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**CARACTERIZAÇÃO QUÍMICA, ATIVIDADES BIOLÓGICAS E**  
**MECANISMOS DE AÇÃO DO ÓLEO ESSENCIAL DE *Piper cyrtopodon* (Miq.)**  
**C.DC (PIPERACEAE) CONTRA *Aedes aegypti* (DIPTERA: CULICIDAE)**

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**MANAUS – AMAZONAS**

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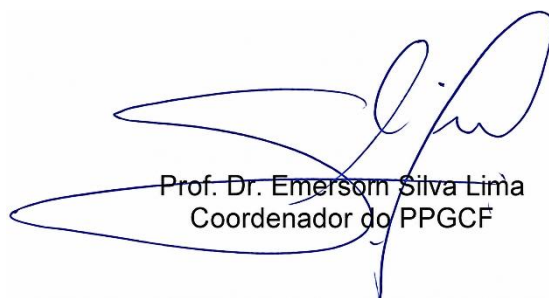
**“CARACTERIZAÇÃO QUÍMICA, ATIVIDADES BIOLÓGICAS E MECANISMOS DE  
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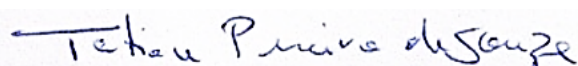
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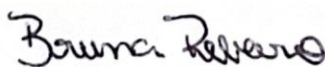
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“Consagre ao Senhor tudo o que você faz,  
e os seus planos serão bem-sucedidos.”

— Provérbios 16:3

## RESUMO

As arboviroses transmitidas pelo mosquito *Aedes aegypti*, como dengue, Zika e chikungunya, continuam a representar um importante desafio em saúde pública, agravado pela resistência do vetor aos inseticidas sintéticos. Nesse contexto, produtos de origem vegetal, especialmente óleos essenciais (OEs), surgem como alternativas promissoras para o controle desse vetor. Assim, o estudo avaliou o potencial larvicida e repelente do OE de *Piper cyrtopodon* e de seus principais constituintes contra *A. aegypti*, bem como seus mecanismos de ação e perfil de segurança. O OE, obtido com rendimento de 1,4%, apresentou germacreno D (57,46%), cadina-1(10),4-dieno (10,57%) e  $\delta$ -Elemeno (8,00%) como constituintes majoritários, os quais exibiram atividade larvicida, com valores de CL<sub>50</sub> de 20,78 a 146,61  $\mu$ g/mL, sendo cadina-1(10),4-dieno o constituinte mais ativo. Essa toxicidade foi associada à indução de estresse oxidativo, evidenciada pelo aumento da produção de espécies reativas de oxigênio (ROS), peroxidação lipídica e alterações nas enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutathione peroxidase (GPx). Nos ensaios de repelência, o OE e seus constituintes apresentaram significativa atividade, com tempo de proteção de até 270 minutos, comparável ao N,N-dietil-meta-toluidina (DEET), destacando-se a cadina-1(10),4-dieno que apresentou o menor valor de CE<sub>50</sub> (2.31 mg/cm<sup>2</sup>). Os estudos de *docking* molecular com as proteínas ligantes de odorantes (OBP1 e OBP22) revelaram significativa afinidade de ligação dos constituintes, especialmente da cadina-1(10),4-dieno, com energias de ligação -8.4 e -7.3 kcal/mol, respectivamente, sustentando o mecanismo de interferência no sistema olfatório do mosquito. As análises de absorção, distribuição, metabolismo, excreção e toxicidade (ADMET), alta lipofilicidade e baixa polaridade dos constituintes, favorecendo a retenção cutânea, com perfil toxicológico variável. Ensaios em células Vero e PBMCs demonstraram alta viabilidade nas menores concentrações, com redução dose-dependente em concentrações mais elevadas observada apenas para o OE e a cadina-1(10),4-dieno. Em conjunto, os resultados evidenciam que o OE de *P. cyrtopodon* atua de forma integrada sobre o vetor, combinando toxicidade larval e efeito repelente, destacando-se como uma fonte promissora de constituintes naturais para estratégias sustentáveis de controle de *A. aegypti*.

Palavras-chave: *Piper*, óleo essencial, larvicida, repelente, *Aedes aegypti*.

## ABSTRACT

Arboviral diseases transmitted by the mosquito *Aedes aegypti*, such as dengue, Zika, and chikungunya, continue to represent a major public health challenge, further aggravated by the vector's resistance to synthetic insecticides. In this context, plant-derived products, particularly essential oils (EOs), have emerged as promising alternatives for vector control. Thus, this study evaluated the larvicidal and repellent potential of the EO from *Piper cyrtopodon* and its major constituents against *A. aegypti*, as well as their mechanisms of action and safety profile. The EO, obtained with a yield of 1.4%, was mainly composed of germacrene D (57.46%), cadina-1(10),4-diene (10.57%), and  $\delta$ -Elemene (8.00%), which exhibited larvicidal activity, with LC<sub>50</sub> values ranging from 20.78 to 146.61  $\mu$ g/mL, with cadina-1(10),4-diene being the most active compound. This toxicity was associated with the induction of oxidative stress, evidenced by increased production of reactive oxygen species (ROS), lipid peroxidation, and alterations in antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). In repellent assays, the EO and its constituents showed significant activity, with a protection time of up to 270 minutes, comparable to N,N-diethyl-meta-toluamide (DEET), with cadina-1(10),4-diene standing out due to its lowest EC<sub>50</sub> value (2.31 mg/cm<sup>2</sup>). Molecular *docking* studies with odorant-binding proteins (OBP1 and OBP22) revealed strong binding affinities of the constituents, particularly cadina-1(10),4-diene, with binding energies of  $-8.4$  and  $-7.3$  kcal/mol, respectively, supporting a mechanism involving interference with the mosquito olfactory system. Absorption, distribution, metabolism, excretion, and toxicity (ADMET) analyses indicated high lipophilicity and low polarity of the constituents, favoring skin retention, with a variable predicted toxicological profile. Cytotoxicity assays in Vero and PBMC cells demonstrated high cell viability at lower concentrations, with dose-dependent reduction at higher levels observed only for the EO and cadina-1(10),4-diene. Overall, the results demonstrate that the EO from *P. cyrtopodon* acts through an integrated mechanism against the vector, combining larvicidal toxicity and repellent effects, highlighting its potential as a promising source of natural compounds for sustainable *A. aegypti* control strategies.

Keywords: *Piper*, essential oil, larvicidal, repellent, *Aedes aegypti*.

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## LISTA DE ABREVIATURAS E SIGLAS

|                  |                                                            |
|------------------|------------------------------------------------------------|
| ADMET            | Absorção, Distribuição, Metabolismo, Excreção e Toxicidade |
| ANOVA            | Análise de Variância                                       |
| ANVISA           | Agência Nacional de Vigilância Sanitária                   |
| BBB              | Barreira hematoencefálica                                  |
| CAT              | Catalase                                                   |
| CE <sub>50</sub> | Concentração efetiva para 50%                              |
| CL <sub>50</sub> | Concentração letal para 50%                                |
| CYP              | Citocromo P450                                             |
| DEET             | N,N-dietil-meta-toluamida                                  |
| DENV             | Vírus da dengue                                            |
| DMSO             | Dimetilsulfóxido                                           |
| FPP              | Farnesil pirofosfato                                       |
| GC-MS            | Cromatografia gasosa acoplada à espectrometria de massas   |
| GC-FID           | Cromatografia gasosa com detector por ionização de chama   |
| GPP              | Geranil pirofosfato                                        |
| GPx              | Glutathione peroxidase                                     |
| GR               | Glutathione reductase                                      |
| GST              | Glutathione S-transferase                                  |
| IPP              | Isopentenil pirofosfato                                    |
| kdr              | Mutação knockdown resistance                               |
| K <sub>i</sub>   | Constante de inibição                                      |
| L1 – L4          | Primeiro ao quarto estágio larval                          |
| MEP              | Via do metileritritol fosfato                              |
| MVA              | Via do ácido mevalônico                                    |
| MTT              | 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio           |
| OBP              | Proteína ligadora de odorante                              |
| OE               | Óleo essencial                                             |
| OR               | Receptor de odorante                                       |
| PAHO             | Organização Pan-Americana da Saúde                         |
| PBMC             | Células mononucleares do sangue periférico                 |
| PDB              | <i>Protein Data Bank</i>                                   |
| RMSD             | <i>Root Mean Square Deviation</i>                          |

|            |                               |
|------------|-------------------------------|
| ROS        | Espécies reativas de oxigênio |
| SOD        | Superóxido dismutase          |
| SUS        | Sistema Único de Saúde        |
| WHO        | Organização Mundial da Saúde  |
| $\Delta G$ | Energia livre de ligação      |

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## 1. INTRODUÇÃO

As arboviroses transmitidas pelo mosquito *Aedes aegypti* (Linnaeus, 1762) (Culicidae) continuam a representar um importante desafio em saúde pública nas regiões tropicais, especialmente no Brasil, onde condições ambientais favoráveis, urbanização desordenada e deficiências no saneamento básico contribuem para a manutenção de elevadas densidades do vetor (Lwande et al., 2020; Ministério da Saúde, 2026a).

Entre essas doenças, destacam-se a dengue, a chikungunya e a Zika, cuja circulação simultânea amplia a carga de morbimortalidade e a pressão sobre os sistemas de saúde (Rückert et al., 2017). Apesar dos avanços no desenvolvimento de vacinas, sobretudo para a dengue, o controle vetorial permanece como a principal estratégia de prevenção das arboviroses, uma vez que ainda não existem imunizantes disponíveis para chikungunya e Zika (Ministério da Saúde, 2026a).

Entretanto, as estratégias atualmente empregadas apresentam limitações relevantes, incluindo dificuldades operacionais e, principalmente, o desenvolvimento de resistência do vetor aos inseticidas sintéticos amplamente utilizados (Konkon et al., 2023; Ministério da Saúde, 2026b). Essa resistência compromete a eficácia do controle químico e está associada tanto a impactos ambientais quanto à ativação de mecanismos adaptativos no mosquito, como alterações nos sistemas antioxidantes e de detoxificação frente ao estresse oxidativo induzido por esses compostos (Zulfa et al., 2022; Pu & Chung, 2024).

Nesse sentido, produtos de origem vegetal têm emergido como alternativas promissoras para o controle do *A. aegypti*, com destaque para os óleos essenciais, devido à sua diversidade química, biodegradabilidade e menor impacto ambiental (Bakkali et al., 2008; Santos et al., 2022). Esses metabólitos apresentam atividade larvicida significativa e atuam por múltiplos mecanismos, o que pode reduzir a seleção de populações resistentes quando comparados aos inseticidas sintéticos convencionais (Azeem et al., 2019; Oliveira et al., 2022b).

Além da toxicidade direta, os óleos essenciais também vêm sendo investigados como repelentes, por interferirem no sistema olfatório do mosquito e comprometerem sua capacidade de localizar o hospedeiro humano, principalmente por meio de interações com proteínas ligadoras de odorantes (*odorant-binding proteins*, OBPs) e receptores de odorantes (*odorant receptors*, ORs), considerados alvos moleculares estratégicos para o desenvolvimento de novos repelentes (Costa et al., 2019; Portilla-Pulido et al., 2022).

Paralelamente, a biodiversidade amazônica constitui uma fonte promissora de espécies vegetais com potencial inseticida e repelente, entre as quais se destacam espécies do gênero *Piper* (Piperaceae), reconhecidas pela produção de óleos essenciais ricos em sesquiterpenos bioativos (Carsono et al., 2022). Dentre essas espécies, *P. cyrtopodon* (Miq.) C.DC., um arbusto amplamente distribuído na região Norte do Brasil, permanece pouco investigado sob os aspectos fitoquímico e biológico (Guimarães et al., 2015).

Diante desse cenário, a avaliação do óleo essencial de *P. cyrtopodon* frente a *A. aegypti* mostra-se relevante por ampliar o conhecimento sobre o potencial bioativo de uma espécie nativa pouco explorada e contribuir para a prospecção de produtos aplicáveis ao controle vetorial.

O presente estudo envolveu a caracterização química do óleo essencial, a avaliação de suas atividades larvicida e repelente e a investigação de seus mecanismos de ação. Para isso, foi adotada uma abordagem integrada, contemplando a análise de parâmetros bioquímicos associados ao estresse oxidativo e à atividade enzimática, estudos *in silico* de interação com proteínas do sistema olfatório por meio de *docking* molecular, além da predição de propriedades farmacocinéticas e toxicológicas (ADMET) e da avaliação do perfil de segurança por ensaios de citotoxicidade *in vitro*. Em conjunto, essas estratégias fornecem bases para o desenvolvimento de abordagens eficazes e ambientalmente sustentáveis no controle de *A. aegypti*.

## 2. FUNDAMENTAÇÃO TEÓRICA

### 2.1 Aspectos gerais do *Aedes aegypti*

O mosquito *A. aegypti* é reconhecido como um dos principais vetores de arboviroses de relevância em saúde pública, especialmente em regiões tropicais e subtropicais (Lwande et al., 2020). Originário do continente africano, esse mosquito expandiu sua distribuição geográfica ao longo dos séculos, impulsionado pela intensificação das atividades humanas, como o transporte marítimo, a urbanização acelerada e o acúmulo de recipientes artificiais propícios à formação de criadouros (Lima et al., 2021).

A espécie encontra-se amplamente distribuída em ambientes urbanos, onde mantém estreita proximidade com o hospedeiro humano, favorecendo a sua elevada importância epidemiológica como vetor de doenças (Oliveira et al., 2022b).

Nesse contexto, a compreensão dos aspectos biológicos e do ciclo de vida do *A. aegypti* torna-se fundamental para o entendimento de sua dinâmica populacional e para o desenvolvimento de estratégias eficazes de controle vetorial (Gubler, 2011). O ciclo de vida do *A. aegypti* compreende quatro fases distintas: ovo, larva, pupa e adulto (Figura 1) (Consoli & Oliveira, 1994). A duração do ciclo é influenciada por fatores ambientais, como temperatura, disponibilidade de água e nutrientes, com um período de desenvolvimento entre sete e dez dias (Consoli & Oliveira, 1994).

Os ovos apresentam elevada resistência à dessecação, podendo permanecer viáveis por até 1 ano em ambientes secos, o que favorece a persistência da espécie no ambiente e dificulta as estratégias de controle (Ahebwa et al., 2023). Após a eclosão, as larvas passam por quatro estádios de desenvolvimento (L1 a L4) até atingirem a fase pupal, que antecede a emergência do adulto (Aguirre-Obando et al., 2016).

Na fase adulta, as fêmeas realizam o repasto sanguíneo, necessário para a maturação dos ovos, comportamento diretamente associado à transmissão dos agentes etiológicos das arboviroses (Lima et al., 2024b). Esse hábito hematofágico, aliado à elevada densidade populacional em áreas urbanas, contribui para a eficiência do *A. aegypti* como vetor de doenças (Farnesi et al., 2017).

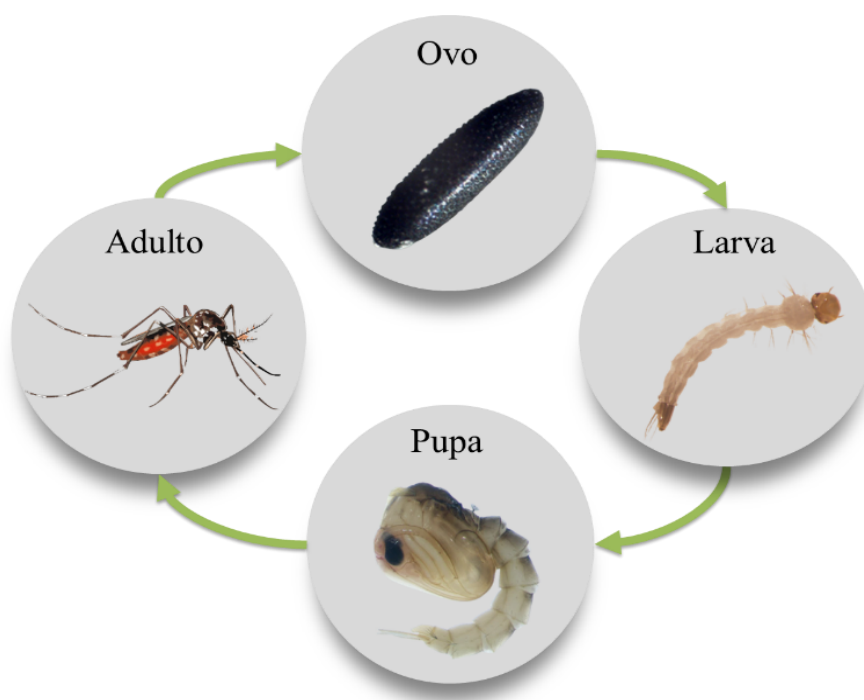


**Figura 1:** Ciclo biológico do *Aedes aegypti*. Fonte: O autor, 2025.

## 2.2 Arboviroses transmitidas pelo *Aedes aegypti*

A dengue é uma das arboviroses mais antigas e amplamente disseminada pelo *A. aegypti*, causada por vírus do gênero *Orthoflavivirus* (*Flaviviridae*), que compreende quatro sorotipos imunologicamente distintos (DENV-1, DENV-2, DENV-3 e DENV-4) (Ministério da Saúde, 2026a).

