proposed by Buege & Aust (1978). Measurements were performed in duplicate at 532 nm, with five replicates per treatment. The results were expressed as malondialdehyde concentration in $mg.L^{-1}$. Detailed methods for the activity of each of these enzymes and the quantification of MDA used in this study can be found in the Supplementary Material.

### 2.5. Effect of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde on the activity of digestive enzymes in adults of *Sitophilus zeamais*

Based on their good performance in the toxicity tests (see results section), the compounds carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde were selected for the analysis of the effects of exposure (to $LC_{50}$s) of *S. zeamais* adults on the digestive enzymes inhibition. All enzymatic analyses were performed in five independent replicates containing 40 insects, totaling 200 individuals for each treatment. After 24 hours of exposure, 10 individuals from each replicate were collected for evaluation of trypsin activity and 10 individuals for evaluation of α-amylase and lipase. For trypsin, each replicate was homogenized with 400 μL of 0.1 M Tris-HCl buffer solution, pH 8.0 containing 20 mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) and for *α*-amylase and lipase, each replicate was homogenized with 400 μL of 0.1 M sodium acetate-acetic acid buffer solution, pH 5.5 (Sigma-Aldrich, St. Louis, MO, USA), according to the methodology adapted from Pimentel et al. (2022). The homogenates were centrifuged at 10,000 g for ten minutes (Multifuge X1R Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). The supernatants were used in subsequent analyses. Trypsin activity (EC 3.4.21.4) was determined by adapting the method proposed by Erlanger et al. (1961), with a reading at 410 nm (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). Laboratory kits (Quibasa-Bioclin, Belo Horizonte, MG, Brazil) were used to evaluate the activity of the enzymes amylase (EC 3.2.1.1) and lipase (EC 3.1.1.3) following the manufacturer's instructions. The determination of the total protein content for normalization of the enzymatic activities was performed according to Bradford (1976). A detailed description of the methods used to determine the activity of each of these enzymes can be found in the Supplementary Material.

### 2.6. Histopathological alterations induced by carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde in exposed adults of *Sitophilus zeamais*

Adults of *S. zeamais* were exposed to $LC_{50}$s of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde, as well as to the control group. Structural changes in the fat body, intestine, exoskeleton, and muscle tissues of the insects after exposure to these compounds were evaluated.

#### 2.6.1. Histopathological and histochemical analyses

For histopathological analysis, thirty adults of *S. zeamais* were exposed to $LC_{50}$ of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde, and control. After 72 h of exposure, five insects were collected from each treatment. The insects were anesthetized and the head and thorax were removed to promote better fixation. The abdomens were fixed in 4% paraformaldehyde solution for 72 h and then were transferred to 70% ethanol solution. The insects were then dehydrated using serial concentrations of ethanol (70, 80, 90, and 95%) and embedded in historesin (Leica Biosystems, Nussloch, Germany) for 24 h at 4 °C before being transferred to plastic molds for embedding. Subsequently, slides containing 4 µm thick sections were prepared using a microtome (Luptec MRP09). Two slides containing at least six sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) (Junqueira and Junqueira, 1983). Finally, the slides were mounted in Entellan® (Merck KGaA, Darmstadt, Germany) for further analysis.

#### 2.6.2. Morphometric analyses

To perform the morphometric analyses, the histological sections were photographed using a DM750 trinocular image capture system (Leica Microsystems, São Paulo, SP, Brazil) and a Flexacam C1 camera (Leica Microsystems, São Paulo, SP, Brazil). Measurements of the thickness of the abdominal muscle fibers, midgut epithelium, diameter of midgut regenerative crypts, abdominal exoskeleton, as well as the area and density of lipid droplets in the fat body were performed using the Image J software (National Institutes of Health, Bethesda, Maryland, USA). For the abdominal muscle fibers, longitudinal histological sections of the mid-portion of adults were selected, where ten measurements of the thickness of the horizontal fibers were made per insect. For the epithelial thickness and diameter of midgut regenerative crypts, ten measurements were made per structure in each of the five insects per treatment. The mean area of adipose cells (trophocytes) in the fat body was calculated with 25 measurements per insect. To determine the density of lipid droplets, specific areas of the fat body were delimited, and the number of droplets was counted, with the results expressed in lipid droplets per 1.000 $\mu m^2$. The analysis of the intensity of regions reactive to the PAS technique, used to highlight glycogen reserves in the fat body, was measured using the Image J histogram tool, with 10 measurements per insect.

## 2.7. Toxicity of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde to the parasitoid Tetrastichus *howardi*

The effect of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde on the mortality, parasitism, emergence, egg-to-adult period (days), and sex ratio of the progeny of the parasitoid *T. howardi* was evaluated using newly emerged (less than 48 h old) and mated females and the previously exposed pupa of its host (Caldeira et al., 2022; Costa et al., 2020). Pupae of *T. molitor*, up to 24 h old and with an average weight of 82.4 ± 3.9 mg, were immersed for ten seconds in 10 mL of $LC_{50}$ solutions of carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde and the control (acetone alone). The pupae were placed on paper towels to remove excess solution before being placed in the bottom of flat-bottomed glass tubes (25 × 85 mm) containing six *T. howardi* females. The tube contained a drop of honey and was sealed with voile fabric, thus constituting a sampling unit. Each treatment had 20 replicates. The treated pupae were exposed to parasitism for 48 hours before being transferred to plastic tubes (8×60 mm). The parasitoid mortality within the 48 h of contact with the treated pupae, parasitism (%), egg-to-adult period (days), emergence (%), progeny, sex ratio (RS = number of emerged females/total number of emerged parasitoids) were evaluated. A change in color of the pupae to brown indicated parasitism. Emergence, progeny, and sex ratio were obtained by counting the total number of emerged females and males per *T. molitor* pupa. The egg-to-adult period was obtained by the number of days between parasitism and the emergence of the new generation of the parasitoid. Furthermore, the survival of females after contact with treated *T. molitor* pupae was assessed by daily mortality counts of *T. howardi* individuals.

### 2.8. Statistical analyses

The toxicity data on adults of *S. zeamais* were subjected to probit analysis (SAS Institute, Cary, NC, USA). Data from enzymatic analyses, malondialdehyde content, morphometric analyses, and biological parameters of *T. howardi* were subjected to the Shapiro-Wilk normality test. They were then compared by Analysis of Variance (ANOVA), followed by Tukey's multiple comparison test ($p < 0.05$), when normal, and Kruskal-Wallis followed by Tukey's post hoc test ($p < 0.05$), when non-normal, using GraphPad Prism software, version 10.2.0 (GraphPad Software, Boston, MA, USA). *T. howardi* survival was determined by Kaplan-Meier (Log-Rank) analysis, using SigmaPlot software (Systat Software Inc., Palo Alto, CA, USA). *T. howardi* parasitism and emergence were analyzed by the generalized linear model (GLM), using R studio software, version 3.6.1 (Posit, Boston, MA, USA).

## 3. Results

### 3.1. Toxicity to adults of *Sitophilus zeamais*

The mortality levels obtained in the dose-response bioassays fitted well with the probit model (goodness-of-fit tests showing $\chi^2$ values <4.5 and p values >0.05) and indicated that (*E*)-anethole, *p*-anisaldehyde, (*E*)-cinnamaldehyde, eugenol, carvacrol, and L-(-)-carvone presented similar $LC_{50}$s indicating similar toxicities to the adults of *S.zeamais*. However, compared at $LC_{90}$s and $LC_{95}$s, (*E*)-cinnamaldehyde and carvacrol were about two-fold more toxic than the other compounds (Table 1). The compound *β*-citronellol was the least toxic presenting an $LC_{50}$ > 10 $\mu L.mL^{-1}$ and thus was not tested in further experiments.

### 3.2. Toxicity of binary mixtures of phenylpropanoids and terpenes on adults of *Sitophilus zeamais*

In general, the effects of all the binary mixtures were positive being either synergistic ($\chi^2$ values > 3.84 and experimental mortality higher than theoretical) or additive ($\chi^2 < 3.84$) except the interaction between eugenol and *p*-anisaldehyde that was antagonistic ($\chi^2 = 37.89$), ($\chi^2 > 3.84$, and experimental mortality lower than theoretical) (Table 2).

(*E*)-anethole was the chemical compound that had the most synergized other substances (*p*-anisaldehyde, carvacrol, and L-(-)-carvone) while the other combinations of chemical compounds generally generated only additive interactions.

### 3.3. Effects of carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde on the oxidative stress-related enzymes' activity in *Sitophilus zeamais* adults

Overall, the exposure of *S.zeamais* adults to the $LC_{50}$s of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde unequally affected the activities of the tested enzymes (Figures 1 and 2). Therefore, the activities of CAT ($F_{(3,16)} = 41.1$ and $p < 0.0001$; Figure 1A), GST ($F_{(3,16)} = 46.0$ and $p < 0.0001$; Figure 1C) and GPx ($F_{(3,16)} = 115.6$ and $p < 0.0001$; Figure 1D) enzymes (Figure 1), as well as the lipid peroxidation ($F_{(3,16)} = 29.5$ and $p < 0.0001$; Figure 2) were statistically increased in *S. zeamais* adults treated with *p*-anisaldehyde compared to the ones treated with carvacrol, (*E*)-cinnamaldehyde and the control. Furthermore, both carvacrol and (*E*)-cinnamaldehyde reduced the activity of CAT compared to the control and (*E*)-cinnamaldehyde. Additionally, while the (*E*)-cinnamaldehyde slightly increased the GPx activity (Figure 1D), it was the compound that the most increased the activity of lipid peroxidation in treated insects (Figure 2). No differences ($F_{(3,16)} = 2.3$ and $p = 0.1119$) between the treatments were found for the SOD enzyme (Figure 1B).