No Brasil, a dengue apresenta comportamento endêmico-epidêmico nas últimas décadas, com elevada carga de morbimortalidade (Costa et al., 2025). Somente em 2025, foram registrados aproximadamente 1,7 milhão de casos prováveis da doença e 1.762 óbitos, evidenciando a persistência da transmissão (Ministério da Saúde, 2026c).

Além da dengue, outras arboviroses transmitidas pelo vetor também apresentam circulação sustentada em território nacional, ampliando o impacto desse vetor sobre a saúde pública (Ministério da Saúde, 2026a).

A febre chikungunya, causada por vírus do gênero *Alphavirus* (*Togaviridae*), apresenta transmissão sustentada no Brasil desde os primeiros registros autóctones, mantendo padrão recorrente de casos (PAHO, 2025a). Em 2025, foram notificados 127.494 casos e 120 óbitos, demonstrando que a doença continua representando um importante problema de saúde pública, especialmente devido às manifestações clínicas crônicas associadas à infecção (Ministério da Saúde, 2026c).

De forma semelhante, a infecção pelo Zika vírus, pertencente ao gênero *Flavivirus* (*Flaviviridae*) ganhou destaque internacional a partir de sua associação com anomalias congênitas, particularmente a microcefalia em recém-nascidos (WHO, 2022). No Brasil, em 2025, foram registrados 4.143 casos prováveis de Zika, com 1 óbito, mantendo-se como uma preocupação relevante em saúde pública, sobretudo para gestantes, em função do risco de complicações fetais (Ministério da Saúde, 2026c; PAHO, 2025b).

A coexistência dessas arboviroses em áreas urbanas, frequentemente de forma simultânea, reforça a persistência dos desafios no controle das doenças e no manejo das populações de *A. aegypti* (Lima et al., 2024a). Esse cenário é agravado por fatores como condições climáticas favoráveis à proliferação do vetor, deficiência no saneamento básico, falhas nas políticas públicas de controle e o desenvolvimento de resistência do mosquito aos inseticidas utilizados em larga escala, evidenciando a necessidade de estratégias alternativas e mais eficazes para o controle vetorial (Oliveira et al., 2022b).

### **2.3 Estratégias de prevenção e suas limitações**

Apesar da relevância clínica das arboviroses transmitidas pelo *A. aegypti*, não existem tratamentos antivirais específicos capazes de interromper a infecção, sendo as abordagens terapêuticas disponíveis voltadas, predominantemente, ao manejo sintomático e à prevenção de complicações (Ministério da Saúde, 2026a).

Entre as estratégias preventivas, o desenvolvimento de vacinas representa um avanço relevante, especialmente no enfrentamento da dengue (Tricou et al., 2024). No Brasil, além da vacina tetravalente Qdenga, incorporada ao Sistema Único de Saúde (SUS) e utilizada com esquema de duas doses, a Agência Nacional de Vigilância Sanitária (ANVISA) aprovou, ao final de 2025, o registro da vacina Butantan-DV, o primeiro imunizante tetravalente de dose única contra a dengue produzido por laboratório nacional (Instituto Butantan, 2025; Ministério da Saúde, 2026a).

Apesar desses avanços, ainda não existem vacinas disponíveis para chikungunya e zika, reforçando que o controle vetorial permanece como a principal estratégia de prevenção das arboviroses transmitidas pelo *A. aegypti* (Ministério da Saúde, 2026a).

Nesse contexto, o controle do vetor baseia-se, tradicionalmente, em um conjunto de estratégias que incluem ações mecânicas, químicas e biológicas (Lepore et al., 2025). As medidas mecânicas envolvem a eliminação de criadouros e a redução de locais propícios à oviposição, enquanto as estratégias biológicas e químicas visam à redução

direta das populações de larvas e adultos (Zara et al., 2016). Embora amplamente empregadas, essas abordagens enfrentam desafios operacionais significativos, como dependência da participação comunitária, dificuldades logísticas e necessidade de reaplicações frequentes (Wilson et al., 2020).

Adicionalmente, o uso intensivo e prolongado de inseticidas químicos tem sido associado ao desenvolvimento de resistência nas populações de *A. aegypti*, bem como a impactos ambientais (Ranatunga et al., 2023; Zulfa et al., 2022). Essas limitações comprometem a eficácia sustentável das estratégias de controle adotadas e evidenciam a necessidade de alternativas mais seguras, eficazes e ambientalmente compatíveis para o manejo do vetor (Lu et al., 2023).

## 2.4 Inseticidas sintéticos e mecanismos associados

A utilização de inseticidas sintéticos é a principal estratégia adotada no Brasil para a redução rápida das populações de larvas e adultos de *A. aegypti*, contribuindo diretamente para a diminuição da transmissão de arboviroses no país (Valle et al., 2019). Esses produtos atuam predominantemente no sistema nervoso dos insetos e pertencem a diferentes classes químicas, cujos mecanismos de ação envolvem a interferência na transmissão sináptica e na excitabilidade neuronal (Figura 2) (Roiz et al., 2018).

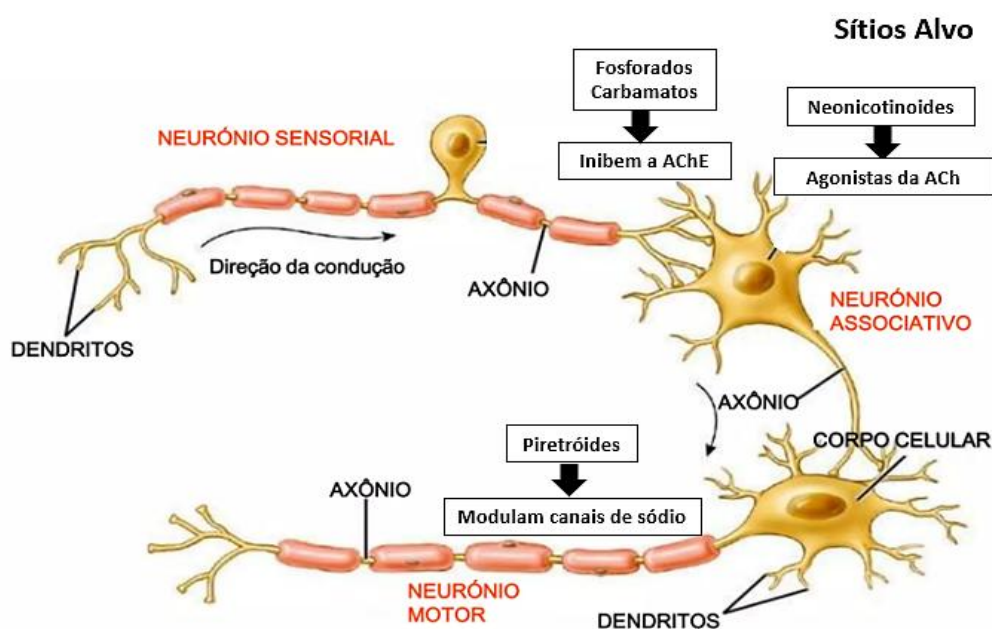


**Figura 2:** Mecanismos de ação de inseticidas no sistema nervoso dos insetos. Fonte: Adaptado de Matias, 2024.

O Ministério da Saúde recomenda piretroides, aos quais interferem nos canais de sódio dependentes de voltagem, provocando hiperexcitação neural e paralisia dos insetos (Ministério da Saúde, 2026d). De forma complementar, os neonicotinoides atuam como agonistas dos receptores nicotínicos da acetilcolina, apresentando elevada eficácia inseticida (Hladik et al., 2018).

Além dos inseticidas químicos neurotóxicos, as estratégias de controle também incluem larvicidas biológicos, como *Bacillus thuringiensis israelensis* (Bti), reguladores de crescimento como o piriproxifeno e adulticidas formulados a partir da combinação de moléculas com diferentes modos de ação, visando ampliar a eficácia e retardar o desenvolvimento de resistência (Ministério da Saúde, 2025d).

No que se refere aos mecanismos de ação, além dos efeitos neurofisiológicos, diversos inseticidas sintéticos podem induzir estresse oxidativo nos insetos, promovendo a geração de espécies reativas de oxigênio (ROS) (Sule et al., 2022). O acúmulo dessas espécies pode exceder a capacidade antioxidante celular, resultando em danos a lipídios, proteínas e ácidos nucleicos, comprometendo a homeostase celular e levando à morte do organismo (Figura 3) (Cutler et al., 2022). Esse processo está diretamente relacionado à ativação de sistemas enzimáticos antioxidantes e de desintoxicação, que desempenham papel central na resposta adaptativa do mosquito à exposição aos inseticidas (Dong et al., 2022).

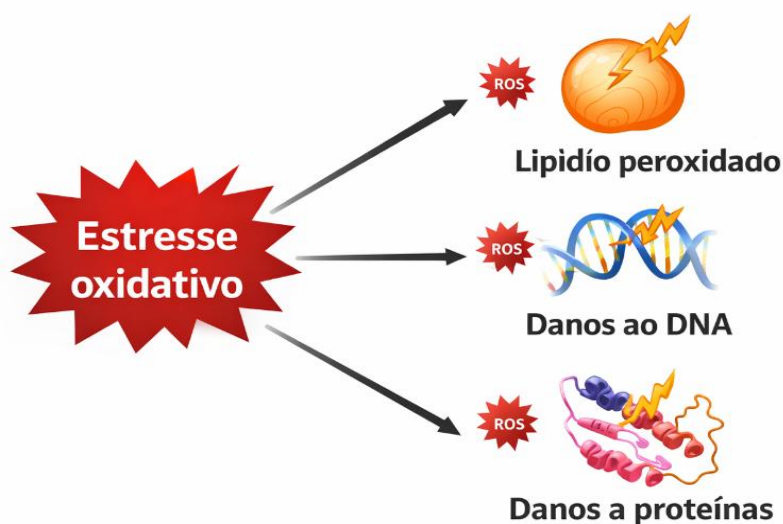


**Figura 3:** Danos a nível celular induzidos por estresse oxidativo decorrente à exposição de inseticidas. Fonte: O autor, 2025.

### 2.4.1 Resistência metabólica

A resistência metabólica é um dos principais mecanismos envolvidos na redução da eficácia dos inseticidas empregados no controle do *A. aegypti* (Konkon et al., 2023). Esse tipo de resistência está relacionado à capacidade do mosquito de metabolizar, sequestrar ou eliminar moléculas tóxicas antes que atinjam seus alvos fisiológicos, permitindo sua sobrevivência mesmo sob exposição contínua a agentes químicos (Moyes et al., 2017).

Esse processo envolve, principalmente, a superexpressão ou o aumento da atividade de sistemas enzimáticos de detoxificação, bem como o fortalecimento dos mecanismos celulares responsáveis pela manutenção da homeostase redox (Pu & Chung, 2024). Em conjunto, essas respostas reduzem os efeitos tóxicos dos inseticidas e contribuem para a adaptação das populações do vetor a ambientes sob intensa pressão química (Figura 4) (Silva et al., 2025).

Entre os sistemas enzimáticos mais frequentemente associados à resistência metabólica destacam-se as monooxigenases do citocromo P450 (CYPs), as esterases e as glutathione S-transferases (GSTs) (Nauen et al., 2022). As enzimas da família CYP catalisam reações oxidativas que facilitam a biotransformação de diferentes classes de inseticidas, incluindo piretroides e neonicotinoides (Wang et al., 2023). As esterases estão relacionadas à hidrólise de moléculas como organofosforados e carbamatos, enquanto as GSTs atuam na conjugação de xenobióticos à glutathione reduzida, favorecendo sua excreção e reduzindo a toxicidade celular (Koirala et al., 2022; Cruse et al., 2023).

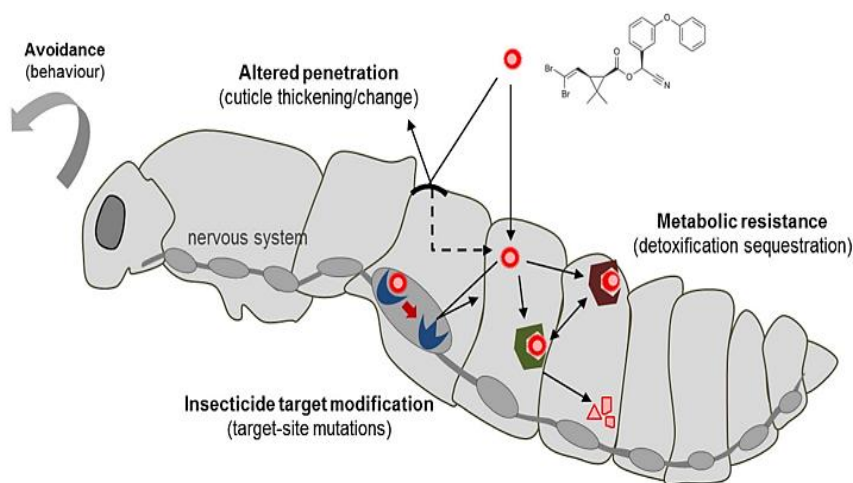


**Figura 4:** Mecanismos de resistência a inseticidas químicos. Fonte: Corbel et al., 2017.

Além da detoxificação direta, a resistência metabólica envolve o fortalecimento dos sistemas antioxidantes celulares, responsáveis por mitigar os efeitos do estresse oxidativo induzido pela exposição a inseticidas (Silva et al., 2025). Enzimas como superóxido dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) e glutathione reductase (GR) desempenham papel central na neutralização das ROS, prevenindo danos oxidativos a lipídios, proteínas e ácidos nucleicos e contribuindo para a manutenção da homeostase celular (García-Caparrós et al., 2021).

Evidências recentes demonstram que o aumento da atividade dessas enzimas antioxidantes ocorre concomitantemente à elevação dos sistemas de detoxificação clássicos, indicando que a resistência metabólica é um processo multifatorial, no qual respostas enzimáticas e antioxidantes atuam de forma integrada (Ranganathan et al., 2022; Zhou et al., 2024).

## **2.5 Repelentes sintéticos: mecanismos de ação e suas limitações**

Repelentes são produtos tópicos, geralmente formulados em veículos oleosos, que atuam formando uma barreira química e sensorial sobre a pele, interferindo na capacidade dos mosquitos hematófagos de localizar e pousar sobre o hospedeiro (Portilla Pulido et al., 2022). Dessa forma, os repelentes desempenham papel importante na redução do contato vetor–hospedeiro, sendo uma estratégia complementar no controle das arboviroses transmitidas pelo *A. aegypti* (Okoli et al., 2021).

Entre os repelentes sintéticos, a N,N-dietil-meta-toluamida (DEET) é a substância química amplamente utilizada e considerada o padrão-ouro, em razão de sua elevada eficácia contra uma ampla variedade de insetos, incluindo o *A. aegypti* (Maia et al., 2022).

Apesar de sua comprovada eficiência, a DEET apresenta limitações relacionadas ao odor desagradável, potencial toxicidade dérmica em altas concentrações e restrições de uso em populações sensíveis, como crianças menores de dois anos e gestantes (Ministério da Saúde, 2026e). No Brasil, a legislação limita sua concentração máxima a 10% para crianças entre 2 e 12 anos, e há relatos de reações adversas, como irritações cutâneas e oculares (Legeay et al., 2018; Ministério da Saúde, 2026e).

Do ponto de vista mecanístico, os repelentes sintéticos atuam principalmente por meio da interferência no sistema olfativo, reduzindo a capacidade do mosquito de detectar substâncias voláteis emitidos pelo hospedeiro humano, como dióxido de carbono, ácido

lático e outros metabólitos liberados pela pele (Dagar & Ramakrishna, 2024). Essa interferência compromete o comportamento de busca e orientação do inseto, diminuindo a probabilidade de pouso e picada (Yan et al., 2021).