### 3.4. Effects of exposure to carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde on the digestive enzymes' activity in *Sitophilus zeamais* adults

The analysis of the digestive enzymes' inhibition after exposure of *S. zeamais* adults to $LC_{50}$s of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde showed distinct trends (Figure 3) depending on the enzyme and the compound used.

Accordingly, the treatment with the $LC_{50}$ of carvacrol and (*E*)-cinnamaldehyde significantly ($F_{(3,16)} = 13.8$ and $p = 0.0001$; Figure 3A) increased the activity of trypsin compared to the control and *p*-anisaldehyde, and although the treatment with the $LC_{50}$ of carvacrol and (*E*)-cinnamaldehyde reduced the activity of *α*-amylase compared to the control, the *p*-anisaldehyde was the compound that the most ($F_{(3,16)} = 59.2$ and $p < 0.0001$) reduced its activity (Figure 3B). Finally, for the lipase, treatment with the $LC_{50}$ of e (*E*)-cinnamaldehyde reduced the enzyme activity compared to the control while *p*-anisaldehyde increased the enzyme's activity ($F_{(3,16)} = 89.7$ and $p < 0.0001$; Figure 3C).

### 3.5. Histopathological changes in adults of *Sitophilus zeamais*

The mean values obtained for the morphometric analyses are presented in Table 3 and illustrative images of the main changes are presented in Figure 4. Exposure to carvacrol promoted the greatest reduction in the thickness of abdominal muscle fibers, differing from the other treatments. (*E*)-cinnamaldehyde also promoted a significant reduction, while *p*-anisaldehyde did not differ from the control ($F_{(3,196)} = 34.71$ and $p < 0.0001$). A significant reduction in the thickness of the midgut epithelium was observed in the treatment with (*E*)-cinnamaldehyde ($F_{(3,196)} = 4.78$ and $p = 0.0031$) as well as in diameter the regenerative crypts of the midgut in the insects treated with *p*-anisaldehyde ($F_{(3,196)} = 5.01$ and $p = 0.0023$) while no difference ($F_{(3,196)} = 1.10$ and $p = 0.3504$) between were found in the thickness of the abdominal exoskeleton. Regarding lipid droplets in the fat body, treatment with (*E*)-cinnamaldehyde reduced the area of these structures, ($F_{(3,496)} = 3.51$ and $p = 0.0152$) but no change ($F_{(3,96)} = 0.21$ and $p = 0.8887$) was detected in the density of lipid droplets between treatments.

Staining with the PAS technique indicated reactive intestinal content (Supplementary Figure 1A), confirming the presence of carbohydrate-rich food both in control and other treatment groups. When analyzing the fat body in the control group, strongly reactive points were observed uniformly distributed throughout this structure, indicating the presence of glycogen reserves (Supplementary Figure 1B). In the treatment with carvacrol $LC_{50}$, although glycogen reserves were observed in 60% of the samples, the number of granules was considerably lower than in the control group. For the $LC_{50}$ of (*E*)-cinnamaldehyde and *p*-anisaldehyde, glycogen reserves were observed in 40% and 20% of the insects, respectively, however, the granules were extremely scarce compared to the control group (Supplementary Figure 1C). The analysis of the intensity of staining in the fat body indicated that the treatment with carvacrol did not differ from the control group, but (*E*)-cinnamaldehyde and especially p-anisaldehyde promoted a significant ($F_{(3,96)} = 11.77$ and $p < 0.0001$) reduction in coloration.

### 3.6. Toxicity on *Tetrastichus howardi*

Exposure to the $LC_{50}$ of carvacrol, (*E*)-cinnamaldehyde, and p-anisaldehyde promoted significant ($F_{(3,16)} = 15.56$ and $p < 0.0001$) parasitoid mortality during the following 48 hours of contact with the treated pupae, especially the treatment with carvacrol (Figure 5A) of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde though this mortality did not exceed 34.17 ± 2.43%. The parasitism (Figure 5B) of *T. howardi* females was mostly (F = 6.99, df = 3 and p = 0.0003) reduced when pupae were treated with *p*-anisaldehyde and the percentage of progeny emergence (Figure 5C) was negatively affected by all treatments (F = 41.26, df = 3 and p < 0.0001) resulting in the reduction of the total number of emerged parasitoids (Figure 5F) compared to the control. Strikingly, the exposure of pupa to carvacrol, although did not affect the % of parasitism by the *T. howardi* females, completely inhibited the development (Figure 5D) and emergence (Figure 5 C, E, F) of the parasitoid progeny. The egg-adult cycle duration ($F_{(3,37)} = 325.60$ and $p < 0.0001$; Figure 5D), the number of offspring ($F_{(3,40)} = 18.72$ and $p < 0.0001$; Figure 5E), and sex-ratio ($F_{(3,37)} = 1{,}637.00$ and $p < 0.0001$; Figure 5F) were not different between the control and the treatments with (*E*)-cinnamaldehyde and *p*-anisaldehyde. Interestingly, the mean survival time was higher in all treatments when compared to the control, especially for *p*-anisaldehyde (Table 4, Supplementary Figure 2) and there was no difference in survival between the carvacrol and (*E*)-cinnamaldehyde treatments ($\chi^2 = 50.509$, df = 3 and $p < 0.0001$).

**4. Discussion**

Various alternative insecticide sources have been recently explored, and plant secondary metabolites like terpenes and phenylpropanoids have been investigated as potential promising tools for Integrated Pest Management. In the present study, we evaluated the effects of L-(-)-carvone, carvacrol, *β*-citronellol, (*E*)-anethole, (*E*)-cinnamaldehyde, *p*-anisaldehyde, and eugenol on adults of *S. zeamais*, an important pest of stored products. Our findings showed that carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde were the most toxic to adults of this pest and that the binary mixtures generally generated additive interactions with (*E*)-anethole presenting the greatest potential for synergistic action. Additionally, the exposure of *S. zeamais* adults to the $LC_{50}$s of carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde altered the activity of enzymes involved in oxidative stress response and the digestive process and generated histomorphological alterations in important internal and external structures of the insects. Furthermore, although the three compounds caused less than 30% of mortality in the pupal parasitoid *T. howardi*, only carvacrol and (*E*)-cinnamaldehyde did not affect the parasitism of this natural enemy.

In terms of *S. zeamais* adults' mortality, carvacrol showed the best performance, followed by (*E*)-cinnamaldehyde, *p*-anisaldehyde, eugenol, (*E*)-anethole, and L-(-)-carvone while *β*-citronellol showed lower insecticidal potential on this pest. Essential oils containing some of these compounds have been previously tested for the control of several stored grain pests, including *S. zeamais*. For example, *Thymus pallescens* essential oil, rich in carvacrol (56.6%), presented $LC_{50}$ equal to 17.7 µL/mL in a contact toxicity test and 15 µL/L of air in a fumigation toxicity test (Moutassem et al., 2024). The *Illicium verum* oil, composed mainly of (*E*)-anethole (77.4%), presented $LC_{95}$ equal to 609.75 µL/L of air in a fumigation toxicity test (Brito et al., 2021). Additionally, the essential oil of *Anethum graveolens*, rich in carvone isomer (66.4%), presented $LC_{50}$ equal to 157.1 mg/L of air in a fumigation toxicity test and 111.3 µg per insect in a topical toxicity test. The isolated carvone isomer presented $LC_{50}$ equal to 51.8 mg/L of air in a fumigation toxicity test and 23.0 µg per insect in a topical toxicity test (Rosa et al., 2020). Additionally, the essential oil of *Cinnamomum zeylanicum*, rich in (*E*)-cinnamaldehyde, and of *Syzygium aromaticum*, rich in eugenol, presented $LC_{95}$ equal to 3.47 µL/cm$^2$ and 3.96 µL/cm$^2$, respectively (Haddi et al., 2015b). Although these studies used different exposure methods, the lethal concentrations obtained in our study were generally lower reinforcing that these compounds isolated from essential oils can be used in formulations to control this pest.

During formulation development, it is important to investigate possible synergistic, additive, or antagonistic effects that may occur between chemical compounds especially when using plants' essential oils known to present variable chemical compositions. Indeed, the synergistic activity of essential oils composed of a mixture of terpenes and phenylpropanoids has been linked to distinct biological activities (Dassanayake et al., 2021; Fouad et al., 2023; Santana et al., 2022). In our study, in a pair-by-pair comparison, we found that (*E*)-anethole was the most promising compound to cause a synergistic effect when combined with other terpenes and phenylpropanoids while L-(-)-carvone participated in the only antagonistic interaction. The insecticidal potential of synergistic interactions of (*E*)-anethole with 23 compounds, including carvacrol, was demonstrated in *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae (Pavela, 2014) and (*E*)-anethole synergistically enhanced the lethal effects of several other compounds, including eugenol, L-carvone, carvacrol, and cinnamaldehyde on *Culex quinquefasciatus* (Diptera: Culicidae) larvae (Pavela, 2015). The mechanisms by which (*E*)-anethole tends to form synergistic interactions and L-(-)-carvone antagonistic ones are not very clear. In general, it is suggested that compounds, like (*E*)-anethole, that have methoxyl groups in their structure normally have the potential for synergistic interactions while the monocyclic compound, like L-(-)-carvone, presenting a carbonyl group favors the occurrence of antagonistic interactions (Pavela, 2015, 2014).

In addition to direct mortality, the evaluation of the sublethal effects of toxic compounds may represent an indicator of possible mechanisms leading to the death of insect pests or even causing long-term control through the generation of metabolically compromised descendants. Thus, histomorphological alterations and/or changes in the activity of detoxifying enzymes related to oxidative stress, and digestive enzymes are important in investigating the toxicological effects in insect pests.

Our findings showed that *p*-anisaldehyde and (*E*)-cinnamaldehyde promoted greater changes in the activity of enzymes related to oxidative stress and induced greater lipid peroxidation in *S. zeamais*, indicated by the increase in MDA concentration. These alterations suggest that the homeostasis balance was not maintained in the cells, which may have caused physiological and morphological damage in organs particularly important for insect survival (El-Ashram et al., 2021; Lennicke and Cochemé, 2021; Li-Byarlay and Cleare, 2020).

Alterations of the natural profile of enzymes participating in the antioxidant defense system such as CAT, SOD, GST, and GPx constitute one of the main mechanisms of the insecticidal activity of essential oils and their constituents (Chaudhari et al., 2021). In our study, *p*-anisaldehyde increased the activity of CAT, GST, and GPx enzymes, while carvacrol reduced CAT activity and (*E*)-cinnamaldehyde reduced CAT activity and increased GPx activity. The change in the activity of these enzymes when compared to the control group is indicative of an imbalance in reactive oxygen species (ROS) present in the organism (Kodrík et al., 2015). The accumulation of ROS due to the inefficiency of the antioxidant defense system can promote lipid peroxidation, which can be estimated by the quantification of malondialdehyde (MDA) (Nam, 2011).

Histopathological analysis demonstrated that the selected compounds significantly affected important structures for *S. zeamais*. The $LC_{50}$ of carvacrol and (*E*)-cinnamaldehyde reduced the thickness of abdominal muscle fibers, (*E*)-cinnamaldehyde reduced the thickness of the midgut epithelium and the area of lipid droplets in the fat body, while p-anisaldehyde reduced the diameter of the regenerative crypts of the midgut. Abdominal contraction in insects, through their muscles, plays an important role in the circulation of hemolymph and their respiration through ventilation in specific metabolic situations that prevent the adequate functioning of the dorsal vessel (Tartes et al., 2002). Considering the reduction in the thickness of abdominal muscle fibers by carvacrol and (*E*)-cinnamaldehyde, insects may have reduced abdominal mobility, which may prevent the activation of this auxiliary mechanism of respiration/hemolymph circulation. Additionally, the midgut of insects is composed of digestive, regenerative, and endocrine cells, and is divided into two parts, the anterior and posterior midgut. The anterior portion is characterized by a high density of regenerative crypts, which are structures that contain cells that promote protein synthesis and have a high concentration of amylases and lipases, indicating that most of the digestion process occurs in this portion of the intestine. The posterior midgut exhibits many gastric ceca and peritrophic membrane, factors that indicate that this portion acts mainly in the absorption of nutrients (Caccia et al., 2019; de Sousa and Conte, 2013; Napoleão et al., 2019). In our study, (*E*)-cinnamaldehyde reduced the thickness of the midgut epithelium and p-anisaldehyde reduced the diameter of the regenerative crypts. Damage caused to these structures can reduce the capacity to produce digestive enzymes and the absorption of nutrients, generating metabolic imbalances in insects. The fat body of insects, in turn, uses lipids, carbohydrates, and proteins as substrates in many metabolic pathways that can be used for energy production or storage of reserves to be used in the most diverse stages of life (Skowronek et al., 2021). (*E*)-Cinnamaldehyde reduced the area of lipid droplets in this tissue, indicating dysregulation in insect metabolism or high energy demand due to the detoxification process. Although some previous studies have demonstrated the potential of essential oils and their constituents to cause damage to muscle, digestive, and fat body tissues in other insect species (de Souza et al., 2024, 2022; Pan et al., 2022; Souza et al., 2021), very few investigations addressed these effects in *S. zeamais*.

The diet of *S. zeamais* is rich in carbohydrates, mainly starch from corn grains (Ojo and Omoloye, 2012). Carbohydrate metabolism is the main energy source for these insects; sugars from the diet are converted to trehalose and stored in the fat body, muscles, and other structures in the form of glycogen (Arrese and Soulages, 2010; Shi et al., 2017). Our results demonstrated that exposure to the selected compounds promoted a significant reduction in glycogen reserves and the intensity of PAS staining, indicating a reduction in the levels of carbohydrates available in the organism. The reduction in carbohydrate reserves can be explained by the high energy demand to promote detoxification of the organism or by the low activity and/or inhibition of digestive enzymes, mainly by *p*-anisaldehyde. Additionally, morphological damage may have caused a lower production of amylase, the main digestive enzyme in *S. zeamais* (Baker, 1983). Furthermore, the insects may have fed less, a hypothesis partially refuted by the evidence of the presence of intestinal contents in the treated groups. However, the exact amount of food consumed by insects in this study was not evaluated. Considering that the insects had their intestines filled with food and that there was a decrease in the stored glycogen content, we hypothesized that these compounds could cause inhibition of digestive enzymes, mainly trypsin, α-amylase, and lipase. Natural products were shown to affect the activity of these enzymes, as is the case of the lectin extracted from *Opuntia ficus-indica* that inhibited the activity of amylase and increased the activity of proteases in *S. zeamais* (Souza et al., 2018). Additionally, extracts from the leaves of *Schinus terebinthifolius* increased amylase activity and strongly inhibited protease activity in this pest (Camaroti et al., 2018). Finally, the essential oil from the leaves of *Croton pulegiodorus* also increased amylase activity and inhibited trypsin activity in *S. zeamais* (dos Santos et al., 2025). Our results demonstrated a reduction in α-amylase activity in all treatments, which corroborates the use of energy reserves due to the impossibility of extracting nutrients from food. The observed increase in trypsin and lipase activity may occur as an attempt to obtain other energy sources since the main energy source was not available.

The search for pesticides that are less harmful to the environment and non-target organisms is a current concern, and natural products usually meet these requirements (Damalas and Koutroubas, 2020). The pupal parasitoid *T. howardi* naturally parasitizes a wide variety of lepidopteran pests and is, therefore, frequently used in integrated pest management programs (Machado et al., 2023; Pereira et al., 2021; Silva-Torres et al., 2010). Our results demonstrate that the selected compounds affected the survival of this parasitoid, especially carvacrol, in which mortality was around 30%. However, the mortality observed during contact with the chemical compounds did not affect the parasitism capacity, except for *p*-anisaldehyde. In pupae treated with carvacrol, there was no emergence of progeny, however, the development of the host was satisfactorily inhibited by the parasitic action of the natural enemy. The parasitoid egg-to-adult development period, progeny, and sex ratio in (*E*)-cinnamaldehyde and *p*-anisaldehyde-exposed individuals did not differ from the control indicating greater compatibility with this insect. Moreover, the mean survival time was higher in all treatments when compared to the control, especially for *p*-anisaldehyde. Positive and stimulatory responses have been reported in several insect pests after exposure to low doses of xenobiotics including plant-based compounds (Haddi et al., 2015b; Leonov et al., 2015; Papanastasiou et al., 2017; Pineda et al., 2023; Rix et al., 2022; Silva et al., 2017). In this sense, (*E*)-cinnamaldehyde appears to be the most promising due to its lower toxicity and induced stimulatory responses in the non-target organism.

In conclusion, the terpenes and phenylpropanoids under study showed considerable potential to promote the control of *S. zeamais*, with carvacrol standing out as the most toxic compound. (*E*)-anethole was the compound that presented the highest number of synergistic interactions, therefore it is a compound to be considered for the development of new plant-based bioinsecticide formulations. Carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde caused sublethal effects on this pest, considerably altering the activity of digestive enzymes and those related to oxidative stress, in addition to causing histopathological damage. *p*-anisaldehyde stands out for having strongly inhibited the activity of the enzyme $\alpha$-amylase and increased the activity of the detoxifying enzymes CAT, GST, and GPx. (*E*)-cinnamaldehyde can be considered the compound most compatible with the natural enemy *T. howardi*, as it affected only the emergence of the progeny and did not alter any other evaluated parameter. Further studies should be carried out with the compounds carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde investigating their performance under real field and grain storage conditions and compatibility with other non-target organisms.