Essa interferência ocorre na etapa inicial do processo de recepção química, em que os odorantes são captados pelas sensilas presentes nas antenas e maxilas do inseto (Rihani et al., 2021). Após a entrada na linfa sensilar, essas moléculas interagem com as OBPs, responsáveis por seu transporte até os ORs localizados na membrana dos neurônios olfativos (Figura 5) (Portilla-Pulido et al., 2019). A ativação desses receptores desencadeia a transdução do sinal químico em impulso nervoso, que orienta o comportamento do mosquito em relação ao hospedeiro (Neto et al., 2022).

Diante da relevância desse sistema, os mecanismos olfativos mediados por OBPs têm sido amplamente explorados como alvos para o desenvolvimento de novos repelentes, uma vez que abordagens baseadas em modelagem molecular e estudos de interação ligante–proteína permitem prever a afinidade de moléculas por essas proteínas, auxiliando na identificação de moléculas com maior potencial repelente e tempo de proteção prolongado (Costa et al., 2019).

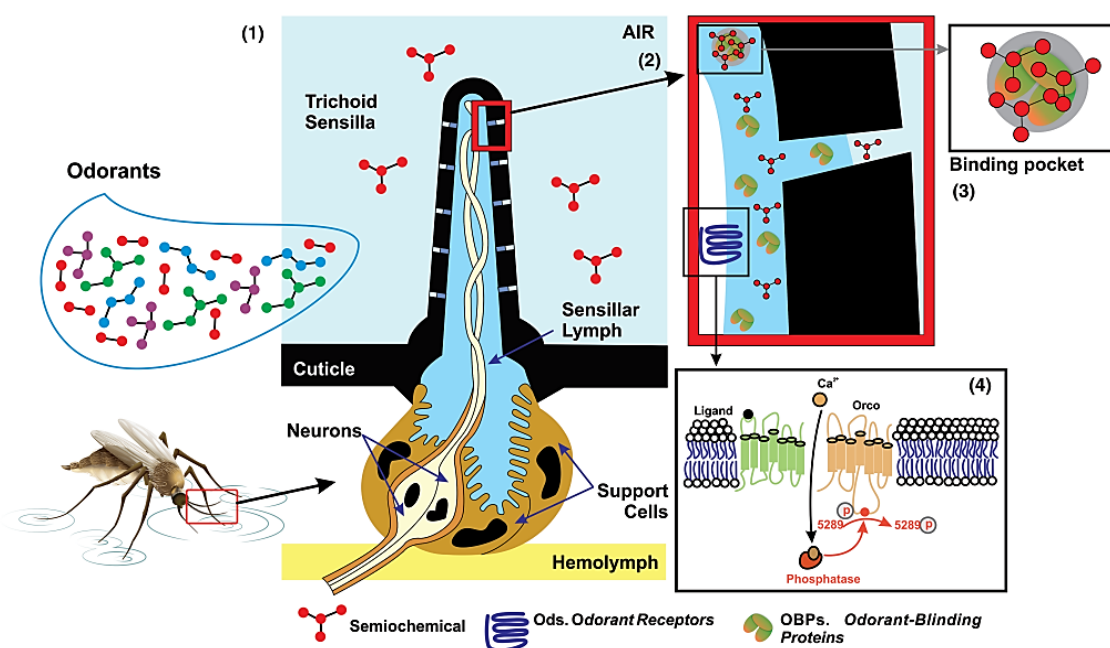


**Figura 5:** Processo de detecção de odorantes em insetos, envolvendo sensilas antenais, proteínas ligadoras de odorantes e receptores de odorantes. Fonte: Portilla-Pulido et al., 2019.

## 2.6 Óleos essenciais no controle de vetores

Os óleos essenciais são misturas de metabólitos secundários, constituídos principalmente por terpenos (monoterpenos e sesquiterpenos) e fenilpropanóides, extraídos de diferentes órgãos vegetais como folhas, frutos, sementes e raízes (Bakkali et al., 2008). Sua composição química pode variar significativamente em função de fatores bióticos e abióticos, incluindo condições ambientais, estágio de desenvolvimento da planta, época de coleta e interações ecológicas (Souza et al., 2025). Essas variações influenciam diretamente suas propriedades físico-químicas e sua eficácia biológica, tornando essencial a caracterização química para a compreensão de seu potencial aplicado (Pathania et al., 2021).

Entre os métodos de obtenção, a destilação por arraste a vapor destaca-se como a técnica amplamente utilizada para a extração de óleos essenciais por permitir a obtenção eficiente de constituintes voláteis utilizando apenas água, sendo amplamente empregada em estudos laboratoriais (Silva et al., 2019a; Bai et al., 2020).

Óleos essenciais têm se destacado como alternativas promissoras aos inseticidas sintéticos, em função de sua atividade larvica e repelente contra mosquitos de importância médica, incluindo o *A. aegypti* (Santos et al., 2022). Além disso, os óleos essenciais apresentam menor impacto ambiental e toxicidade reduzida para animais não-alvo, o que os torna mais seguros para o uso ambiental (Oliveira et al., 2022b).

Diversos estudos evidenciam o potencial larvica de óleos essenciais, como por exemplo, o óleo essencial extraído das folhas de *Croton linearis* Jacq. (Euphorbiaceae), que apresentou toxicidade significativa contra *A. aegypti*, *An. albitalis* Lynch Arribálzaga, 1878 e *Cx. quinquefasciatus* Say 1823 (Culicidae), com valores de CL<sub>50</sub> entre 63,54 e 64,63 mg/L (García-Díaz et al., 2023). De forma semelhante, o óleo essencial de *Kaempferia galanga* L. (Zingiberaceae) demonstrou elevada atividade larvica contra *A. vittatus* (Bigot, 1861) e *An. Maculatus* Theobald, 1901 com CL<sub>50</sub> de 39,22 a 41,36 µg/mL (AlSalhi et al., 2020).

Adicionalmente, diversos óleos essenciais também apresentam atividade repelente, como os extraídos de *Croton tetradenius* Baill. (Euphorbiaceae), *Erigeron canadensis* L. (Asteraceae), *Mentha spicata* L. (Lamiaceae) e *Parthenium hysterophorus* L. (Asteraceae), que apresentaram eficácia repelente contra mosquitos de *A. aegypti* (Azeem et al., 2019, Sousa et al., 2023).

### 2.6.1 Rotas biossintéticas dos constituintes de óleos essenciais

Os principais constituintes dos óleos essenciais, especialmente monoterpenos e sesquiterpenos, são biossintetizados nas plantas a partir de duas rotas metabólicas distintas e complementares: via do ácido mevalônico (MVA) e a via do metileritritol fosfato (MEP) (de Sousa et al., 2023). Essas rotas são responsáveis pela formação das unidades isoprênicas fundamentais, isopentenil pirofosfato (IPP) e dimetilalil pirofosfato (DMAPP), que atuam como precursores universais dos terpenóides que constituem os óleos essenciais (Figura 6) (Shan & Jin, 2025).

A via do MVA ocorre predominantemente no citosol e está associada, principalmente, à biossíntese de sesquiterpenos, enquanto a via do MEP ocorre nos plastídios e é a principal responsável pela formação de monoterpenos (Yang et al., 2018, Chen et al., 2021). A partir da condensação sequencial de IPP e DMAPP, são formados intermediários como o geranyl pirofosfato (GPP) e o farnesil pirofosfato (FPP), que dão origem a uma ampla diversidade estrutural de terpenos por meio da ação de enzimas terpeno sintases (Ninkku et al., 2021).

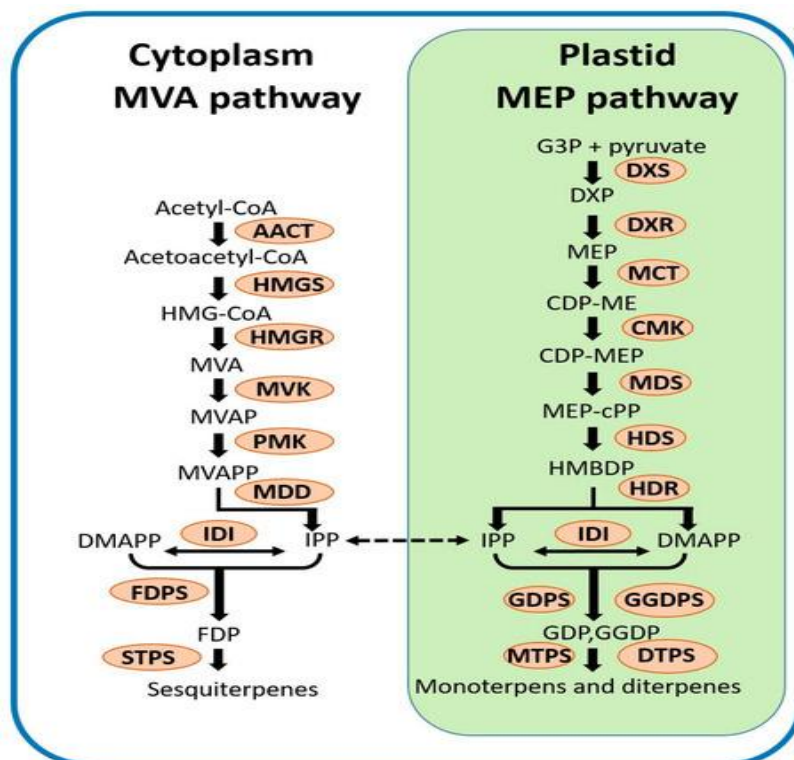


**Figura 6:** Vias MVA e MEP responsáveis pela biossíntese de terpenóides voláteis de plantas. Fonte: Huang et al., 2021.

A compreensão das rotas biossintéticas é essencial para a interpretação da composição química dos óleos essenciais e para a avaliação da atividade biológica de seus constituintes isolados, uma vez que variações estruturais nos terpenos podem influenciar significativamente os efeitos larvicida e repelente, refletindo ainda a flexibilidade metabólica das plantas na produção desses constituintes em resposta a demandas de defesa e sinalização química (Henry et al., 2018, Zeni et al., 2021).

## **2.7 Potencial larvicida e repelente do gênero *Piper***

O gênero *Piper* é um grupo botânico amplamente distribuído nas regiões tropicais e subtropicais, apresentando inflorescências do tipo espiga como uma de suas principais características morfológicas (Villamizar et al., 2017). No Brasil, espécies desse gênero são encontradas em abundância, especialmente em florestas de várzea, áreas sombreadas e bordas de florestas, com maior riqueza registrada na Amazônia e na Mata Atlântica, o que confere ao país papel estratégico na exploração biotecnológica desse grupo vegetal (Guimarães et al., 2015).

Do ponto de vista fitoquímico, óleos essenciais extraídos de espécies desse gênero são ricos em monoterpenos, sesquiterpenos e fenilpropanóides (Guimarães et al., 2015), com atividade promissora contra espécies de mosquitos vetores (Tabela 1).

**Tabela 1.** Atividade inseticida de óleos essenciais de espécies do gênero *Piper*.

| <b>Espécie</b>            | <b>Constituinte majoritário</b> | <b>Classe química</b>           | <b>Atividade biológica</b>                                                                              | <b>Referência</b>                                   |
|---------------------------|---------------------------------|---------------------------------|---------------------------------------------------------------------------------------------------------|-----------------------------------------------------|
| <i>P. purusanum</i>       | $\beta$ -Cariofileno            | Sesquiterpeno<br>hidrocarboneto | Larvicida contra <i>A. aegypti</i> e <i>An. darlingi</i> (CL <sub>50</sub> de 53,41 e 49,84 $\mu$ g/mL) | (Oliveira et al., 2022a)                            |
| <i>P. hispidinervum</i>   | Nerolidol                       | Sesquiterpeno<br>oxigenado      | Larvicida contra <i>A. aegypti</i> (CL <sub>50</sub> de 84,80 $\mu$ g/mL)                               | (Silva et al., 2019b)                               |
| <i>P. tuberculatum</i>    | $\beta$ -Cariofileno            | Sesquiterpeno<br>hidrocarboneto | Larvicida contra <i>A. aegypti</i> (CL <sub>50</sub> de 48,41 $\mu$ g/mL)                               | (Lima et al., 2024a)                                |
| <i>P. alatipetiolatum</i> | Ishwarone                       | Sesquiterpeno<br>oxigenado      | Larvicida contra <i>A. aegypti</i> (CL <sub>50</sub> de 33,74 ppm)                                      | (Oliveira et al., 2020)                             |
| <i>P. capitarianum</i>    | <i>trans</i> -cariofileno       | Sesquiterpeno<br>hidrocarboneto | Larvicida contra <i>A. aegypti</i> (CL <sub>50</sub> de 76,13 $\mu$ g/mL)                               | (França et al., 2020)                               |
| <i>P. baccans</i>         | Eudesma-7(11)-en-4-ol           | Sesquiterpeno<br>oxigenado      | Larvicida contra <i>A. aegypti</i> (CL <sub>50</sub> de 10,68 $\mu$ g/mL)                               | (Souza et al., 2025)                                |
| <i>P. aduncum</i>         | Apiol                           | Fenilpropanóide                 | Repelência contra <i>A. albopictus</i> e <i>A. aegypti</i>                                              | (Mamood et al., 2017;<br>Soto-Cáceres et al., 2022) |
| <i>P. betle</i>           | Eudesma-7(11)-en-4-ol           | Sesquiterpeno<br>oxigenado      | Repelência contra <i>A. aegypti</i>                                                                     | (Vasanth-Srinivasan et al., 2018)                   |

### 2.7.1 *Piper cyrtopodon* (Miq. C.DC)

*P. cyrtopodon* é uma espécie arbustiva nativa da região amazônica, com ocorrência em ambientes de floresta tropical úmida, especialmente no sub-bosque sombreado, onde fatores ambientais podem influenciar a biossíntese de metabólitos secundários (Guimarães et al., 2015). Morfologicamente, apresenta caule verde, inflorescências do tipo espiga e folhas com base arredondada a levemente cordada, além de discreta pilosidade ao longo das nervuras (Figura 7) (Guimarães et al., 2020).

Embora amplamente distribuída na região Norte do Brasil, a espécie ainda é pouco investigada sob os aspectos fitoquímico e biológico, o que limita a compreensão de seu potencial aplicado. Em contraste, outras espécies do gênero *Piper* já apresentam perfil químico bem estabelecido, marcado pela presença de terpenóides e fenilpropanóides com reconhecida atividade biológica.

Diante disso, a caracterização do óleo essencial de *P. cyrtopodon* mostra-se relevante, especialmente considerando o papel desses metabólitos na toxicidade e na modulação comportamental de insetos vetores. Essa abordagem contribui para a prospecção de substâncias bioativas com potencial aplicação no desenvolvimento de estratégias alternativas de controle.



**Figura 7:** Espécie vegetal *Piper cyrtopodon*. Fonte: O autor, 2025.

### 3. OBJETIVOS

#### 3.1 Geral

Avaliar a atividade larvícida e repelente do óleo essencial das folhas de *P. cyrtopodon* e de seus constituintes majoritários contra *A. aegypti*, bem como investigar seus mecanismos de ação e perfil de segurança por abordagens *in vitro* e *in silico*.

#### 3.2 Específicos

- Caracterizar a composição química do óleo essencial de *P. cyrtopodon*;
- Determinar a atividade larvícida e o efeito residual do óleo essencial e de seus constituintes majoritários sobre larvas de *A. aegypti*;
- Avaliar a atividade repelente e o tempo de proteção do óleo essencial e de seus constituintes majoritários frente a fêmeas adultas de *A. aegypti*;
- Elucidar os mecanismos de ação relacionados ao efeito larvícida, com ênfase na indução de estresse oxidativo e nas alterações das enzimas antioxidantes;
- Investigar o mecanismo de ação associado ao efeito repelente, com foco na interação dos constituintes com proteínas ligadoras de odorantes (OBPs), por meio de *docking* molecular;
- Avaliar o perfil de segurança por meio de ensaios de citotoxicidade *in vitro* em células Vero e PBMCs.
- Analisar as propriedades farmacocinéticas e toxicológicas (ADMET) dos constituintes majoritários por ferramentas *in silico*.

## 4. MATERIAL E MÉTODOS

### 4.1 Químicos e reagentes

Todos os reagentes foram adquiridos dos fornecedores Merck e Sigma-Aldrich, garantindo a qualidade e a confiabilidade dos materiais utilizados durante o estudo. Foram utilizados, sulfato de sódio anidro ( $\text{Na}_2\text{SO}_4$ ), acetato de etila P.A. (EtOAc), acetona P.A., etanol P.A., dimetilsulfóxido (DMSO, 99%), dimetilformamida (DMF), ácido fórmico (50%), ácido clorídrico (HCl, 2 M), peróxido de hidrogênio ( $\text{H}_2\text{O}_2$ , 300 mM) e iodeto de potássio (KI, 10 M). Para análise de composição química do óleo essencial foram utilizados *n*-alcanos ( $\text{C}_8$ – $\text{C}_{30}$ ) e  $\alpha$ -Cipermetrina (PESTANAL®, padrão analítico).

Nos ensaios bioquímicos foram empregados ainda albumina sérica bovina (BSA), soro fetal bovino (SFB/FBS), penicilina–estreptomicina, fungizona (anfotericina B), nicotinamida adenina dinucleotídeo fosfato reduzido (NADPH), glutathione reduzida (GSH), glutathione redutase (GR), N,N,N',N'-tetrametiletilenodiamina (TEMED), além de malondialdeído (MDA), 2,4-dinitrofenilhidrazina (DNPH), ácido tricloroacético (TCA), ácido tiobarbitúrico (TBA) e guanidina (6 M).

Para os ensaios celulares, foram utilizados os meios de cultura Dulbecco's Modified Eagle Medium (DMEM) e Roswell Park Memorial Institute (RPMI), além de tampões como fosfato de potássio (0,1 M, pH 7,3), HEPES (20 mM, pH 7,0) e fosfato de sódio com EDTA (0,025 M/0,1 mM, pH 10), bem como solução salina tamponada com fosfato (PBS). O ensaio de viabilidade celular foi realizado utilizando o reagente MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio brometo) e Ficoll-Hypaque (GE Healthcare). A linhagem celular Vero CCL-81, derivada do rim do macaco-verde-africano (*Chlorocebus sabaeus*, Cercopithecidae), foi adquirida da American Type Culture Collection (ATCC, Manassas, VA, EUA).

O repelente comercial N,N-dietil-meta-toluamida (DEET, 7,125%, SC Johnson) foi adquirido em farmácia local. Os padrões químicos germacreno D,  $\delta$ -Elemeno e cadina-1(10),11-dieno foram obtidos junto ao Laboratório de Princípios Ativos da Amazônia (LAPAAM/INPA).

### 4.2 Licenças e registros

A coleta da espécie vegetal foi autorizada pelo Sistema de Informação e Autorização da Biodiversidade (SISBIO, nº 96329-1). O repasto sanguíneo das fêmeas de *A. aegypti* realizado em hamsters da espécie *Mesocricetus auratus* (Cricetinae) foi previamente aprovado pela Comissão de Ética de Uso de Experimentação Animal

(CEUA/INPA, nº 11/2022). Além disso, o estudo está devidamente registrado no Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado (SisGen, nº A357D96).

#### **4.3 Coleta e identificação da espécie vegetal**

A espécie *P. cyrtopodon* foi coletada na Base de Manejo Florestal do Instituto Nacional de Pesquisas da Amazônia (ZF2/INPA), localizada na cidade de Manaus, Amazonas, Brasil (2°38'18.5"S, 60°09'29.7"W). O espécime vegetal foi coletado manualmente em seu habitat natural, utilizando tesouras de poda para obtenção de galhos com folhas, flores e/ou frutos. Após a coleta, as amostras foram transportadas para o Laboratório de Inovação e Desenvolvimento em Tecnologia Farmacêutica (LIDETEF) da Universidade Federal do Amazonas (UFAM). A identificação taxonômica foi realizada pelo botânico Gabriel Mendes Marcusso, do Instituto de Pesquisas do Jardim Botânico do Rio de Janeiro, Brasil. Uma exsicata da espécie foi confeccionada e depositada no Herbário da universidade, sob o registro HUAM nº 12630.

#### **4.4 Processamento do material vegetal**

O material vegetal foi inicialmente tratado para remover resíduos provenientes da coleta, com as folhas sendo separadas dos ramos e galhos. Posteriormente, amostras foram retiradas para a análise do teor de umidade, e o material com umidade superior a 14% foi submetido à secagem em estufa de ar circulante (modelo SSDcr, SolidSteel) a 40 °C por 7 dias. Após a secagem, o material foi cominuído em moinho de facas tipo Willey (modelo SL-31, Solab), resultando em um pó vegetal que foi devidamente armazenado para análises subsequentes (ANVISA, 2019).

#### **4.5 Extração do óleo essencial**

O óleo essencial foi extraído por hidrodestilação, utilizando um aparelho de Clevenger modificado (modelo TE-2762, Tecnal), interligado a um sistema de refrigeração com água mantida a 10 °C. Para o processo, 50 g das folhas pulverizadas de *P. cyrtopodon* foram submersas em 4 L de água destilada e submetidas à destilação por 3 horas (Oliveira et al., 2020). Posteriormente, o óleo essencial obtido foi coletado, centrifugado e desidratado utilizando sulfato de sódio anidro (Na<sub>2</sub>SO<sub>4</sub>). Em seguida, foi acondicionado em frasco de vidro âmbar e armazenado a 4 °C até a realização das análises cromatográficas, biológicas, bioquímicas e enzimáticas (Huong et al., 2023). O

rendimento (%) foi calculado pela equação: (%) = (volume do óleo essencial obtido/massa seca das folhas) x 100.

#### 4.6 Identificação dos constituintes voláteis

As análises qualitativas e quantitativas do óleo essencial foram realizadas através de Cromatografia Gasosa Acoplada à Espectrometria de Massas (GC-MS) e Cromatografia Gasosa Acoplada ao Detector de Ionização de Chama (GC-FID), seguindo a metodologia descrita por Oliveira et al., (2020). Antes das análises, 1 mg do óleo essencial foi diluído em 1 mL de acetato de etila, e 1 µL da solução foi injetado nos sistemas cromatográficos.

Para a análise por GC-MS, foi utilizado um sistema Trace GC Ultra acoplado a um espectrômetro de massas ISQ (Thermo Scientific) equipado com uma coluna capilar TR-5 (30 m × 0,25 mm × 0,25 µm). A temperatura do forno foi programada de 60 a 240 °C, aumentando 3 °C/min, usando hélio como gás de arraste, e com uma temperatura de injeção de 240 °C e uma temperatura de interface de 250 °C. O espectrômetro de massa foi ajustado com temperatura da fonte de íons, 220 °C; faixa de massa, m/z 40–600; e velocidade de varredura, 2 s.

Já a análise por GC-FID foi realizada em um sistema G-1860 Plus (Agilent), equipado com uma coluna capilar HP-5 (30 m × 0,32 mm × 0,25 µm). A temperatura do forno foi programada de 60 a 240 °C, aumentando 3 °C/min, usando hidrogênio como gás carreador a 4 mL/min. A temperatura de injeção foi ajustada para 250 °C com razão de divisão de 1:2. Os detectores de ionização de chama foram ajustados para 280 °C com taxas de fluxo de hidrogênio, ar sintético e nitrogênio a 30, 300 e 30 mL/min, respectivamente.

Os índices de retenção (IR) foram determinados utilizando uma série homóloga de *n*-alcanos (C<sub>8</sub>–C<sub>30</sub>) e aplicando a equação de Vandendool & Kratz (1963). A identificação de cada constituinte foi realizada comparando os valores de IR obtidos com os valores relatados em bancos de dados da literatura, como o *National Institute of Standards and Technology* e a biblioteca interna.

#### 4.7 Criação da colônia de *Aedes aegypti* em laboratório

A colônia de *A. aegypti* foi mantida no Laboratório de Malária e Dengue do INPA, sob condições controladas de temperatura (26 ± 3°C), umidade (70 ± 10%) e fotoperíodo (12 h de luz e 12 h de escuro), conforme metodologia descrita por Silva et al.,

(2014). Ovos de *A. aegypti*, depositados em tiras de papel filtro, foram imersos em bacias plásticas contendo água destilada para a eclosão das larvas. Após a eclosão, as larvas foram separadas conforme os estágios larvais (L1, L2, L3 e L4) e alimentadas com uma mistura na proporção de 1:1 (m/m) de ração para gatos (Whiskas®) e roedores (Zootekna®) até alcançarem o estágio L4. As pupas foram transferidas para copos plásticos contendo 50 mL de água destilada e, em seguida, colocadas em gaiolas entomológicas (30 × 30 × 30 cm) para a emergência dos mosquitos adultos. Os adultos foram alimentados com solução aquosa de sacarose a 10% (m/v), enquanto as fêmeas receberam repasto sanguíneo em hamsters anestesiados da espécie *Mesocricetus auratus* (Cricetidae), para assegurar a manutenção e reprodução da colônia.

#### 4.8 Atividade larvicida

O ensaio larvicida do óleo essencial de *P. cyrtopodon* e seus principais constituintes germacreno D,  $\delta$ -Elemeno e cadina-1(10),11-dieno foi realizado seguindo as diretrizes para Testes de Laboratório e Campo de Larvicidas de Mosquitos da Organização Mundial da Saúde (WHO, 2005).

Para cada tratamento, grupos de 20 larvas de terceiro estágio ( $n = 500$ ) foram distribuídos em 25 recipientes de 250 mL, cada um contendo 100 mL de água destilada e concentrações variando de 20 a 100  $\mu\text{g/mL}$  do óleo essencial, germacreno D,  $\delta$ -Elemeno e cadina-1(10),11-dieno, preparados em 1 mL de DMSO. Como controle negativo, DMSO foi utilizado nas mesmas concentrações, enquanto o inseticida sintético  $\alpha$ -Cipermetrina, foi avaliado pela  $\text{CL}_{50}$  de 0,14  $\mu\text{g/mL}$  identificada em estudo anterior (Oliveira et al., 2022b), como controle positivo.

Todos os ensaios foram avaliados em quintuplicata e repetidos três vezes, sob condições controladas de temperatura ( $27 \pm 3^\circ\text{C}$ ) e umidade relativa (70–80%). Após 24 e 48 h de exposição, a porcentagem de atividade larvicida em cada concentração foi calculada utilizando a equação: Atividade larvicida (%) = (número de larvas mortas/número total de larvas)  $\times 100$ . A potência relativa (RP) foi identificada usando a seguinte equação:  $\text{RP} = \text{CL}_{50} \text{ do larvicida padrão} / \text{CL}_{50} \text{ do produto natural}$  (Cheng et al., 2009).

#### 4.9 Efeito residual

O efeito residual foi avaliado sob as mesmas condições do ensaio larvicida previamente descrito. Após 48 h do teste inicial, as larvas mortas na maior concentração

(100 µg/mL) do óleo essencial e dos constituintes majoritários foram removidas e substituídas por um novo grupo de larvas nos mesmos recipientes tratados, sem renovação do meio ou reposição dos produtos. Esse procedimento foi repetido diariamente, com a substituição das larvas mortas a cada 24 h, durante um período de 5 dias. O efeito residual foi considerado significativo quando a mortalidade larval excedeu 90% (WHO, 2005).

#### **4.10 Preparação do homogenato larval**

O homogenato foi preparado usando larvas que morreram após 48 horas de exposição à maior concentração (100 µg/mL) do óleo essencial, germacreno D, cadinal(10),11-dieno e  $\delta$ -Elemeno durante o ensaio larvicida. Larvas tratadas com  $\alpha$ -Cipermetrina (0,14 µg/mL) e DMSO (100 µg/mL) foram usadas como controles positivo e negativo, respectivamente.

As larvas mortas foram coletadas e transferidas para tubos falcon contendo 2 mL de tampão fosfato de potássio 0,1 M (pH 7,3) e mantidas refrigeradas a 4 °C para minimizar a degradação enzimática durante a preparação da amostra. Posteriormente, as amostras foram homogeneizadas em um misturador vórtex (modelo G-560, Scientific Industries) por 5 min e centrifugadas a 4000 rpm por 5 min. Aliquotas do homogenato foram coletados para as análises de peroxidação lipídica e oxidação de proteínas.

O homogenato remanescente foi então submetido a uma nova centrifugação nas mesmas condições (4000 rpm por 5 min) para a obtenção dos sobrenadantes, os quais foram cuidadosamente transferidos para microtubos de 3 mL e armazenados a 4 °C, para à determinação de marcadores enzimáticos associados à exposição aos produtos testados.

Os níveis de absorbância específicos para cada enzima foram medidos utilizando um leitor de microplacas equipado com o software Gen5 Data Analysis (modelo Epoch, BioTek Instruments), em comprimentos de onda otimizados para cada ensaio. Os valores médios de absorbância foram calculados a partir de três réplicas, garantindo a precisão e reprodutibilidade dos dados (Costa et al., 2024).

#### **4.11 Testes bioquímicos e enzimáticos**

##### **4.11.1 Determinação da concentração de proteína**

O conteúdo de proteína nos sobrenadantes larvais foi quantificado usando albumina sérica bovina (BSA, 1 mg/mL) como padrão, seguindo a metodologia descrita por Lowry et al., (1951).

#### **4.11.2 Quantificação do conteúdo de espécies reativas de oxigênio (ROS)**

A quantificação de ROS foi realizada utilizando peróxido de hidrogênio ( $H_2O_2$ ) como indicador, seguindo o protocolo estabelecido por Velikova et al., (2000) e adaptado para homogenato larval por Silva et al., (2025). Para o ensaio, 45  $\mu$ L de cada sobrenadante larval foi misturado com 45  $\mu$ L de solução de iodeto de potássio 10M. A absorbância foi medida a 390 nm. As concentrações de  $H_2O_2$  foram determinadas com base em uma curva padrão preparada com uma solução estoque de 250 mM de  $H_2O_2$ , diluída para uma faixa de 0 a 45  $\mu$ mol. Os resultados foram expressos como  $\mu$ mol de  $H_2O_2$  por grama<sup>-1</sup> em cada amostra. Todas as medições foram conduzidas em triplicatas para garantir a precisão e a reprodutibilidade dos dados.

#### **4.11.3 Danos por peroxidação lipídica**

Os danos oxidativos aos componentes celulares, particularmente aqueles induzidos por  $H_2O_2$ , foram avaliados pela quantificação dos níveis de malondialdeído (MDA), um importante marcador da peroxidação lipídica. A quantificação foi realizada usando substâncias reativas ao ácido tiobarbitúrico (TBARS), seguindo o método de Buege & Aust (1978), com modificações para homogenato larval conforme descrito por Silva et al., (2025)

Para o ensaio, 125  $\mu$ L de cada homogenato larval foram misturados com 250  $\mu$ L de uma solução contendo 10% de ácido tricloroacético (TCA) e 0,5% (m/v) de ácido tiobarbitúrico (TBA). As absorbâncias foram registradas a 535 nm para determinar o conteúdo de MDA, com correções de turbidez aplicadas subtraindo-se as absorbâncias a 600 nm. Os resultados foram expressos como nmol de MDA por g<sup>-1</sup> em cada amostra. Todos os ensaios foram realizados em triplicatas para garantir a confiabilidade e precisão.

#### **4.11.4 Danos por oxidação de proteínas**

A quantificação dos danos oxidativos às proteínas foi realizada de acordo com a metodologia de Levine et al., (1994). Este método se baseia na reação entre grupos carbonila, formados em proteínas danificadas pelo estresse oxidativo causado por radicais hidroxila ( $OH^\bullet$ ) derivados de  $H_2O_2$ , e 2,4-dinitrofenilhidrazina (DNPH), resultando na formação de 2,4-dinitrofenilhidrazona, que pode ser detectada e quantificada.

Para o ensaio, 100  $\mu$ L do homogenato larval foram misturados com 150  $\mu$ L de DNPH 10 mM preparado em ácido clorídrico 2 M e incubados por 1 h. Posteriormente, 250  $\mu$ L de ácido tricloroacético a 6% foram adicionados, e a mistura foi centrifugada a

1000 rpm por 5 min. O *pellet* resultante foi lavado três vezes com uma solução de etanol e acetato de etila, seguido pela adição de 1 mL de guanidina 6 M dissolvida em ácido fórmico 50%. As leituras de absorbância foram realizadas a 366 nm e os resultados foram expressos como nM de carbonilas reativas por mg de proteína em cada amostra. Todos os procedimentos foram realizados em triplicata para garantir a reprodutibilidade dos resultados.