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**Table 1 -** Toxicological performance of terpenes and phenylpropanoids on adults of *Sitophilus zeamais*.

| Chemical Compounds | Class of natural products | Number of insects | $LC_{50}$ (95% FI) (µL.mL$^{-1}$) | $LC_{90}$ (95% FI) (µL.mL$^{-1}$) | $LC_{95}$ (95% FI) (µL.mL$^{-1}$) | $\chi^2$ | *P* | TR $LC_{95}$ (95% FI) (µL.mL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| (*E*)-anethole | Phenylpropanoids | 240 | 5.30(4.79-5.83) | 8.75(7.73-10.53) | 10.09(8.72-12.65) | 1.5761 | 0.4547 | 1.87(1.81-1.96) |
| *p*-anisaldehyde | Phenylpropanoids | 200 | 4.48(4.06-4.90) | 6.93(6.20-8.15) | 7.84(6.89-9.56) | 1.9150 | 0.3838 | 1.45(1.43-1.48) |
| (*E*)-cinnamaldehyde | Phenylpropanoids | 200 | 4.15(3.96-4.38) | 5.35(4.91-6.37) | 5.74(5.18-7.13) | 1.7717 | 0.4129 | 1.10(1.08-1.11) |
| eugenol | Phenylpropanoids | 240 | 5.14(4.57-5.71) | 9.71(8.42-11.97) | 11.62(9.82-15.08) | 1.4060 | 0.7041 | 2.16(2.04-2.34) |
| carvacrol | Terpenes | 200 | 3.31(3.07-3.56) | 4.84(4.39-5.59) | 5.39(4.81-6.44) | 2.2919 | 0.3179 | * |
| L-(-)-carvone | Terpenes | 200 | 5.75(5.24-6.28) | 9.07(8.06-10.89) | 10.32(8.98-12.90) | 4.3689 | 0.1125 | 1.91(1.87-2.00) |
| *β*-citronellol | Terpenes | 200 | > 10 | - | - | - | - | - |

Where: $LC_{50}$ is the lethal concentration for 50% of individuals. $LC_{90}$ is the lethal concentration for 90% of individuals and $LC_{95}$ is the lethal concentration for 95% of individuals; (95% FI) represents the 95% fiducial interval; $\chi^2$ is the chi-square for lack of fit to the probit model; *P* is the probability associated with the chi-square statistic; TR $LC_{50}$ (95% LC) is the toxicity rate based on the $LC_{50}$, determined by the ratio of the $LC_{50}$ of the chromatographic standard to the $LC_{50}$ of the lowest value, with a 95% confidence limit; * chromatographic standard used as a reference for calculating the TR $LC_{50}$.

**Table 2 -** Analysis of toxicity caused by the interaction between binary mixtures of the compounds studied on adults of *Sitophilus zeamais*.

| Pairs of Chemical Compounds | Number of insects | Theoretical mortality (%) | Experimental mortality (%) | $\chi^2$ | Type of interaction |
|---|---|---|---|---|---|
| (*E*)-anethole + *p*-anisaldehyde | 40 | 72.50 | 100.00 | 10.43 | Synergistic |
| (*E*)-anethole + (*E*)-cinnamaldehyde | 40 | 75.25 | 85.00 | 1.26 | Additive |
| (*E*)-anethole + eugenol | 40 | 73.88 | 75.00 | 0.02 | Additive |
| (*E*)-anethole + carvacrol | 40 | 71.13 | 97.50 | 9.78 | Synergistic |
| (*E*)-anethole + L-(-)-carvone | 40 | 73.88 | 95.00 | 6.04 | Synergistic |
| *p*-anisaldehyde+ (*E*)-cinnamaldehyde | 40 | 77.50 | 75.00 | 0.08 | Additive |
| *p*-anisaldehyde+ eugenol | 40 | 76.25 | 77.50 | 0.02 | Additive |
| *p*-anisaldehyde+ carvacrol | 40 | 73.75 | 75.00 | 0.02 | Additive |
| *p*-anisaldehyde+ L-(-)-carvone | 40 | 76.25 | 22.50 | 37.89 | Antagonist |
| (*E*)-cinnamaldehyde + eugenol | 40 | 78.63 | 95.00 | 3.41 | Additive |
| (*E*)-cinnamaldehyde + carvacrol | 40 | 76.38 | 87.50 | 1.62 | Additive |
| (*E*)-cinnamaldehyde + L-(-)-carvona | 40 | 78.63 | 87.50 | 1.00 | Additive |
| eugenol + carvacrol | 40 | 75.06 | 85.00 | 1.32 | Additive |
| eugenol + L-(-)-carvone | 40 | 77.44 | 97.50 | 5.20 | Synergistic |
| carvacrol + L-(-)-carvone | 40 | 75.06 | 90.00 | 2.97 | Additive |

Where: $\chi^2$ is the chi-square (if < 3.84 the interaction is additive, if > 3.84 and experimental mortality is greater than theoretical the interaction is synergistic and if > 3.84 and experimental mortality is less than theoretical the interaction is antagonistic, considering degrees of freedom equal to 1 and 95% probability).

**Table 3 -** Morphometric analysis of structures in adults of *Sitophilus zeamais* exposed for 24 hours to the control groups and to the $LC_{50}$ of the compounds carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde.

| Structures | Control | carvacrol | (*E*)-cinnamaldehyde | *p*-anisaldehyde |
|---|---|---|---|---|
| Abdominal muscle fiber thickness (μm) | 11.9 ± 1.2[a] | 9.1 ± 2.2[c] | 10.3 ± 1.8[b] | 12.2 ± 1.4[a] |
| Midgut epithelium thickness (μm) | 63.8 ± 11.6[a] | 60.6 ± 10.4[ab] | 57.4 ± 12.2[b] | 64.6 ± 7.8[a] |
| Diameter of regenerative crypts (μm) | 61.4 ± 13.3[a] | 56.1 ± 10.4[ab] | 56.2 ± 12.1[ab] | 52.8 ± 8.7[b] |
| Abdominal exoskeleton thickness (μm) | 14.0 ± 1.9[a] | 14.0 ± 2.4[a] | 14.3 ± 3.1[a] | 14.9 ± 2.6[a] |
| Lipid droplet area of the fat body($μm^2$) | 101.6 ± 39.0[a] | 100.9 ± 29.3[a] | 90.0 ± 28.2[b] | 94.5 ± 33.8[ab] |
| Density of lipid droplets in the fat body (lipid droplets in1000 $μm^2$) | 4.2 ± 0.4[a] | 4.2 ± 0.4[a] | 4.2 ± 0.4[a] | 4.2 ± 0.4[a] |
| Intensity of PAS staining in the fat body (a.u.) | 134.1 ± 1.9[a] | 132.7 ± 2.2[ab] | 131.2 ± 3.1[b] | 127.9 ± 6.4[c] |

Data normality was verified by the Shapiro-Wilk test. Means in the same row followed by the same letters do not differ from each other by Analysis of Variance (ANOVA) followed by Tukey's post hoc test ($p > 0.05$). LC = lethal concentration, μm = micrometer. a.u. = arbitrary units.

**Table 4 -** Survival of *Tetrastichus howardi* females exposed to *Tenebrio molitor* pupae treated with the $LC_{50}$ of the compounds carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde.

| Treatment | Median survival time (days) | Standard error |
|---|---|---|
| **Control** | 11.276 (10.386 – 12.167)[a] | 0.454 |
| **carvacrol** | 12.388 (10.315 – 14.461)[b] | 1.058 |
| **(E)-cinnamaldehyde** | 12.689 (11.217 – 14.162)[b] | 0.751 |
| ***p*-anisaldehyde** | 17.830 (15.290 – 20.371)[c] | 1.296 |

Survival was analyzed by the Kaplan-Meier test (n=120). Means in the same column followed by the same letters do not differ from each other ($p > 0.05$). LC = lethal concentration.

**Figure 1 -** Enzyme activity related to oxidative stress in *Sitophilus zeamais* adults exposed for 24 hours to the control groups and to the $LC_{50}$ of the compounds carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde. (One-Way ANOVA followed by Tukey's post hoc test, n=5/treatment).