#### **4.11.5 Atividade da superóxido dismutase (SOD)**

A atividade da SOD foi avaliada usando o método originalmente descrito por Afanas'ev et al., (1989), com modificações para sobrenadante larval, conforme descrito por Janner et al., (2021). Inicialmente, os sobrenadantes foram centrifugados a 15.000 rpm por 10 min a 4 °C após a adição de 250 µL de tampão HEPES 20 mM (pH 7,0) para obter sobrenadantes clarificados. A mistura de reação consistiu em tampão fosfato de sódio (0,025 M/0,1 mM EDTA, pH 10), TEMED e 10 µL dos sobrenadantes clarificados. A reação foi iniciada pela adição de 0,15% de quercetina dissolvida em dimetilformamida, e a autooxidação da quercetina foi monitorada por 2 min a 406 nm.

A atividade da SOD foi determinada com base na inibição de autooxidação da quercetina, conforme descrito por Franco et al., (2009). Os resultados foram expressos como a quantidade de proteína necessária para atingir 50% de inibição da oxidação da quercetina. Todos os procedimentos foram conduzidos em quatro experimentos independentes, cada um usando 10 larvas por grupo. A atividade enzimática foi expressa como mU/mg de proteína em cada amostra.

#### **4.11.6 Atividade da catalase (CAT)**

A atividade da enzima catalase foi realizada seguindo o método de Aebi (1984), com adaptações de Abolaji et al., (2015). O experimento foi realizado em triplicata para garantir a precisão dos resultados. A mistura de reação incluiu 1,8 mL de tampão de fosfato de potássio (pH 7,0), 180 µL de 300 mM de H<sub>2</sub>O<sub>2</sub> e 20 µL de sobrenadantes larvais diluídos (1:50, v/v). A redução de H<sub>2</sub>O<sub>2</sub> foi monitorada a 240 nm por 2 min com leituras feitas em intervalos de 10 segundos a 25 °C e expressas como mmol de H<sub>2</sub>O<sub>2</sub> consumido/min/mg de proteína em cada amostra.

#### 4.11.7 Atividade da glutathiona peroxidase (GPx)

A atividade da GPx foi medida monitorando a diminuição da absorbância de NADPH a 340 nm, seguindo a metodologia originalmente descrita por Paglia & Valentine (1967) e recentemente adaptada por Olufemi-Salami et al., (2024) para sobrenadantes larvais. Para o ensaio, 100 µL de glutathiona (GSH) foram combinados com uma mistura de solução enzimática e tampão em tubos de ensaio contendo nicotinamida adenina dinucleotídeo fosfato (NADPH) e glutathiona redutase (GR). A reação foi iniciada adicionando 100 µL de H<sub>2</sub>O<sub>2</sub> à mistura contendo sobrenadantes larvais. O progresso da reação foi monitorado a 340 nm por 3 min em um leitor de microplacas (modelo Epoch, Biotek Instruments) equipado com o software Gen5 Data Analysis. A atividade da GPx foi expressa como mmol de NADPH/min/mL em cada amostra, e todos os procedimentos foram realizados em triplicatas.

#### 4.12 Atividade repelente

A atividade repelente foi realizada seguindo as Diretrizes da Organização Mundial de Saúde para a Avaliação da Eficácia de Repelentes Espaciais (WHO, 2013), utilizando um olfatômetro de dupla escolha adaptado (Sousa et al., 2023). O aparato consistiu em um cilindro central para liberação dos mosquitos, conectado a dois cilindros laterais: um contendo o tratamento e o outro servindo como controle negativo.

Papéis de filtro (4 cm<sup>2</sup>) foram tratados com 1, 2, 4, 6, 8 e 10 mg/cm<sup>2</sup> do óleo essencial de *P. cyrtopodon* e de seus constituintes majoritários germacreno D, cadina-1(10),4-dieno e  $\delta$ -Elemeno, solubilizados em 1 mL de acetona. Após 10 min de evaporação do solvente à temperatura ambiente, os papéis tratados foram colocados na extremidade do cilindro de tratamento.

Para cada concentração, foram realizadas 10 repetições utilizando 20 fêmeas de *A. aegypti* não alimentadas (privação de 24 h;  $n = 200$  por tratamento). Após 1 min de aclimação no cilindro central, as tampas laterais foram abertas simultaneamente, permitindo que os mosquitos se movessem livremente entre todos os cilindros por 10 min. Ao final do ensaio, os mosquitos foram contabilizados em cada cilindro.

DEET e acetona foram utilizados como controles positivo e negativo, respectivamente. Todos os ensaios foram conduzidos sob condições ambientais controladas de temperatura ( $27 \pm 3$  °C) e umidade relativa (70–80%).

A porcentagem de repelência foi calculada utilizando a equação: Porcentagem de repelência (%) =  $(Mc - Mt) / (Mc + Mt) \times 100$ , onde Mc é o número de mosquitos no

cilindro não tratado (controle negativo) e  $M_t$  é o número de mosquitos no cilindro de tratamento (Liang et al., 2017).

#### 4.13 Tempo de proteção repelente

O tempo de proteção repelente foi avaliado utilizando a concentração efetiva ( $CE_{90}$ ) do óleo essencial e de seus constituintes majoritários, previamente testados no ensaio de repelência. O delineamento experimental foi o mesmo, utilizando 20 fêmeas de *A. aegypti* por repetição e três repetições por tratamento ( $n = 60$ ).

A  $CE_{90}$  foi aplicada em papéis de filtro e posicionada no cilindro de tratamento do olfatômetro. Após 1 min de aclimatação no cilindro central, as tampas foram abertas para uma exposição de 3 min, repetida a cada 30 min até 270 min. O tempo de proteção foi definido como o intervalo entre a aplicação e o primeiro pouso de mosquito no cilindro de tratamento. O DEET e a acetona foram incluídos como controles positivo e negativo, respectivamente.

#### 4.14 Estudos de *docking* molecular

##### 4.14.1 Preparação e validação das estruturas proteicas

As estruturas da OBP1 de *An. gambiae* (PDB ID: 3N7H) e da OBP22 de *A. aegypti* (PDB ID: 6OGH) foram obtidas no RCSB Protein Data Bank (<https://www.rcsb.org/>) com resoluções de 1,60 Å e 1,85 Å, respectivamente. As estruturas foram preparadas por meio da remoção de moléculas de água, íons e ligantes co-cristalizados, seguida da adição de hidrogênios polares e cargas de Kollman utilizando o AutoDockTools v1.5.6 (The Scripps Research Institute, La Jolla, CA, EUA) (Morris et al., 2009).

A validação do protocolo de *docking* foi realizada por meio do redocking dos ligantes nativos (DEET para OBP1 e ácido linoleico para OBP22). Para cada ligante, foram geradas 20 poses de *docking* utilizando parâmetro de exaustividade de 20 e energy range de 20, com as dimensões e coordenadas do grid box selecionadas para cobrir toda a proteína. Valores de *Root Mean Square Deviation* (RMSD)  $\leq 2,0$  Å foram considerados indicativos de reprodução confiável da pose experimental.

##### 4.14.2 Seleção das estruturas tridimensionais dos ligantes

Os constituintes majoritários do óleo essencial de *P. cyrtopodon*, germacreno D (CID 5317570), cadina-1(10),4-dieno (CID 10223) e  $\delta$ -Elemeno (CID 89316), juntamente com o repelente sintético DEET (CID 4284), foram obtidos no banco de dados

*PubChem* (<https://pubchem.ncbi.nlm.nih.gov>) em formato SDF. As estruturas dos ligantes foram submetidas à minimização de energia e convertidas para o formato PDBQT utilizando o *AutoDockTools* v1.5.6 (Affonso et al., 2013).

#### 4.14.3 Simulações de *docking*

Os estudos de *docking* molecular foram realizados utilizando o *AutoDock Vina* (v1.2.0), com a grid box ajustada para cobrir completamente o sítio ativo. Para cada ligante, foram geradas 20 poses de *docking*, com exaustividade igual a 20 e *energy range* de 20. A melhor pose foi selecionada com base na menor energia livre de ligação ( $\Delta G$ , kcal/mol) e analisada no *BIOVIA Discovery Studio Visualizer* (Dassault Systèmes, Waltham, MA, EUA) para identificação dos resíduos interagentes e dos modos de ligação.

As constantes de inibição ( $K_i$ ,  $\mu M$ ) foram calculadas a partir da energia livre de ligação ( $\Delta G$ , kcal/mol) utilizando a equação:  $K_i = e^{(\Delta G \times 1000) / (R \times T)}$ , onde  $R$  é a constante universal dos gases ( $1,987 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ) e  $T$  é a temperatura absoluta (298,15 K).

#### 4.15 Previsão das propriedades de ADMET

Germacreno D, cadina-1(10),4-dieno,  $\delta$ -Elemeno e DEET selecionados para estudos de acoplamento molecular, também foram avaliados quanto aos seus perfis de absorção, distribuição, metabolismo, excreção e toxicidade (ADMET) utilizando o servidor *ADMETlab* 3.0 (<https://admetlab3.scbdd.com/>). Seus SMILES canônicos foram recuperados do banco de dados *PubChem* e analisados no modo padrão da plataforma para estimar parâmetros físico-químicos e toxicológicos, visando correlacionar os perfis previstos com a atividade comportamental do mosquito (Okoli et al., 2021).

#### 4.16 Ensaio de citotoxicidade

Ensaio de viabilidade celular foram realizados para avaliar os níveis de toxicidade do óleo essencial e seus principais constituintes, utilizando o ensaio colorimétrico MTT, seguindo a metodologia de Mosmann (1983) com modificações descritas por Niksic et al. (2021). Células mononucleares do sangue periférico (PBMCs) foram obtidas de doadores saudáveis após consentimento informado por escrito, isoladas por centrifugação em gradiente de densidade utilizando Ficoll-Hypaque, lavadas três vezes com RPMI e mantidas em cultura sob condições padrão.

Para os ensaios, PBMCs ( $5 \times 10^5$  células/poço) e linhagens de células Vero ( $1 \times 10^5$  células/poço) foram semeadas em placas de 96 poços contendo 200  $\mu L$  de RPMI ou

DMEM, respectivamente, suplementados com 10% de soro fetal bovino (SFB), penicilina-estreptomicina e fungizona, e incubadas a 37 °C com 5% de CO<sub>2</sub> por 24 h para permitir a adesão celular e a formação de monocamadas subconfluentes.

As células foram então expostas ao óleo essencial e seus constituintes em concentrações de 3, 6, 12, 25, 50 e 100 µg/mL e incubadas por 24, 48 e 72 h. Células não tratadas serviram como controle negativo e DMSO a 100% como controle positivo. Ao final de cada ponto de tempo, o meio foi substituído por 10 µL de solução de MTT (5 mg/mL em PBS) diluída em 100 µL de DMEM sem vermelho de fenol e incubada por 4 h a 37 °C. O sobrenadante foi removido, 50 µL de tampão de lise foram adicionados e as placas foram agitadas suavemente para dissolver os cristais de formazan, seguido por uma incubação adicional de 10 min a 37 °C.

A absorbância foi medida a 492 nm usando um leitor de microplacas e a viabilidade celular foi expressa como a porcentagem de absorbância nas amostras tratadas em relação aos controles não tratados, de acordo com a equação:  $(A_{492} \text{ tratado} / A_{492} \text{ não tratado}) \times 100$  (Ramos et al., 2025).

#### **4.17 Análise estatística**

Os valores de CL<sub>50</sub> e CL<sub>90</sub>, bem como de CE<sub>50</sub> e CE<sub>90</sub>, foram estimados por análise *Probit* por meio de regressão linear (Finney, 1971), utilizando o *software* IBM SPSS *Statistics*. Os parâmetros de inclinação ( $\pm$  erro padrão) e o teste do qui-quadrado também foram obtidos a partir desse modelo.

Para a avaliação de diferenças significativas, os valores de CL<sub>50</sub>, CL<sub>90</sub>, CE<sub>50</sub> e CE<sub>90</sub>, assim como os dados bioquímicos, de viabilidade celular e de tempo de proteção repelente, foram submetidos à análise de variância (ANOVA), seguida do teste *post hoc* de Tukey para comparações múltiplas ( $p < 0,05$ ), utilizando o *GraphPad Prism* versão 9. Todas as análises estatísticas foram conduzidas com intervalo de confiança de 95%. (Oliveira et al., 2024).

# CAPÍTULO 1

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Atividade larvívica, estresse oxidativo e alterações enzimáticas induzidas pelo óleo essencial de *Piper cyrtopodon* (Miq.) C.DC (Piperaceae) em *Aedes aegypti* (Culicidae)

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(ANEXO 1)

Larvicidal activity, oxidative stress, and enzymatic alterations induced by essential oil from *Piper cyrtopodon* (Miq.) C.DC (Piperaceae) on *Aedes aegypti* (Culicidae)

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## Highlights

- The essential oil from *P. cyrtopodon* exhibited high larvicidal activity;
- Cadina-1(10),4-diene was the most active compound against *Ae. aegypti*;
- The toxic effect induces oxidative stress and cellular damage.

## Abstract

Dengue, Zika, and chikungunya are mosquito-borne diseases transmitted by *Aedes aegypti*, with over 13 million dengue cases, 431,223 chikungunya cases, and 44,242 Zika cases reported in the Americas in 2024. The resistance of *Ae. aegypti* to synthetic insecticides has driven the search for botanical alternatives, such as essential oils (EOs) from *Piper* species. This study evaluated the EO from *Piper cyrtopodon* and its main compounds as larvicides, investigating their mechanism of action. The EO, extracted by hydrodistillation (yield:  $1.4 \pm 0.2\%$ ), contained germacrene D (57.46%), cadina-1(10),4-diene (10.57%), and  $\delta$ -Elemene (8.0%). It showed significant larvicidal activity against *Ae. aegypti*, with  $LC_{50}$  values ranging from 20.78 to 146.61  $\mu\text{g/mL}$  over 24 and 48 h. Cadina-1(10),4-diene was the most active compound ( $p < 0.0001$ ). Biochemical analyses revealed oxidative stress in larvae, with increased reactive oxygen species production ( $11 \pm 2$  to  $26 \pm 2$   $\mu\text{mol H}_2\text{O}_2/\text{g}$ ), lipid peroxidation ( $0.54 \pm 0.06$  to  $2.8 \pm 0.22$   $\eta\text{mol MDA/g}$ ), and protein oxidation ( $1.62 \pm 0.04$  to  $4.54 \pm 0.51$   $\text{nmol/mg protein}$ ) compared to controls ( $p < 0.0001$ ). Antioxidant enzymes superoxide dismutase ( $10.33 \pm 3$  to  $28.33 \pm 6$   $\text{mU/mg protein}$ ), catalase ( $1.04 \pm 0.09$  to  $7.08 \pm 2.42$   $\text{mmol H}_2\text{O}_2/\text{min/mg protein}$ ), and glutathione peroxidase ( $5.40 \pm 1.37$  to  $14.67 \pm 1.52$   $\text{mmol NADPH/min/mL}$ ) were altered, suggesting redox imbalance as a toxicity mechanism. These findings highlight the potential of the EO from *P. cyrtopodon* as a natural alternative for *Ae. aegypti* control.

**Keywords:** *Piper*, sesquiterpenes, oxidative stress, detoxification enzymes.

## Introduction

In recent years, dengue, zika, and chikungunya have emerged as major global public health threats (Lwande et al., 2020). These arboviral diseases, caused by viruses from the genera *Flavivirus* (Flaviviridae) and *Alphavirus* (Togaviridae), are transmitted by *Aedes aegypti* (Culicidae), a vector highly adapted to urban environments (Ahebwa et al., 2023).

In 2024, countries in the Americas experienced an unprecedented epidemic, with over 13 million reported cases of dengue, resulting in 8,348 deaths (PAHO, 2024a). Additionally, 431,223 cases of chikungunya were reported, with 243 deaths, along with 44,242 cases of Zika, with 1 fatality (PAHO, 2024b; 2024c). Brazil was the epicenter of this health crisis, leading the notifications with 6.6 million dengue cases and 6,264 deaths (Ministry of Health, 2024). Chikungunya also had a significant impact, with 226,732

reported cases and 243 deaths, while Zika accounted for 5.337 reported cases with 1 fatality (Ministry of Health, 2024).

The rapid spread of these diseases is directly linked to the widespread distribution of *Ae. aegypti*, which proliferates in urban areas, especially in artificial containers such as tires, flower pots, and water tanks (de Oliveira et al., 2022a). Factors such as unplanned urban growth and climate change, including rising temperatures, high humidity, and irregular rainfall, further promote the mosquito's expansion and complicate control efforts (Carvalho & Moreira, 2017).

Chemical control, primarily through the use of insecticides such as pyrethroids, has been the main strategy adopted by health authorities (Reis et al., 2017; Braga et al., 2020). However, the intensive and continuous use of these compounds has accelerated the development of resistance in mosquito populations, compromising the effectiveness of interventions (Zulfa et al., 2020; Jangir & Prasad, 2022). Oxidative stress is one of the mechanisms involved in insecticide toxicity, occurring when there is an imbalance between the production of reactive oxygen species (ROS) and the organism's antioxidant capacity (Johnson et al., 2021a; Dong et al., 2022; Konkon et al., 2023).

Under normal conditions, enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST) maintain this balance (García-Caparrós et al., 2021; Silva et al., 2025). However, prolonged exposure to chemical compounds can impair these defenses, leading to ROS accumulation and structural damage to proteins, lipids, and DNA, which may affect insect physiology and reduce the efficacy of chemical control (Cutler et al., 2022a; Ranjith et al., 2023).

Given the increasing resistance to synthetic insecticides, essential oils (EOs) have emerged as a promising alternative, particularly for larval control of *Ae. aegypti* (da Costa et al., 2024). Composed of mixtures of secondary metabolites rich in mono- and sesquiterpenes, EOs exhibit insecticidal, repellent, and physiological-disrupting activities against insects (Budiman et al., 2021; Giunti et al., 2023).

Their mechanisms of action include disruption of cell membrane permeability, cell lysis, inhibition of oxidative enzymes, induction of oxidative stress, and interference with mitochondrial electron transport and respiration (Moldogazieva et al., 2020; Ercan et al., 2022). Some compounds, such as terpenoids, also have neurotoxic effects, acting on neuronal ion channels, blocking nerve transmission, and leading to larval paralysis and death (Silvério et al., 2020; Lima et al., 2024).

Studies on species of the genus *Piper* (Piperaceae) have demonstrated larvicidal efficacy against medically important vectors such as *Aedes*, *Anopheles*, and *Culex*, with lower environmental impact compared to conventional synthetic insecticides (França et al., 2021; Santos et al., 2023). *Piper cyrtopodon* (Miq.) C.DC., a shrub native to the northern region of Brazil, is found in shaded tropical forests. However, its biological properties remain poorly explored (Guimarães et al., 2015; 2020). Therefore, the present study aims to evaluate the larvicidal activity of *P. cyrtopodon* essential oil against *Ae. aegypti*, identify its main chemical constituents, and investigate the mechanisms involved. The findings may contribute to the development of effective, biodegradable, and sustainable natural insecticides.

## **Material and methods**

### **Chemicals and reagents**

All reagents were purchased from reputable suppliers such as Merck and Sigma-Aldrich, ensuring the quality and reliability of the materials used throughout the study. Various reagents and standards were employed in the described methodologies, including distilled water, anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), ethyl acetate (EtOAc), dimethyl sulfoxide (DMSO, 99%), *n*-alkanes ( $\text{C}_8$  -  $\text{C}_{30}$ ),  $\alpha$ -Cypermethrin (PESTANAL®, analytical standard), potassium phosphate buffer (0.1 M, pH 7.3), HEPES buffer (20 mM, pH 7.0), and sodium phosphate buffer with EDTA (0.025 M/0.1 mM, pH 10), as well as quercetin (0.15%), dimethylformamide (DMF), hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 300 mM), formic acid (50%), hydrochloric acid (HCl, 2 M), and potassium iodide (KI, 10 M).

Additionally, bovine serum albumin (BSA), malondialdehyde (MDA), 2,4-dinitrophenylhydrazine (DNPH), trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethanol HPLC, guanidine (6 M), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (GSH), N,N,N',N'-tetramethylethylenediamine (TEMED), and glutathione reductase (GR) were also used. The standard compounds germacrene D,  $\delta$ -Elemene, and cadin-1(10),4-diene, were not isolated in the present study but were obtained from the Laboratório de Princípios Ativos da Amazônia for use in the assays.

### **Collection and identification of *P. cyrtopodon***

The specimen *P. cyrtopodon* was collected at the Base de Manejo Florestal do Instituto Nacional de Pesquisas da Amazônia, located in Manaus, Amazonas, Brazil (2°38'18.5"S and 60°09'29.7"W). The plant specimen was collected manually in its

natural habitat, using pruning shears to obtain branches with leaves, flowers, and/or fruits. Immediately after collection, the material was pressed between newspaper sheets and placed in a plant press.

The plant material was dried at room temperature, mounted on an herbarium sheet, and identified by botanist Gabriel Mendes Marcusso (Instituto de Pesquisas Jardim Botânico do Rio de Janeiro). The voucher specimen was deposited in the Herbarium of the Universidade Federal do Amazonas (HUAM), under accession number 12630 (de Oliveira et al. 2020).

### **Essential oil extraction**

Prior to the EO extraction, *P. cyrtopodon* leaves were dried in an air-circulating oven at 40 °C for 7 days and on the ground using a knife mill. Subsequently, 50 g of the powdered leaves were subjected to hydrodistillation for 3 h in a Clevenger apparatus containing 3 L of distilled water, as detailed by de Oliveira et al. (2020). The obtained EO was collected, dried with anhydrous sodium sulfate, and stored in an amber glass vial at 4 °C until further analysis. The yield of the EO (%) was calculated as the ratio between the mass of EO obtained and the mass of the dry sample multiplied by 100 (Huong et al., 2023).

### **GC-MS and GC-FID analyses**

Qualitative and quantitative analyses of the EO were performed using GC-MS (Trace GC Ultra/ISQ, Thermo Scientific, TR-5 column) and GC-FID (Agilent G-1860 Plus, HP-5 column), as described by de Oliveira et al. (2020). For GC-MS, a Trace GC Ultra system coupled to an ISQ mass spectrometer (Thermo Scientific) equipped with a TR-5 capillary column (30 m × 0.25 mm × 0.25 µm) was used. For GC-FID analysis, an Agilent G-1860 Plus system equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 µm) was employed. Prior to analysis, 1 mg of EO was diluted in 1 mL of ethyl acetate, and 1 µL of the solution was injected into the systems for analysis.

Retention indices (RI) were calculated using a homologous series of *n*-alkanes (C<sub>8</sub> - C<sub>30</sub>) and applying the Van Den Dool and Dec. Kratz (1963) equation. Each compound was identified by comparing the calculated RI with values reported in the literature databases such as the National Institute of Standards and Technology and in-house library.

### **Rearing mosquitoes**

The *Ae. aegypti* mosquitoes were reared following the methodology described by Silva et al. (2014). Eggs were obtained from successive generations of colonies previously maintained under laboratory conditions. For larval hatching, *Ae. aegypti* eggs were immersed in distilled water and subsequently fed cat food (Whiskas®) until they reached the third instar. At this stage, samples were selected for larvicidal bioassays. The remaining larvae were maintained until the pupal stage and then transferred to entomological cages (30 × 30 × 30 cm) for mosquito emergence. Adults were provided with 10% sucrose solution, and females were blood meals from hamsters *Mesocricetus auratus* (Cricetidae) to ensure colony reproduction. All procedure was conducted under controlled conditions of temperature ( $27 \pm 3$  °C), relative humidity ( $70 \pm 10\%$ ), and photoperiod (12 h of light and 12 h of dark).

### **Larvicidal activity**

The potential larvicidal activity of the EO from *P. cyrtopodon* and its major constituents germacrene D,  $\delta$ -Elemene, and cadina-1(10),4-diene was evaluated following the World Health Organization (WHO, 2005) guidelines for Laboratory and Field Testing of Mosquito Larvicides. For each treatment, groups of 20 third-instar larvae ( $n = 500$ ) were distributed into 25 containers of 250 mL, each containing 100 mL of distilled water and concentrations ranging from 20 to 100  $\mu\text{g/mL}$  of the EO, germacrene D,  $\delta$ -Elemene, and cadina-1(10),4-diene, prepared in 1 mL of DMSO.

DMSO, at concentrations ranging from 20 to 100  $\mu\text{g/mL}$ , was used as the negative control. Conversely,  $\alpha$ -Cypermethrin, with a concentration of 0.14  $\mu\text{g/mL}$  identified in our previous study (de Oliveira et al., 2022b), served as the positive control. All assays were performed in quintuplicate with five repetitions each, under controlled conditions of temperature ( $27 \pm 3$  °C) and relative humidity (70–80%). After 48 h of exposure, the percentage of larvicidal activity at each concentration was calculated using the equation 1: Larvicidal activity (%) = number of dead larvae / total number of larvae used  $\times 100$ . The relative potency (RP) of the EO was identified using the equation:  $\text{RP} = \text{LC}_{50} \text{ of standard larvicidal} / \text{LC}_{50} \text{ of EO}$  (Cheng et al., 2009).

### **Residual effect**

The residual effect was evaluated under the same conditions as the previously described larvicidal assay. After 48 h of the initial test, dead larvae from the highest concentration (100  $\mu\text{g/mL}$ ) of the EO and the major compounds were removed and

replaced with a new batch of larvae in the same treated containers, without renewing the medium or replenishing the products. This procedure was repeated daily, with dead larvae being replaced every 24 h over the course of 5 days. The residual effect was considered significant when larval mortality exceeded 90% (WHO, 2005).

### **Preparation of the homogenate**

The homogenate was prepared using larvae that died after 48 h of exposure to the highest concentration (100 µg/mL) of the EO, germacrene D, cadina-1(10),4-diene, and  $\delta$ -Elemene during the larvicidal assay. Larvae treated with  $\alpha$ -Cypermethrin (0.14 µg/mL) and DMSO (100 µg/mL) were used as controls. Dead larvae were collected and transferred to 5 mL tubes containing 2 mL of 0.1 M potassium phosphate buffer (pH 7.3) and kept refrigerated at 4 °C to minimize enzymatic degradation during sample preparation.

Subsequently, the samples were homogenized using a vortex mixer for 5 min and centrifuged at 4000 rpm for 5 min. The resulting supernatants were carefully transferred to tubes and stored at 4 °C for determination of biochemical markers, including the evaluation of oxidative stress markers and enzymatic activity associated with exposure to the tested compounds.

Specific enzyme absorbance levels were measured using a microplate reader at optimized wavelengths for each enzyme. Mean absorbance values were calculated from three replicates to ensure data accuracy and reproducibility.

### **Determination of protein concentration**

The protein content in the larval supernatants was quantified using bovine serum albumin (BSA, 1 mg/mL) as a standard, following the methodology described by Lowry et al. (1951).

### **Quantification of Reactive Oxygen Species content (ROS)**

The quantification of ROS was performed using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as an indicator, following the protocol established by Velikova et al. (2000) and adapted for larval supernatants by Silva et al. (2025). For the assay, 45 µL of each larval supernatant was combined with 45 µL of 10 M potassium iodide solution. The absorbance was measured at 390 nm using an Epoch microplate reader equipped with Gen5 Data Analysis software (BioTek Instruments, Inc., Vermont, USA). H<sub>2</sub>O<sub>2</sub> concentrations were

determined based on a standard curve prepared with a stock solution of 250 mM H<sub>2</sub>O<sub>2</sub>, diluted to a range of 0 to 45 µmol. Results were expressed as µmol of H<sub>2</sub>O<sub>2</sub> per gram<sup>-1</sup>. All measurements were conducted in technical triplicate to ensure data accuracy and reproducibility.

### **Lipid peroxidation damage**

Oxidative damage to cellular components, particularly lipid peroxidation, was assessed by measuring total malondialdehyde (MDA) levels, a key marker for lipid degradation. The quantification was performed using thiobarbituric acid reactive substances (TBARS), following the original method of Buege and Aust (1978), with modifications for larval supernatants introduced by Silva et al. (2025).

For the assay, 125 µL of each larval supernatant was combined with 250 µL of a solution containing 10% trichloroacetic acid (TCA) and 0.5% (w/v) thiobarbituric acid (TBA). Absorbance was measured at 535 nm to quantify MDA. To correct for turbidity caused by protein precipitates or other suspended particles in the larval homogenates, readings at 600 nm, where the MDA-TBA complex does not absorb. This correction enhances accuracy and is a standard procedure in oxidative stress assays.

A microplate reader (Epoch model, BioTek Instruments, Inc., Vermont, USA) and Gen5 Data Analysis software were used for the measurements. The results were expressed as nmol of MDA per gram<sup>-1</sup> of sample, and all assays were conducted in triplicate to ensure reliability and precision.

### **Protein oxidation damage**

The quantification of oxidative damage to proteins was performed according to the protocol by Levine et al. (1994). This method relies on the reaction between carbonyl groups, formed in proteins damaged by oxidative stress caused by hydroxyl radicals (OH•) derived from H<sub>2</sub>O<sub>2</sub>, and 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of 2,4-dinitrophenylhydrazone, which can be detected and quantified. For the assay, 100 µL of larval supernatants were mixed with 150 µL of 10 mM DNPH prepared in 2 M hydrochloric acid and incubated for 1 h. Subsequently, 250 µL of 6% trichloroacetic acid were added, and the mixture was centrifuged at 1000 rpm for 5 min. The resulting pellet was washed three times with a solution of ethanol and ethyl acetate, followed by the addition of 1 mL of 6 M guanidine dissolved in 50% formic acid. Absorbance readings were performed at 366 nm, and the results were expressed as

nanomoles of nmol/mg protein sample. All procedures were performed in triplicate to ensure the reproducibility of the results.

### **Determination of Superoxide Dismutase (SOD) activity**

SOD activity was evaluated using the method originally described by Afanas'ev et al. (1989), with modifications for mosquito supernatants as outlined by Janner et al. (2021). Initially, larval supernatants were centrifuged at 15,000 rpm for 10 min at 4 °C after the addition of 250 µL of 20 mM HEPES buffer (pH 7.0) to obtain clarified supernatants.

The reaction mixture consisted of sodium phosphate buffer (0.025 M/0.1 mM EDTA, pH 10), TEMED, and 10 µL of the clarified supernatants. The reaction was initiated by adding 0.15% quercetin dissolved in dimethylformamide, and quercetin auto-oxidation was monitored for 2 min at 406 nm. SOD activity was determined based on the inhibition of quercetin auto-oxidation, as described by Franco et al. (2009), with further modifications. Results were expressed as the amount of protein required to achieve 50% inhibition of quercetin oxidation. All procedures were conducted in four independent experiments, each using 10 larvae per group. Enzyme activity was reported in milliunits per milligram of protein (mU/mg protein).

### **Catalase activity**

The enzyme activity was calculated as mmol of H<sub>2</sub>O<sub>2</sub> consumed per minute per milligram of protein, following the method of Aebi (1984), with adaptations by Abolaji et al. (2015). Each procedure was performed in triplicate to ensure accuracy. The reaction mixture included 1.8 mL of potassium phosphate buffer (pH 7.0), 180 µL of 300 mM H<sub>2</sub>O<sub>2</sub>, and 20 µL of diluted larval supernatants (1:50). The reduction of H<sub>2</sub>O<sub>2</sub> was monitored spectrophotometrically at 240 nm for 2 min, with readings taken at 10-second intervals at 25 °C, and expressed as mmol of H<sub>2</sub>O<sub>2</sub> consumed/min/mg of protein.

### **Glutathione peroxidase (GPx) activity assay**

The GPx activity was measured by monitoring the decrease in absorbance of NADPH at 340 nm, following the methodology originally described by Paglia and Valentine (1967) and recently adapted by Olufemi-salami et al. (2024) for larval supernatants. For the assay, 100 µL of glutathione (GSH) were combined with a mixture of enzyme solution and buffer in test tubes containing nicotinamide adenine dinucleotide

phosphate (NADPH) and glutathione reductase (GR). The reaction was initiated by adding 100  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  to the mixture containing larval supernatants. The reaction progress was monitored spectrophotometrically at 340 nm for 3 min. GPx activity was expressed as mmol NADPH/min/mL. All procedures were performed in triplicate to ensure accuracy.

### Statistical analyses

The  $\text{LC}_{50}$  and  $\text{LC}_{90}$  values, along with Chi-square and slope  $\pm$  SE, were determined using Probit analysis through linear regression (Finney, 1971) in IBM® SPSS® Statistics software. To evaluate significant differences,  $\text{LC}_{50}$  and  $\text{LC}_{90}$  values, as well as biochemical data, were subjected to One-Way ANOVA followed by Tukey's post-hoc test ( $p < 0.05$ ). Prior to parametric tests, data normality and homogeneity of variances were verified using Shapiro-Wilk and Brown-Forsythe tests, performed using GraphPad Prism version 9 (de Oliveira et al., 2024).