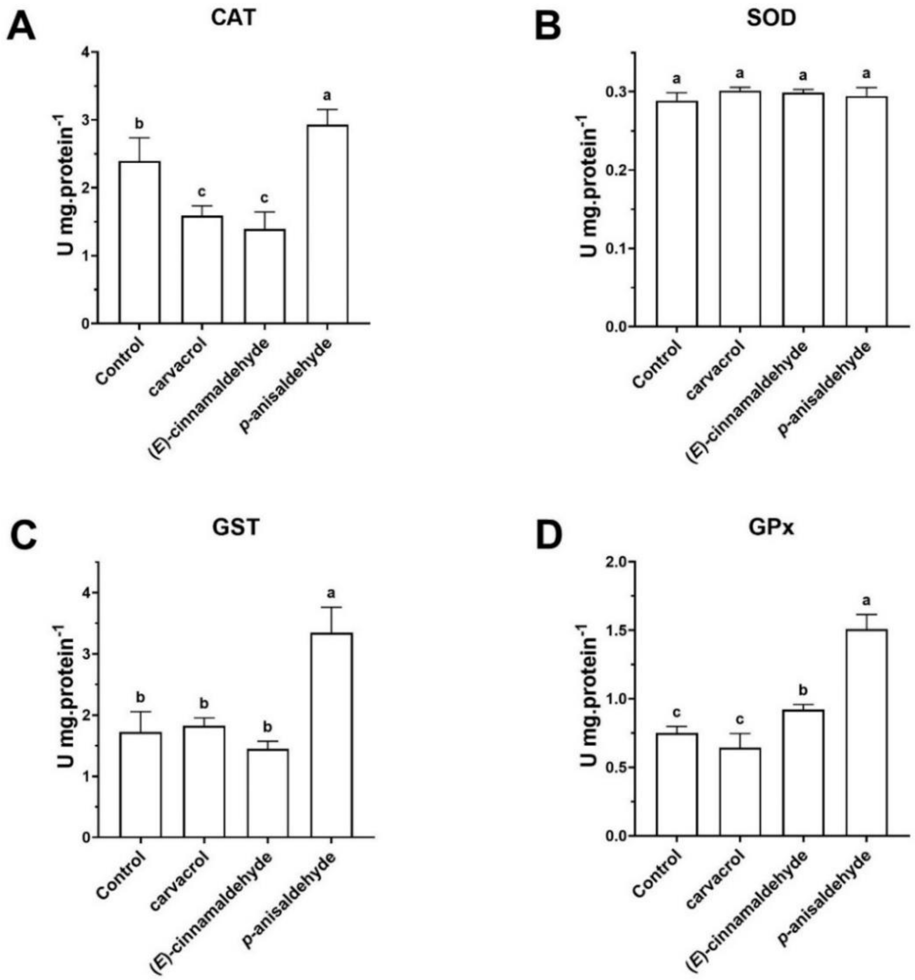


**Figure 2 -** Lipid peroxidation determined by the concentration of malondialdehyde (MDA) in *Sitophilus zeamais* adults exposed for 24 hours to the control groups and to the $LC_{50}$ of the compounds carvacrol, (*E*)-cinnamaldehyde and *p*-anisaldehyde. (One-Way ANOVA followed by Tukey's post hoc test, n=5/treatment).

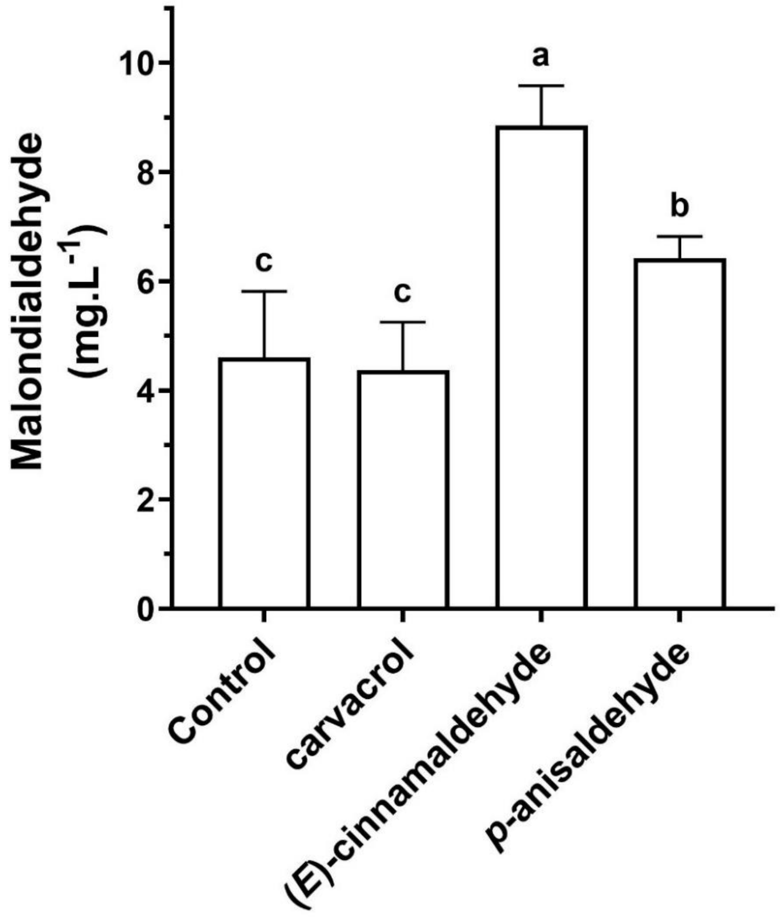


**Figure 3 -** Digestive enzyme activity in *Sitophilus zeamais* adults exposed for 24 hours to the control groups and the $LC_{50}$ of the compounds, carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde. (One-Way ANOVA followed by Tukey's post hoc test, n=5/treatment).

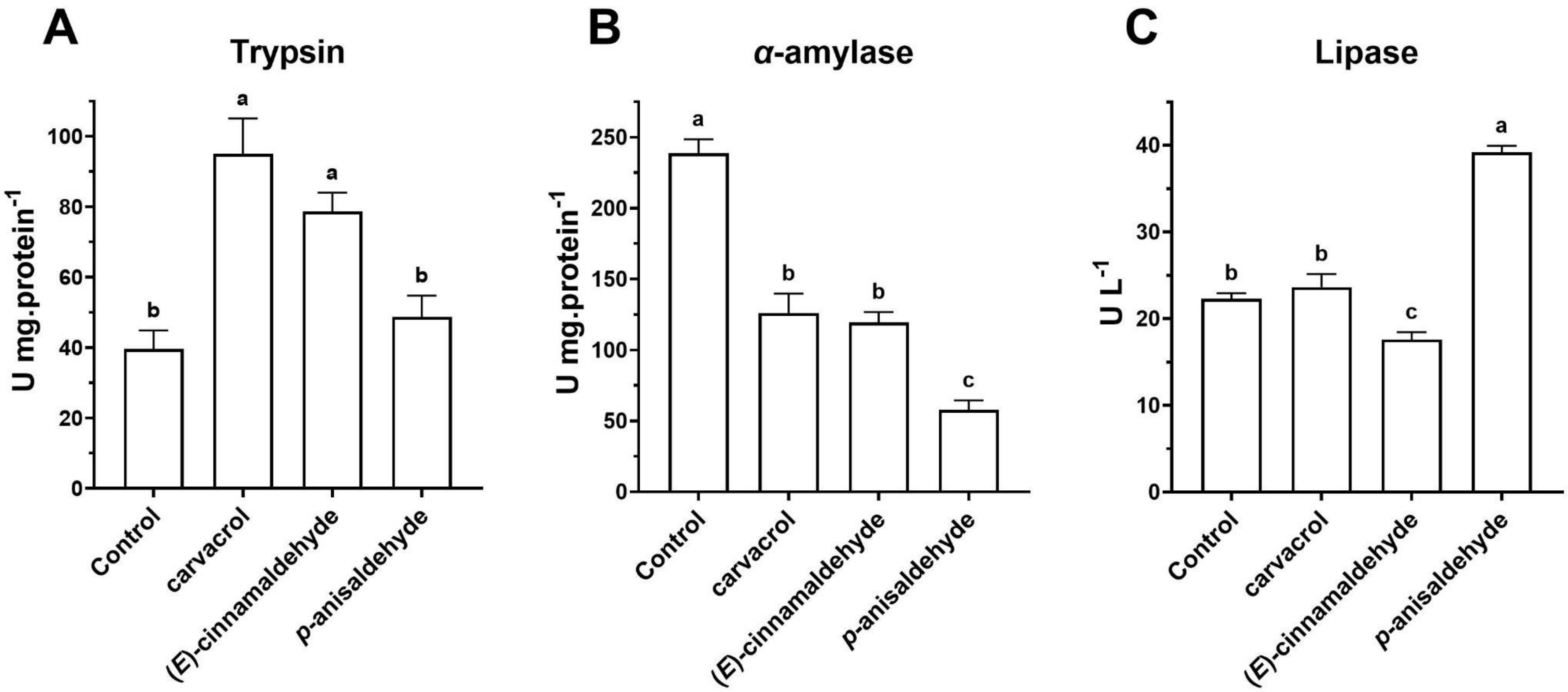


**Figure 4 -** Representation of the main histological alterations in *Sitophilus zeamais* adults. All histological sections were stained with hematoxylin and eosin. Control group (A and B); $LC_{50}$ carvacrol group (C and D); $LC_{50}$ (*E*)-cinnamaldehyde group (E and F) and $LC_{50}$ *p*-anisaldehyde group (G and H). Scale bars: 50 µm. Captions: AD – adipocyte; DIL – diet in the intestinal lumen; DRC – diameter of regenerative crypts; E – exoskeleton; RAD – reduction in adipocyte area; RDC – reduction in the diameter of regenerative crypts; RTM – reduction in the thickness of abdominal muscle fibers; RTI – reduction in the thickness of intestinal epithelium; TIE – thickness of intestinal epithelium; TMF – thickness of abdominal muscle fiber.

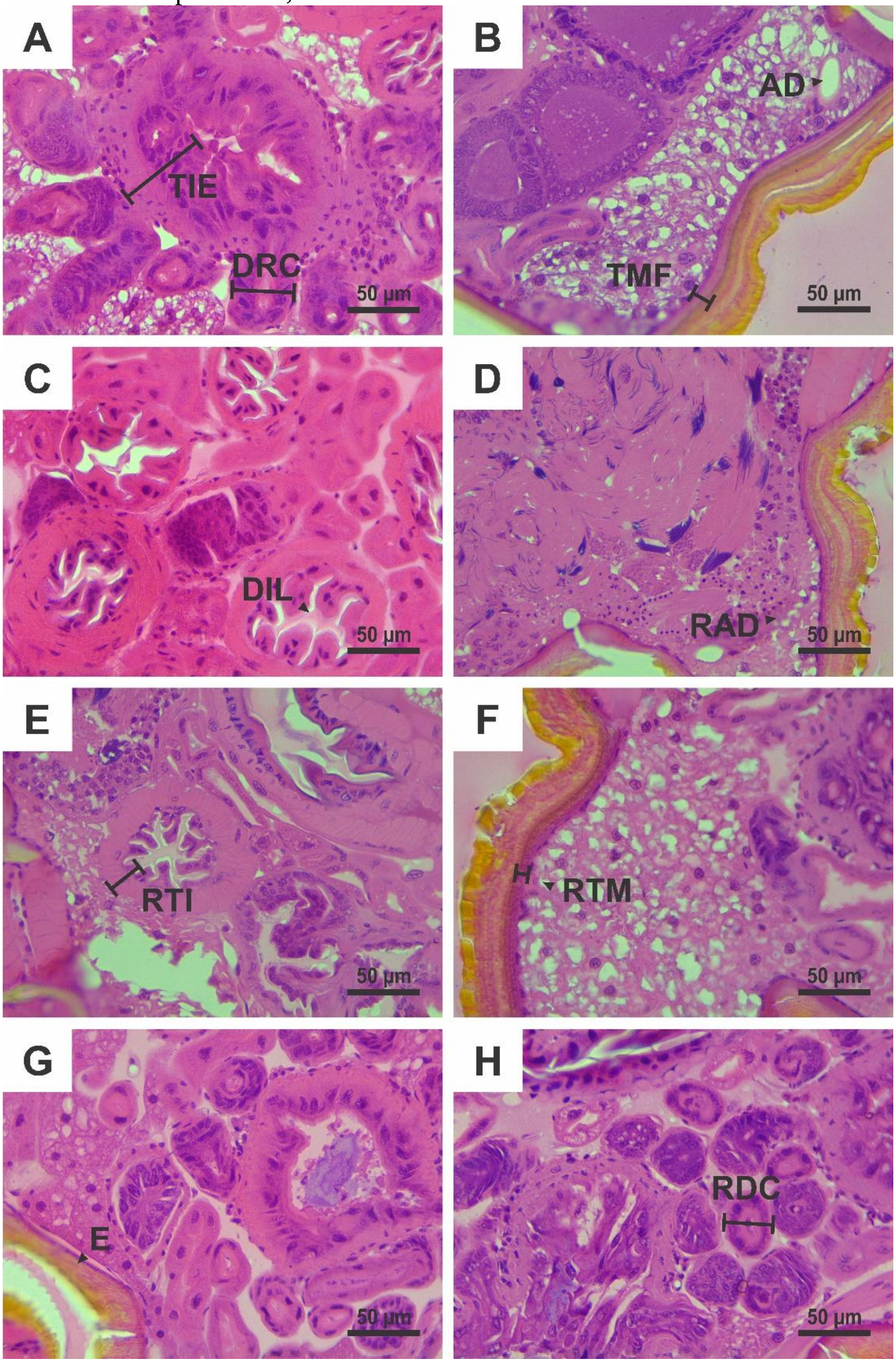
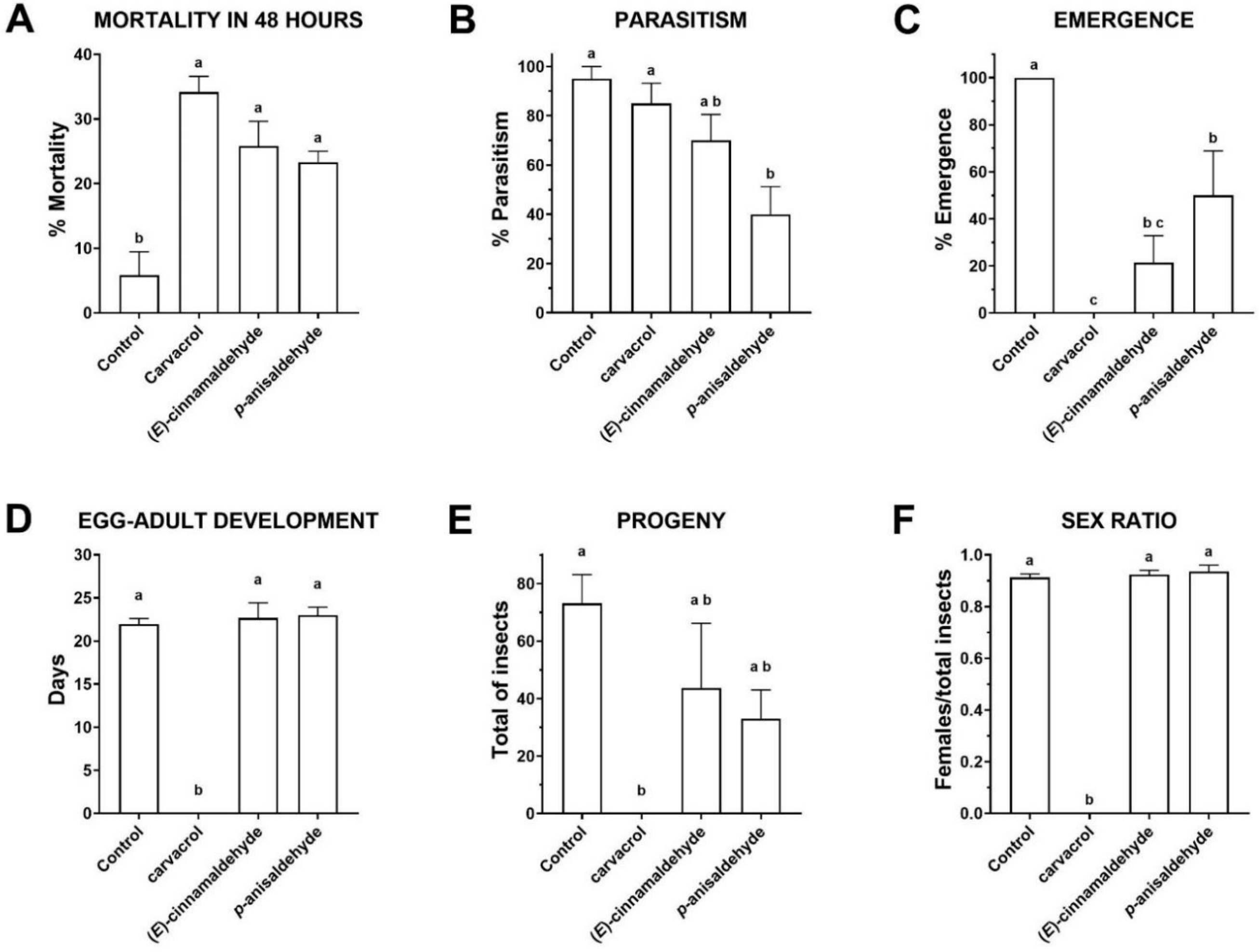


**Figure 5 -** Biological parameters of *Tetrastichus howardi* in *Tenebrio molitor* pupae treated with the $LC_{50}$ of the compounds carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde. (One-Way ANOVA followed by Tukey's post hoc test, n=20/treatment).



# Synergism, biochemical changes and morphophysiological impacts induced by terpenes and phenylpropanoids in *Sitophilus zeamais*

## Supplementary material

## 1. Material and Methods

### 1.1. Analysis of enzymes related to oxidative stress

#### 1.1.1. Superoxide dismutase enzyme activity

Superoxide dismutase (SOD, EC 1.15.1.1) enzyme activity was assessed following an adaptation of the method described by Madesh & Balasubramanian (1997). For dilution, 40 μL of the homogenate and 132 μL of PBS (Sigma-Aldrich, St. Louis, MO, USA) were added to a microplate. Subsequently, 8 μL of 0.6 g/L (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) and 20 μL of 1.25 mM pyrogallol (Sigma-Aldrich, St. Louis, MO, USA) were added. The plate was then incubated at 37 °C for 5 min. After incubation, 150 μL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added, and the absorbance was measured in duplicate for each replicate using a microplate reader at 570 nm (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). Negative controls included all components except pyrogallol and the sample. Total SOD activity was expressed in enzyme activity unit (U) per milligram of protein, where one unit of SOD activity corresponds to the quantity required to perform 50% of the dismutation of the superoxide radical per minute.