## Results

### Essential oil analyses

The yield of the EO obtained from *P. cyrtopodon* leaves was  $1.4 \pm 0.2\%$  relative to the dry weight of the plant material. Chromatographic analyses by GC-MS and GC-FID identified 12 compounds in the EO (Table 1), of which 94.35% corresponded to sesquiterpenes and 5.65% to oxygenated sesquiterpenes. The main compounds identified were germacrene D (57.46%), cadina-1(10),4-diene (10.57%),  $\delta$ -Elemene (8.0%),  $\delta$ -Selinene (5.09%) and silphiperfol-5-en-3-one B (4.25%), while (*E*)-Caryophyllene (2.93%),  $\gamma$ -Elemene (2.02%),  $\beta$ -Elemene (1.95%), spathulenol (1.40%),  $\alpha$ -Humulene (1.21%), eudesma-4(14),11-diene (1.05%) and  $\gamma$ -Muurolene (0.87%) were also detected in smaller proportions.

### Larvicidal activity

The results demonstrate that the EO extracted from the leaves of *P. cyrtopodon* exhibited significant larvicidal activity against *Ae. aegypti*, with mortality rates ranging from 46% to 100%. The  $\text{LC}_{50}$  values were 26.14  $\mu\text{g/mL}$  at 24 h and 23.03  $\mu\text{g/mL}$  at 48 h (Table 2). Among the tested compounds, cadina-1(10),4-diene showed larvicidal activity ranging from 50% to 100%, with  $\text{LC}_{50}$  values of 23.38  $\mu\text{g/mL}$  (24 h) and 20.78  $\mu\text{g/mL}$  (48 h), presenting results closely aligned with those of the EO. This suggests a significant

contribution of this compound to the overall larvicidal effect of the oil. In contrast, germacrene D, despite being the major constituent of the EO, exhibited lower larvicidal activity, ranging from 10% to 50%, with LC<sub>50</sub> values of 35.65 µg/mL and 31.70 µg/mL at 24 and 48 h, respectively.  $\delta$ -Elemene showed the weakest activity, with mortality rates between 3% and 43%, and LC<sub>50</sub> values of 124.52 µg/mL (24 h) and 146.61 µg/mL (48 h).

In the control groups, the synthetic larvicide  $\alpha$ -Cypermethrin (0.14 µg/mL) caused 100% larval mortality at both time points, while the DMSO control group exhibited no larval mortality. Statistical analysis using ANOVA followed by Tukey's post hoc test revealed significant differences among all treatments ( $F_{(7, 8)} = 236.35$ ,  $p < 0.0001$ ).

### Residual effect

The EO of *P. cyrtopodon* demonstrated high initial larvicidal activity, achieving 100% mortality of *Ae. aegypti* larvae on days 1 and 2, matching the activity of  $\alpha$ -Cypermethrin. However, the residual effect of the EO declined sharply after day 2, with mortality rates dropping to 46.66% on day 3, 26.66% on day 4, and only 6.66% by day 5. Conversely,  $\alpha$ -Cypermethrin maintained 100% larvicidal activity throughout all five days, highlighting its superior residual efficacy. Among the isolated compounds, cadina-1(10),4-diene exhibited the best residual activity. It caused 33.33% mortality on day 3, 20% on day 4, and 13.33% on day 5, outperforming germacrene D and  $\delta$ -Elemene. Germacrene D and  $\delta$ -Elemene showed limited efficacy, with mortality rates below 43.33% on day 1 and a consistent decline over time, reaching 6.66% by day 5 (Fig. 1).

ANOVA confirmed significant differences among treatments ( $F_{(4, 20)} = 6.358$ ,  $p = 0.0018$ ), indicating substantial variation in larvicidal efficacy across the tested compounds. The Brown-Forsythe test ( $p = 0.0428$ ) further revealed heterogeneity in variances among treatments, meaning that the different treatments showed inconsistencies in the variability of the results. For instance, the residual effect of some treatments, such as EO, varied more over the days, while others, like  $\alpha$ -Cypermethrin, maintained greater consistency in the results. Post-hoc comparisons using Tukey's test showed no significant differences between the EO and cadina-1(10),4-diene ( $p > 0.9999$ ),  $\delta$ -Elemene ( $p = 0.4275$ ), or germacrene D ( $p = 0.2670$ ). However,  $\alpha$ -Cypermethrin displayed significantly higher efficacy than germacrene D ( $p = 0.0019$ ) and  $\delta$ -Elemene ( $p = 0.0039$ ).

## Quantification of Reactive Oxygen Species content (ROS)

The quantification of H<sub>2</sub>O<sub>2</sub> was used as an indicator of ROS in the supernatant of *Ae. aegypti* larvae, is presented in Fig. 2a. The EO from *P. cyrtopodon* induced an H<sub>2</sub>O<sub>2</sub> production equivalent to  $23.5 \pm 2$   $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per gram<sup>-1</sup>, demonstrating its ability to stimulate ROS generation. Among its major compounds, cadina-1(10),4-diene ( $26 \pm 2$   $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per gram<sup>-1</sup>) was the most active, inducing H<sub>2</sub>O<sub>2</sub> production, followed by germacrene D ( $16.0 \pm 1$   $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per gram<sup>-1</sup>) and  $\delta$ -Elemene ( $11.0 \pm 2$   $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per gram<sup>-1</sup>), all tested at a concentration of 100  $\mu\text{g}/\text{mL}$ . In comparison, the synthetic insecticide  $\alpha$ -Cypermethrin, at a concentration of 0.14  $\mu\text{g}/\text{mL}$ , induced the highest H<sub>2</sub>O<sub>2</sub> production ( $40.6 \pm 1.5$   $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per gram<sup>-1</sup>), whereas the negative control, DMSO at 100  $\mu\text{g}/\text{mL}$ , exhibited the lowest production ( $6.0 \pm 1$   $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per gram<sup>-1</sup>). Statistical analysis confirmed significant differences among the treatments ( $F_{(5, 12)} = 154.9$ ,  $p < 0.0001$ ).

## Lipid peroxidation damage

The exposure to the EO at a concentration of 100  $\mu\text{g}/\text{mL}$  resulted in an MDA content of  $2.78 \pm 0.32$   $\eta\text{mol}$  of MDA per gram<sup>-1</sup>, indicating a significant increase compared to compounds. Among the major components of the EO, cadina-1(10),4-diene showed the highest MDA content ( $2.80 \pm 0.22$   $\eta\text{mol}$  of MDA per gram<sup>-1</sup>), followed by germacrene D ( $1.04 \pm 0.05$   $\eta\text{mol}$  of MDA per gram<sup>-1</sup>) and  $\delta$ -Elemene ( $0.54 \pm 0.06$   $\eta\text{mol}$  of MDA per gram<sup>-1</sup>). In comparison, the insecticide  $\alpha$ -Cypermethrin, tested at 0.14  $\mu\text{g}/\text{mL}$ , induced the highest level of lipid peroxidation ( $4.93 \pm 0.4$   $\eta\text{mol}$  of MDA per gram<sup>-1</sup>), while the negative control (DMSO at 100  $\mu\text{g}/\text{mL}$ ) exhibited the lowest MDA content ( $0.41 \pm 0.11$   $\eta\text{mol}$  of MDA per gram<sup>-1</sup>), with substantial differences among the treatments ( $F_{(5,12)} = 247.50$ ,  $p = 0.0001$ ; Fig. 2b).

## Protein oxidation damage

The EO from *P. cyrtopodon* at 100  $\mu\text{g}/\text{mL}$  resulted in protein oxidation levels of  $4.44 \pm 0.51\%$ , while the major compounds cadina-1(10),4-diene, germacrene D, and  $\delta$ -Elemene exhibited oxidation levels of  $4.54 \pm 0.51$ ,  $2.30 \pm 0.16$ , and  $1.62 \pm 0.04$  nmol/mg protein, respectively (Fig. 2c). On the other hand, the positive control  $\alpha$ -Cypermethrin at 0.14  $\mu\text{g}/\text{mL}$  showed the highest level of protein oxidation, reaching  $6.08 \pm 0.10$  nmol/mg protein, while the negative control (DMSO, 100  $\mu\text{g}/\text{mL}$ ) displayed the lowest level of oxidation at  $0.94 \pm 1.18$  nmol/mg protein, with significant differences among treatments

( $F_{(5, 12)} = 118.9, p < 0.0001$ ), highlighting the oxidative damage variation induced by the tested compounds and controls.

### Determination of Superoxide Dismutase activity

The exposure of EO from *P. cyrtopodon* at a concentration of 100 µg/mL increased the SOD activity ( $21.67 \pm 6$  mU/mg protein) followed by cadina-1(10),4-diene ( $28.33 \pm 6$  mU/mg protein), germacrene D ( $14.67 \pm 1$  mU/mg protein), and  $\delta$ -Elemene ( $10.33 \pm 3$  mU/mg protein) (Fig. 3a). The positive control,  $\alpha$ -Cypermethrin at 0.14 µg/mL, caused the highest increase ( $37.00 \pm 2$  mU/mg protein), underscoring its potent oxidative impact. Conversely, the negative control DMSO at 100 µg/mL showed the lowest levels ( $12.37 \pm 2$  mU/mg protein), demonstrating the varying levels of oxidative damage induced by the EO, its constituents, and the controls ( $F_{(5, 12)} = 46.07, p < 0.0001$ ).

### Catalase activity

ANOVA, followed by Tukey's test, revealed significant differences in catalase enzyme activity among the treatments ( $F_{(5, 12)} = 36.91, p < 0.0001$ ). Larvae treated with the EO from *P. cyrtopodon* at 100 µg/mL showed a catalase activity of  $7.08 \pm 2.42$  mmol of  $H_2O_2$  consumed/min/mg of protein, followed by those treated with the isolated compounds cadina-1(10),4-diene ( $5.96 \pm 0.46$  mmol of  $H_2O_2$  consumed/min/mg of protein), germacrene D ( $1.55 \pm 0.23$  mmol of  $H_2O_2$  consumed/min/mg of protein) and  $\delta$ -Elemene ( $1.04 \pm 0.09$  mmol of  $H_2O_2$  consumed/min/mg of protein). The negative control (DMSO at 100 µg/mL) exhibited the lowest enzymatic activity ( $0.87 \pm 0.09$  mmol of  $H_2O_2$  consumed/min/mg of protein). The positive control,  $\alpha$ -Cypermethrin at 0.14 µg/mL, resulted in the highest catalase activity, reaching  $9.56 \pm 0.68$  mmol of  $H_2O_2$  consumed/min/mg of protein (Fig. 3b). These findings demonstrate the differential effects of the EO, its isolated constituents, and the synthetic insecticide on oxidative stress in mosquito larvae.

### Glutathione peroxidase (GPx) activity assay

Exposure of *Ae. aegypti* larvae to various treatments significantly increased GPx enzymatic activity compared to the DMSO negative control ( $2.80 \pm 0.23$  mmol NADPH/min/mL). Treatment with EO of *P. cyrtopodon* at 100 µg/mL resulted in a GPx activity of  $10.95 \pm 1.84$  mmol NADPH/min/mL. Among the compounds, cadina-1(10),4-diene showed the highest GPx activity ( $14.67 \pm 1.52$  mmol NADPH/min/mL), followed

by  $\delta$ -Elemene ( $5.40 \pm 1.37$  mmol NADPH/min/mL) and germacrene D ( $5.35 \pm 1.16$  mmol NADPH/min/mL). In contrast, larvae exposed to the positive control,  $\alpha$ -Cypermethrin at  $0.14$   $\mu$ g/mL, exhibited the greatest increase in GPx activity ( $23 \pm 2\%$ ). One-way ANOVA revealed statistically significant differences in GPx activity among the treatments and the control group ( $F_{(5, 12)} = 78.88$ ,  $p < 0.0001$ ) (Fig. 3c). These results highlight the varying effects of the EO, its isolated constituents, and the synthetic insecticide on the oxidative stress response in mosquito larvae.

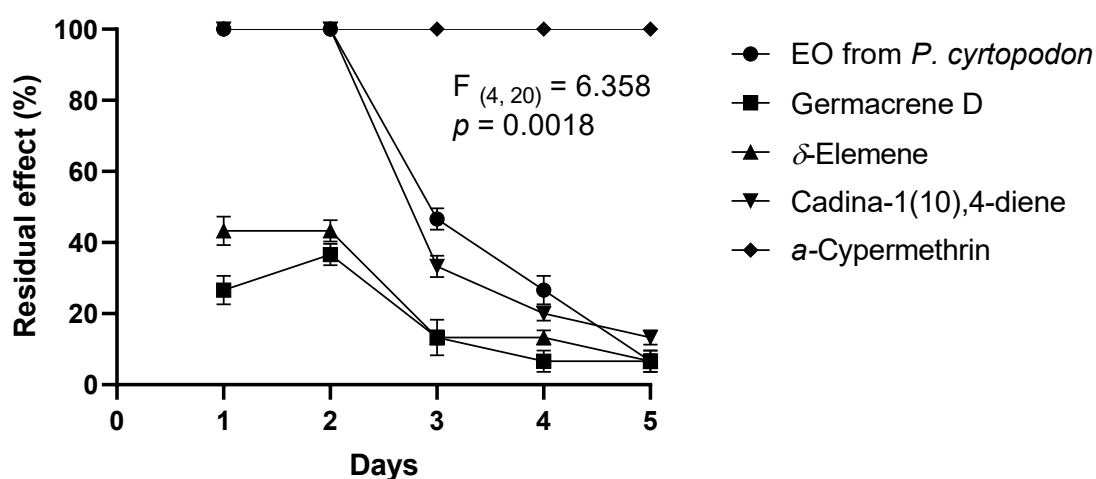


**Fig. 1** Residual effect of EO from *P. cyrtopodon* and its major compounds against *Ae. aegypti* larvae, compared to the positive control  $\alpha$ -Cypermethrin, over 5 days of exposure.

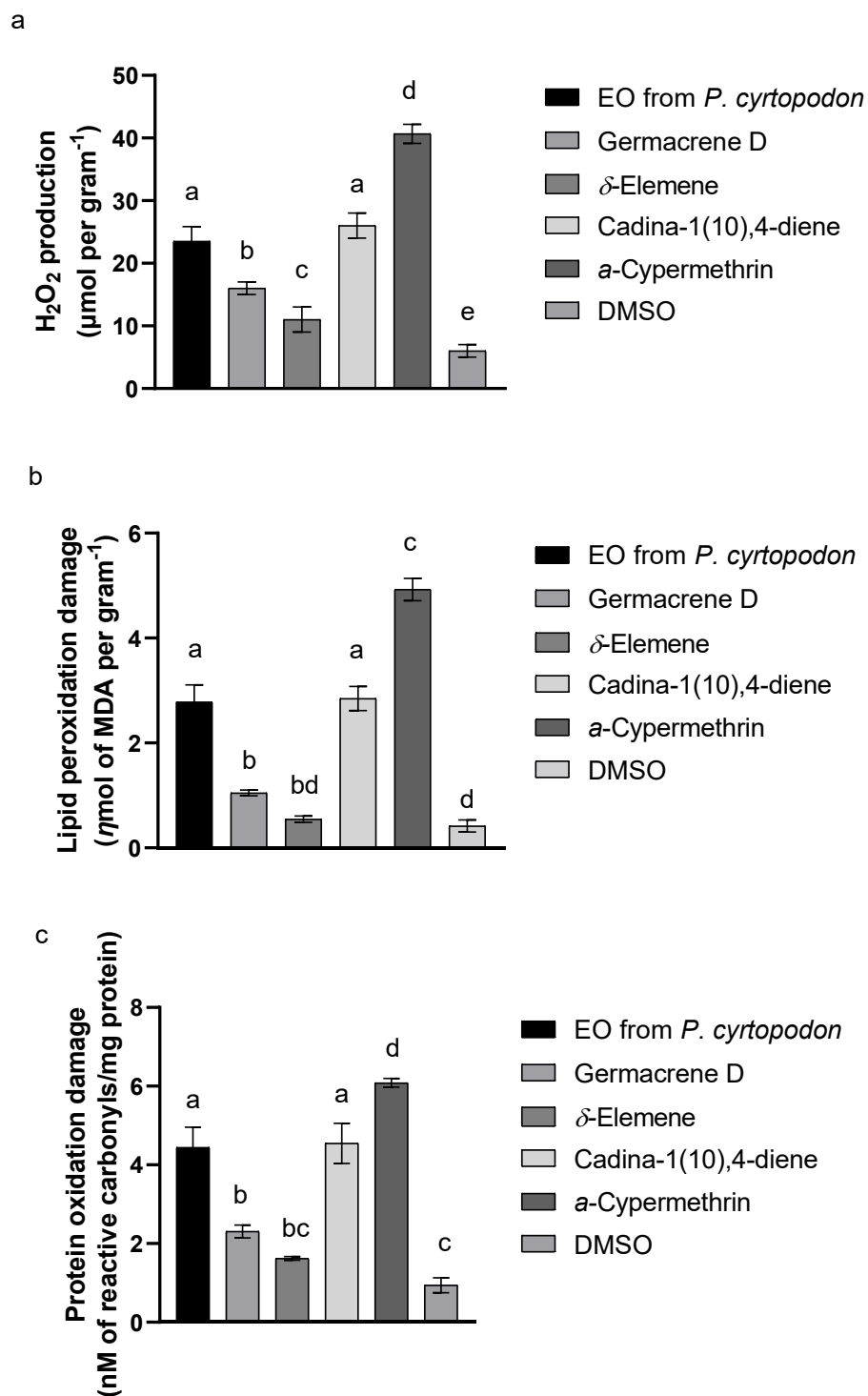


**Fig. 2** Production of reactive oxygen species, specifically H<sub>2</sub>O<sub>2</sub> (a), along with the lipid peroxidation (b) and protein oxidation (c) damages in *Ae. aegypti* larvae compared with the control groups  $\alpha$ -Cypermethrin and DMSO. Data are expressed as mean  $\pm$  standard deviation.