#### 1.1.2. Glutathione S-transferase enzyme activity

The activity of the glutathione S-transferase enzyme (GST, EC 2.5.1.18) was evaluated following the method described by Habig et al. (1974). This technique uses 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) as substrates. In a microplate, 25 μL of the homogenate were added to 125 μL of reaction medium, composed of 380 μL of 0.1 M CDNB in ethanol (Sigma-Aldrich, St. Louis, MO, USA), 2.3 mL of GSH at 30.7 $mg.mL^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA) and 12.32 mL of PBS (Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured every 20 seconds, for a total of 15 measurements, in a microplate reader adjusted to 340 nm, with duplicate readings. The negative control consisted only of PBS. GST activity was expressed in enzymatic activity units (U) per milligram of protein.

### 1.1.3. Glutathione peroxidase enzyme activity

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined according to the method described by Günzler et al. (1984). The reaction mixture for the assay consisted of 10 mL of PBS, 2.6 mL of 10 mM GSH solution, 2.6 mL of 1.6 mM NADPH solution, 2.6 mL of 10 mM $NaN_3$ solution and 1 μL of GR enzyme (0.5 $U.mL^{-1}$). In each well of the microplate, 40 μL of the homogenate, 200 μL of the reaction mixture and 25 μL of 10 mM hydrogen peroxide solution were then added. The reaction was monitored at 340 nm, and GPx activity was expressed in enzyme activity units (U) per milligram of protein.

### 1.1.4. Catalase enzyme activity

The activity of the catalase enzyme (CAT, EC 1.11.1.6) was measured based on the method of Aebi (1984). This method determines the enzyme activity through the consumption of $H_2O_2$, evidenced by the reduction of absorbance at 240 nm. For the assay, 10 μL of the supernatant was diluted in 90 μL of PBS, resulting in a 10-fold dilution. Then, 10 μL of the diluted supernatant and 140 μL of 10 mM $H_2O_2$ (Sigma-Aldrich, St. Louis, MO, USA) were added to a microplate well. The reduction in absorbance of the reaction mixture was monitored at 240 nm every 10 seconds for two minutes, with measurements performed in triplicate. The specific activity of CAT was expressed in units of enzymatic activity (U) per milligram of protein.

### 1.1.5. Quantification of protein content

The quantification of protein content in the homogenates was performed based on an adaptation of the method described by Bradford (1976). For the assay, 10 µL of the supernatant was mixed with 200 µL of Bradford reagent. An analytical curve was generated using albumin as a standard. Absorbance was measured at 595 nm in duplicate. The protein content obtained was used to normalize the results of the enzymatic tests described above.

### 1.2. Evaluation of lipid peroxidation in *Sitophilus zeamais* adults

For the assay, 150 µL of the homogenate (section 2.5) was mixed with 200 µL of the TBARS solution. The mixture was then vortexed and incubated in a water bath at 37 °C for 15 minutes. After cooling to room temperature, 420 µL of butyl alcohol was added to the solution, which was again vortexed and centrifuged (Multifuge X1R Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes at 5000 g. The supernatant of all samples was read in 96-well flat-bottom microplates, and readings were performed at 532 nm. Measurements were made in duplicate at 532 nm, with five samples per treatment. The results were expressed as malondialdehyde concentration in $mg.L^{-1}$.

### 1.3. Evaluation of digestive enzyme activity

#### 1.3.1. Trypsin activity

The activity of the enzyme trypsin (EC 3.4.21.4) was determined by adapting the method proposed by Erlanger et al. (1961). The enzyme was extracted with tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$. In a 96-well plate, 20 µL of the supernatant, 40 µL of buffer and 80 µL of reaction substrate consisting of α-benzoylarginine-4-nitroanilide hydrochloride at 0.00054 $g.mL^{-1}$ and DMSO at 12.4 µL were added. $mL^{-1}$ solubilized in tris-HCl buffer. The samples were analyzed in triplicate at 410 nm in kinetic mode, with each reading performed every 10 seconds, totaling 50 readings. The results were expressed as specific enzyme activity (U $mg.protein^{-1}$).

#### 1.3.2. *α*-Amylase Activity

The activity of the α-amylase enzyme (EC 3.2.1.1) was determined according to the protocol described by the manufacturer of the kit used (Quibasa-Bioclin, Belo Horizonte, MG, Brazil). The enzyme was extracted with 0.1 M sodium acetate-acetic acid buffer, pH 5.5. In a 96-well plate, 4 µL of the supernatant and 200 µL of reaction substrate (200 mM MES buffer and 5 mM α-(2-chloro-4-nitrophenyl)-β-1,4-galactopyranosylmaltoside) were added. The samples were analyzed in triplicate at 405 nm in kinetic mode, with each reading performed every 1 minute, totaling 4 readings. The results were expressed as specific enzyme activity (U mg.protein$^{-1}$).

### 1.3.3. Lipase activity

The activity of the lipase enzyme (EC 3.1.1.3) was determined according to the protocol described by the manufacturer of the kit used (Quibasa-Bioclin, Belo Horizonte, MG, Brazil). The enzyme was extracted with 0.1 M sodium acetate-acetic acid buffer, pH 5.5. In a 96-well plate, 5 μL of the supernatant, 100 μL of 100 mM Tris buffer, pH 8.5, 2 μL of the reaction inhibitor phenylmethyl sulfonyl fluoride at 8 mM and 10 μL of color reagent (3 mM dithionitrobenzoic acid and 100 mM sodium acetate) were added. The plate was incubated at 37 °C for 2 minutes. Next, 10 μL of 20 mM dithiopropanol tributyrate were added. The plate was incubated at 37 °C for 30 minutes. Finally, 200 μL of acetone was added to stop the reaction. The samples were analyzed in duplicate at 410 nm. A blank was made for each sample, which consisted of the addition of all reagents except 2 μL of the reaction inhibitor phenylmethyl sulfonyl fluoride at 8 mM and 10 μL of 20 mM dithiopropanol tributyrate. The activity was determined by the following equation and the results were expressed as enzyme activity (U.L$^{-1}$).

$$\text{Lipase activity} = ((\text{Sample Abs.} - \text{Blank Abs.}) \times 1.000)/7$$

### 1.3.4. Determination of protein content

To normalize the results, the protein content was determined as described in section 1.1.5 in Supplementary Materials.

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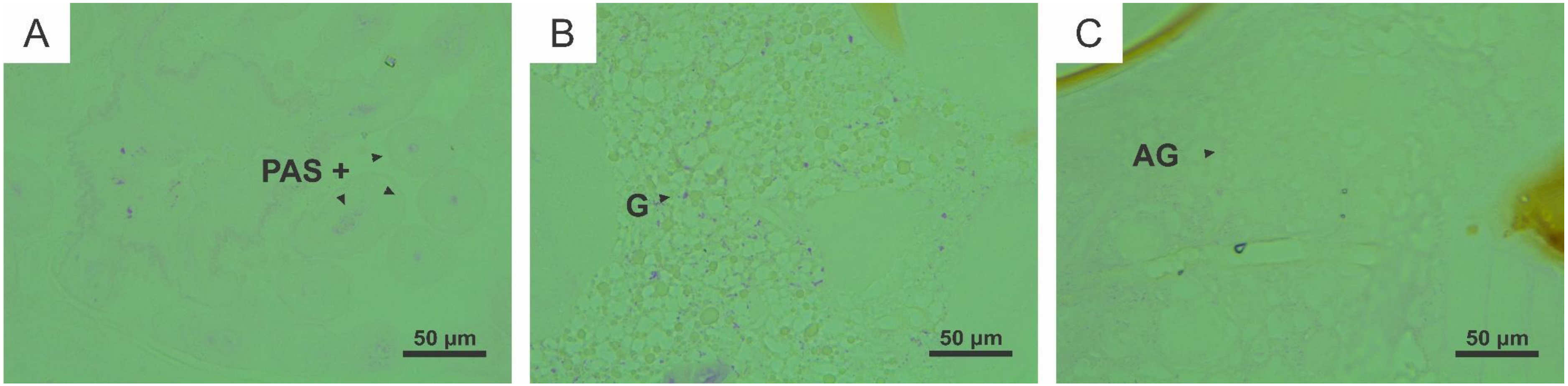
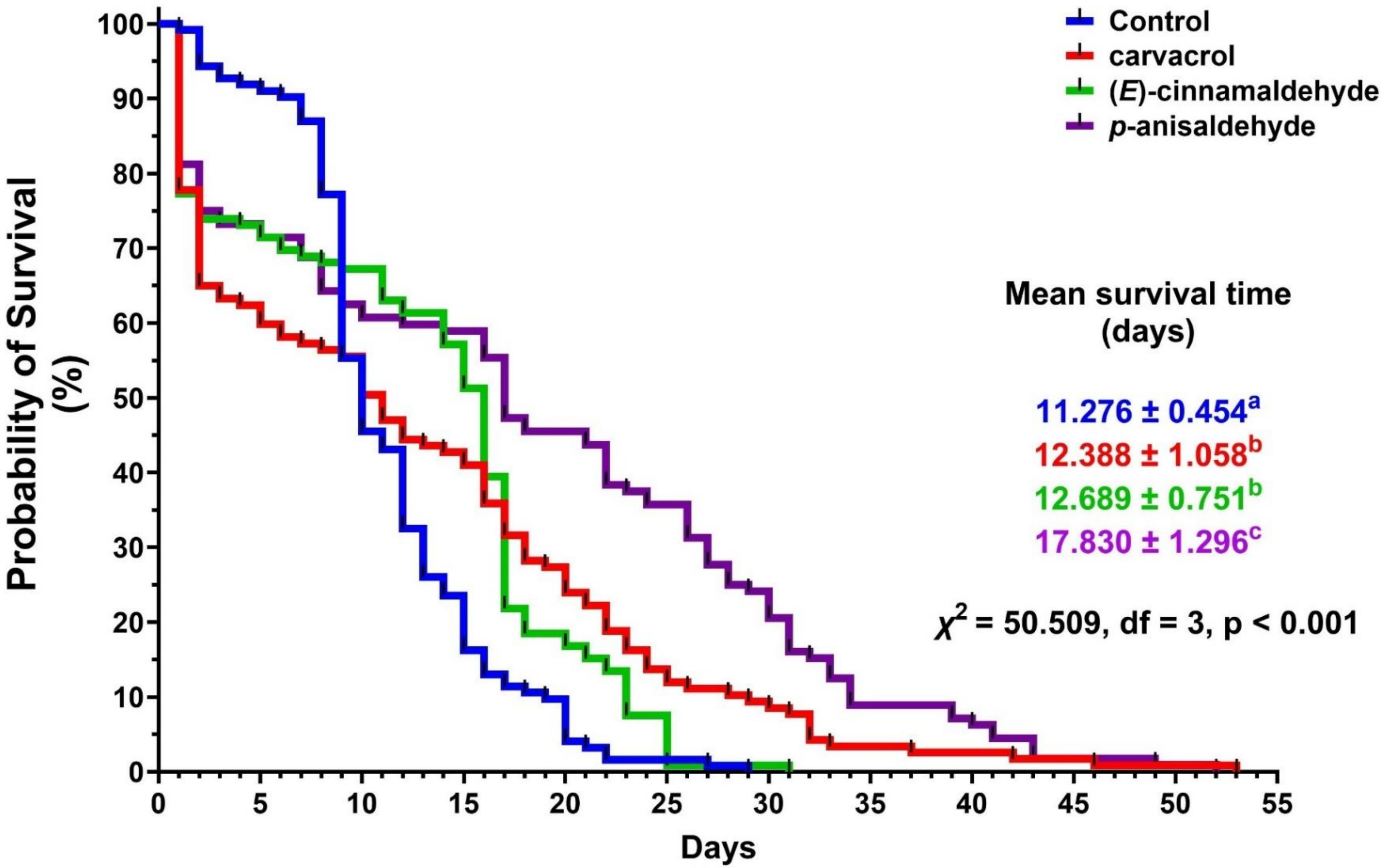
**Supplementary figure 1 -** Representation of the main histological alterations in adults of *Sitophilus zeamais* in histological sections stained with periodic acid-Schiff (PAS). (A) – Representative image of the intestinal content reactive to the PAS staining technique in the *p*-anisaldehyde group; (B) – Fat body of the control group; (C) – Fat body of the *p*-anisaldehyde group. Scale bars: 50 µm. Captions: AG – absence of glycogen granules; G – glycogen granules; PAS+ – structures reactive to the PAS staining technique.



**Supplementary figure 2 -** Survival analysis curve of *Tetrastichus howardi* females exposed to *Tenebrio molitor* pupae treated with the $LC_{50}$ of the compounds carvacrol, (*E*)-cinnamaldehyde, and *p*-anisaldehyde. (Log-rank analysis followed by the Holm-Sidak test, n=120/treatment).



# CONSIDERAÇÕES FINAIS

O desenvolvimento desse projeto comprovou o potencial que produtos naturais, especialmente terpenos e fenilpropanoides, apresentam como alternativas ecologicamente corretas e sustentáveis para o manejo de pragas agrícolas. Este estudo demonstra o ótimo desempenho inseticida desses compostos sobre os insetos-praga *Drosophila suzukii* e *Sitophilus zeamais*, e fornece informações valiosas sobre impactos da exposição a doses sub-letais na morfofisiologia e possíveis mecanismos de ação dessas substâncias nos organismos em estudo.

Os terpenos, L-(-)-carvona e carvacrol, e os fenilpropanoides, (*E*)-anetol e (*E*)-cinamaldeído apresentaram a melhor atividade inseticida sobre adultos de *D. suzukii*, doses subletais desses compostos promoveram elevada mortalidade de larvas e deformação em indivíduos expostos, induziram o estresse oxidativo e alterações morfológicas em estruturas internas e externas dessa espécie. Além disso, esses compostos não afetaram parâmetros biológicos do predador natural *Doru luteipes*.

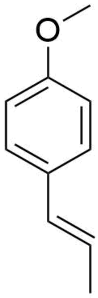
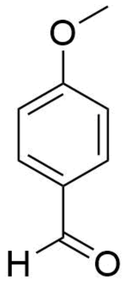
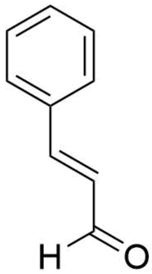
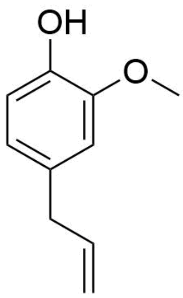
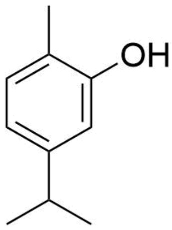
Buscando contornar as limitações impostas pelas propriedades físico-químicas desses produtos naturais, os terpenos e fenilpropanoides de melhor desempenho inseticida sobre adultos de *D. suzukii* foram incorporados a nanopartículas. A incorporação dos compostos em nanopartículas do polímero biodegradável PCL prolongou a atividade inseticida desses compostos ao longo do tempo, particularmente os compostos PCL-carvacrol e PCL-(*E*)-cinamaldeído. Os compostos associados às nanopartículas demonstraram maior potencial em ativar as defesas enzimáticas do estresse oxidativo, especialmente o PCL-carvacrol, indicando maior potencial para gerar danos ao organismo dos insetos-praga. Os compostos em sua forma isolada promoveram maior peroxidação lipídica, indicada pela maior concentração de malondialdeído, possivelmente por não terem gerado alta atividade das enzimas detoxificantes. Apesar de terem promovido a elevação da atividade das enzimas que combatem o estresse oxidativo, os compostos nanoparticulados demonstraram maior potencial para gerar danos histopatológicos em diversas estruturas de fêmeas adultas de *D. suzukii*; comprovando que os compostos em estudo não afetaram parâmetros biológicos do organismo não alvo *Palmistichus elaeisis*.

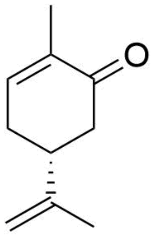
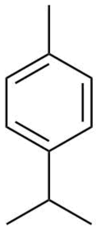
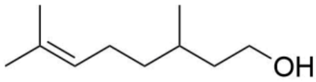
Por fim, os terpenos e fenilpropanoides em estudo apresentaram apreciável atividade inseticida sobre *S. zeamais*, destacando-se o carvacrol como o composto mais tóxico. O (*E*)-anetol foi o composto que apresentou o maior número de interações sinérgicas quando combinado com outros compostos, portanto é uma substância a ser considerada para elaboração de novos produtos inseticidas. O carvacrol, (*E*)-cinamaldeído e *p*-anisaldeído causaram efeitos subletais nessa praga, alterando consideravelmente a atividade das enzimas digestivas e as relacionadas ao estresse oxidativo, além de causar danos morfológicos. *p*-anisaldeído se destaca por ter inibido fortemente a atividade da enzima $\alpha$-amilase e elevado a atividade das enzimas detoxificantes CAT, GST e GPx. (*E*)-cinamaldeído pode ser considerado o composto mais compatível com o inimigo natural *Tetrastichus howardi*, por ter afetado apenas a emergência da progênie e não ter alterado nenhum outro parâmetro avaliado.

Estudos posteriores devem ser realizados com esses terpenos e fenilpropanoides, investigando o desempenho em condições reais de campo e armazenamento de grãos, além de se avaliar a compatibilidade com outros organismos.

# APÊNDICE

Quadro dos compostos químicos em estudo

| Composto | Estrutura | Fórmula molecular | Classe de produto natural |
|---|---|---|---|
| (*E*)-anetol |   | $C_{10}H_{12}O$ | Fenilpropanoide |
| *p*-anisaldeído |   | $C_8H_8O_2$ | Fenilpropanoide |
| (*E*)-cinamaldeído |   | $C_9H_8O$ | Fenilpropanoide |
| eugenol |   | $C_{10}H_{12}O_2$ | Fenilpropanoide |
| carvacrol |   | $C_{10}H_{14}O$ | Terpeno com características fenólicas |

| | | | |
|---|---|---|---|
| L-(-)-carvona |  | $C_{10}H_{14}O$ | Terpeno cíclico |
| *p*-cimeno |  | $C_{10}H_{14}$ | Terpeno aromático |
| 1,8-cineol |  | $C_{10}H_{18}O$ | Terpeno bicíclico |
| *β*-citronelol |  | $C_{10}H_{20}O$ | Terpeno |