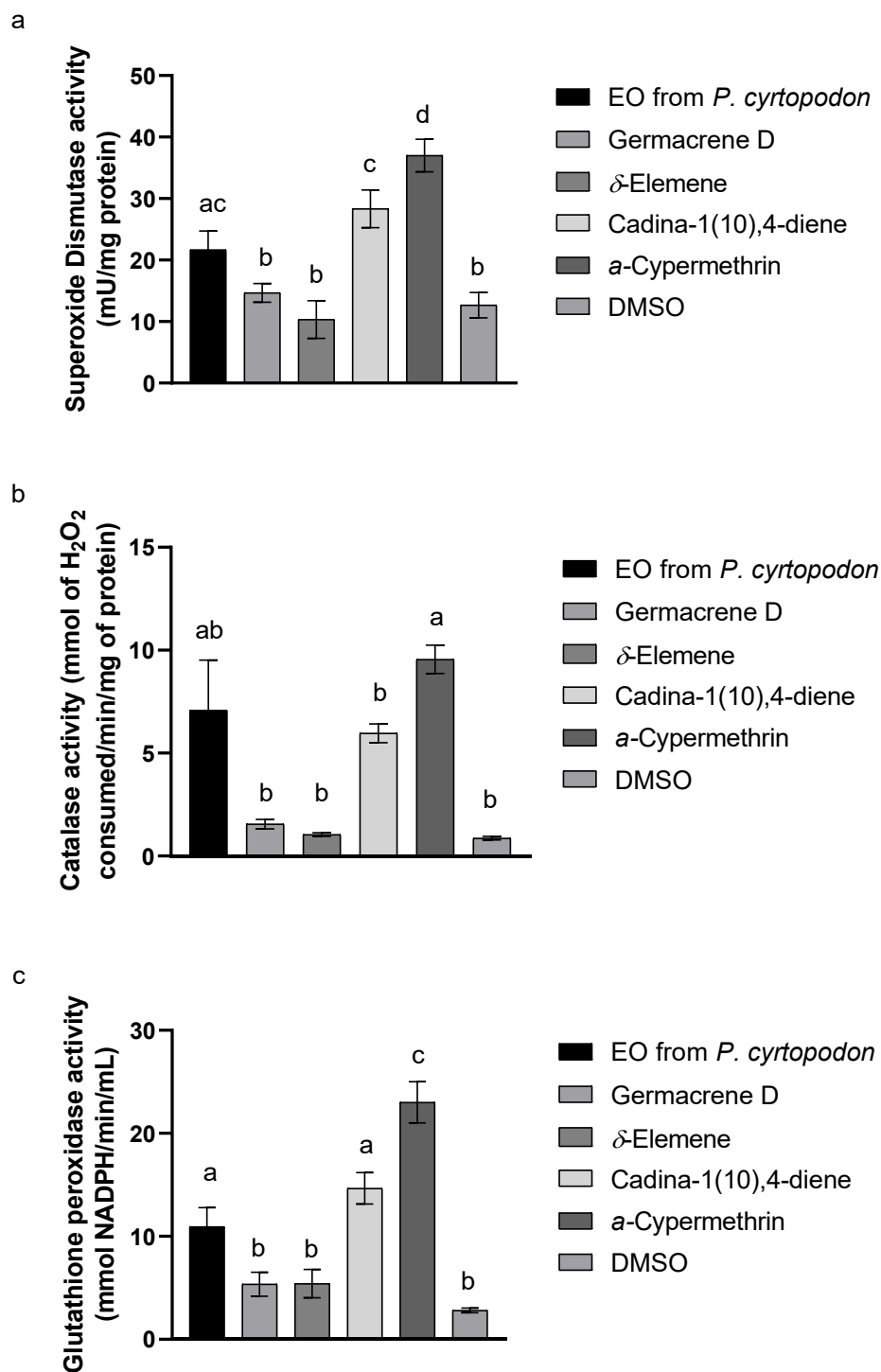


**Fig. 3** Changes in superoxide dismutase (a), catalase (b), and glutathione peroxidase activities (c) in *Ae. aegypti* larvae compared with the control groups  $\alpha$ -Cypermethrin and DMSO. Data are expressed as mean  $\pm$  standard deviation.

**Table 2.** Chemical compounds of the EO extracted from *P. cyrtopodon* leaves.

| N. | Compounds                 | RI <sup>calc</sup> | RI <sup>lit</sup> | Area (%) | Identification | Chemical class           |
|----|---------------------------|--------------------|-------------------|----------|----------------|--------------------------|
| 1  | $\delta$ -Elemene         | 1332               | 1338              | 8.00     | RI, MS         | Sesquiterpene            |
| 2  | $\beta$ -Elemene          | 1386               | 1386              | 1.95     | RI, MS         | Sesquiterpene            |
| 3  | (E)-Caryophyllene         | 1416               | 1419              | 2.93     | RI, MS         | Sesquiterpene            |
| 4  | $\gamma$ -Elemene         | 1427               | 1436              | 2.02     | RI, MS         | Sesquiterpene            |
| 5  | $\alpha$ -Humulene        | 1452               | 1454              | 1.21     | RI, MS         | Sesquiterpene            |
| 6  | $\gamma$ -Muurokene       | 1474               | 1479              | 0.87     | RI, MS         | Sesquiterpene            |
| 7  | Germacrene D              | 1479               | 1481              | 57.46    | RI, MS         | Sesquiterpene            |
| 8  | Eudesma-4(14),11-diene    | 1487               | 1490              | 1.05     | RI, MS         | Sesquiterpene            |
| 9  | $\delta$ -Selinene        | 1493               | 1492              | 5.09     | RI, MS         | Sesquiterpene            |
| 10 | Cadina-1(10),4-diene      | 1515               | 1523              | 10.57    | RI, MS         | Sesquiterpene            |
| 11 | Silphiperfol-5-en-3-ol B  | 1557               | 1551              | 4.25     | RI, MS         | Oxygenated Sesquiterpene |
| 12 | Spathulenol               | 1574               | 1578              | 1.40     | RI, MS         | Oxygenated Sesquiterpene |
|    | Sesquiterpenes            |                    |                   | 94.35    |                |                          |
|    | Oxygenated Sesquiterpenes |                    |                   | 5.65     |                |                          |
|    | Total identified          |                    |                   | 100      |                |                          |

RI<sup>calc</sup> - Retention index calculated using *n*-alkanes (C<sub>8</sub>-C<sub>30</sub>) on the TR5 column. RI<sup>lit</sup> - Retention index literature databases such as the National Institute of Standards and Technology (NIST) and in-house library. Relative area calculated from the peak area relative to the total peak area in the GC-FID chromatogram average of three replicates. MS - Mass spectra were compared with literature databases such as NIST and in-house library.

**Table 2.** LC<sub>50</sub> and CL<sub>90</sub> of EO from *P. cyrtopodon* and its major compounds against *Ae. aegypti* larvae in comparison with  $\alpha$ -Cypermethrin.

| Sample                 | Time | LC <sub>50</sub> (μg/mL) (LCL-UCL)    | LC <sub>90</sub> (μg/mL) (LCL-UCL)     | $\chi^2$ (Df) | <i>p</i> -value* | Slope ± SE          | Relative potency |
|------------------------|------|---------------------------------------|----------------------------------------|---------------|------------------|---------------------|------------------|
| EO                     | 24   | 26.14 (22.286-29.601) <sup>a</sup>    | 70.43 (61.975-83.155) <sup>a</sup>     | 7.264(3)      | 0.064            | Y = - 4.220 + 2.977 | 0.0053           |
|                        | 48   | 23.03 (19.279-26.365) <sup>a</sup>    | 60.91 (53.838-71.293) <sup>b</sup>     | 3.916(3)      | 0.271            | Y = - 4.134 + 3.035 | 0.0060           |
| Germacrene D           | 24   | 35.65 (15.052-59.089) <sup>b</sup>    | 97.67 (58.462-113.448) <sup>c</sup>    | 3.462(3)      | 0.326            | Y = - 5.304 + 2.868 | 0.0039           |
|                        | 48   | 31.70 (10.756-61.395) <sup>c</sup>    | 88.26 (51.381-101.937) <sup>d</sup>    | 7.517(3)      | 0.057            | Y = - 4.105 + 2.284 | 0.0044           |
| Cadina-1(10),4-diene   | 24   | 23.38 (20.694-25.827) <sup>a</sup>    | 46.39 (41.932-52.629) <sup>c</sup>     | 3.028(3)      | 0.387            | Y = - 5.896 + 4.307 | 0.0059           |
|                        | 48   | 20.78 (17.936-23.276) <sup>a</sup>    | 42.50 (38.212-48.578) <sup>c</sup>     | 3.938(3)      | 0.268            | Y = - 5.436 + 4.125 | 0.0067           |
| $\delta$ -Elemene      | 24   | 124.52 (106.872-159.613) <sup>d</sup> | 310.99 (222.422-559.178) <sup>f</sup>  | 2.860(3)      | 0.414            | Y = - 6.755 + 3.224 | 0.0011           |
|                        | 48   | 146.61 (116.317-220.051) <sup>c</sup> | 569.06 (335.000-1532.956) <sup>g</sup> | 6.329(3)      | 0.097            | Y = - 6.329 + 2.176 | 0.0009           |
| $\alpha$ -Cypermethrin | 24** | 0.14 (0.126-0.159) <sup>f</sup>       | -                                      | -             | -                | -                   | 1                |

LC<sub>50</sub> and <sub>90</sub> - Concentrations needed to kill 50 and 90% of larvae. LCL - Lower confidence limit of 95%. UCL - Upper confidence limit of 95%.

Df - Degree of freedom. Letters (a to g) in same column indicate statistical difference between LC<sub>50</sub> values F (7, 16) = 18.90, *p* < 0.0001, as well as LC<sub>90</sub> values (F (7, 8) = 236.35, *p* < 0.0001). *p*-value obtained for  $\chi^2$  shown no significant. No have larvae died in the negative control (DMSO) (20 to 100 μg/mL). \*\* The positive control  $\alpha$ -Cypermethrin evaluated at 0.14 μg/mL resulted in 100% larvae mortality.

## Discussion

The yield of the EO extracted from the leaves of *P. cyrtopodon* in our study, characterized by a high concentration of germacrene D (57.46%), was comparable to the yields reported by Andrade et al. (2006), who used the aerial parts of the plant (leaves and thin stems). They documented EO yields of 0.8% and 0.9% from samples collected in Manaus, Amazonas, and Santarém, Pará, respectively, both in Brazil. The EO from the aerial parts collected in Manaus was predominantly composed of epi- $\alpha$ -bisabolol (26.3%),  $\alpha$ -Cadinol (9.5%), bicylogermacrene (8.3%), germacrene D (7.5%), and spathulenol (0.5%). In contrast, the EO from the aerial parts collected in Santarém was mainly constituted of bicylogermacrene (23.3%), germacrene D (17.9%), spathulenol (6.9%), (*E*)-nerolidol (6.6%), *p*-Cymene (6.3%),  $\beta$ -Elemene (0.7%), and  $\delta$ -Elemene (0.2%).

The sesquiterpenes germacrene D e  $\delta$ -Elemene were identified in all EO of *P. cyrtopodon* described above, while cadina-1(10),4-diene, a precursor of  $\alpha$ -Cadinol, through a series of chemical reactions, such as oxidation, was identified only in sample collected in Manaus. These findings suggest that the chemical profile of the EO in this species may be influenced by geographic location and a variety of environmental factors, including both intrinsic and extrinsic aspects of the plant (Martianasari and Hamid, 2019).

Intrinsic factors refer to the plant's biological characteristics, such as genetic variability, developmental stage, physiological age, and metabolic status at the time of collection (Manfron et al., 2021). These elements directly influence the expression of biosynthetic pathways involved in the production of secondary metabolites, such as terpenes, phenolics, and alkaloids (Filho et al., 2021). In contrast, extrinsic factors comprise external environmental conditions, including soil composition, altitude, temperature, relative humidity, photoperiod, solar radiation, rainfall patterns, and the presence of biotic stresses (Ghavam et al., 2021).

The interaction between these intrinsic and extrinsic factors can result in substantial qualitative and quantitative variations in the chemical constitution of EOs, even among individuals of the same species (Lima et al., 2025). Such variations directly influence the bioactivity of EOs, including their biological effects on target organisms, such as medically important vectors (Ainane et al., 2020).

Compared with other studies, the larvicidal activity demonstrated by EO from *P. cyrtopodon* against *Ae. aegypti* was superior to other species within the *Piper* genus,

such as the EOs from *P. capitarianum* (Miq.) C. DC. (CL<sub>50</sub> of 139.50 µg/mL) (França et al., 2021), *P. tuberculatum* Jacq. (CL<sub>50</sub> of 48.61 µg/mL) (Lima et al., 2024), *P. betle* L. (CL<sub>50</sub> of 59.80 µg/mL) (Martianasari and Hamid, 2019), and *P. purusanum* C. DC. (CL<sub>50</sub> of 53.41 µg/mL) (de Oliveira et al., 2022a). However, it was similar to EOs from *P. alatipetiolatum* Yunck (CL<sub>50</sub> of 33.74 µg/mL) (de Oliveira et al., 2020), and *P. corcovadensis* (Miq.) C.DC. (CL<sub>50</sub> of 30.52 µg/mL) (da Silva et al., 2016).

The larvicidal activity of germacrene D, isolated EO from *P. purusanum*, was initially documented against *Ae. aegypti*, *Ae. albopictus* Skuse, 1894 (CL<sub>50</sub> of 33.51 and 35.96 µg/mL), *An. darlingi* Skuse, 1926, *An. nuneztovari* Gabaldón, 1940, *An. triannulatus* Neiva & Pinto, 1922, and *An. albitarsis* Lynch-Arribálzaga, 1878 (CL<sub>50</sub> of 24.49 to 32.36 µg/mL), as described by de Oliveira et al. (2022a). These values are comparable to those reported in Table 2 in our study. Later, the larvicidal activity of germacrene D, isolated EO from *Matricaria chamomilla* (Asteraceae) was documented against *Ae. vittatus* Bigot, 1861 and *An. subpictus* Grassi, 1899 (CL<sub>50</sub> of 9.11 and 10.95 µg/mL) (Al-Ghanim et al., 2023), confirming the effectiveness of this compound.

The residual activity results highlight the potential of EO from *P. cyrtopodon* as a natural larvicidal agent, with strong initial efficacy but limited persistence. Among its major constituents, cadina-1(10),4-diene exhibited the best residual performance, although still less effective than  $\alpha$ -Cypermethrin. While the rapid decline in efficacy limits its use in long-term larval control, the EO may serve as a short-term solution or adjunct to synthetic insecticides. Moreover, EOs like that of *P. cyrtopodon* present ecological benefits, such as biodegradability and lower risk of resistance development, which support their relevance in integrated vector management strategies.

EOs are known to contain lipophilic compounds such as sesquiterpenes and monoterpenes that can integrate into the lipid bilayers of cell membranes (Cutler et al., 2022). This interaction can lead to mitochondrial dysfunction, disruption of electron transport chains, and leakage of electrons that reduce molecular oxygen to form ROS, particularly H<sub>2</sub>O<sub>2</sub> (Mao et al., 2020). In the case of  $\alpha$ -Cypermethrin, its insecticidal activity is mainly due to the disruption of voltage-gated sodium channels in neuronal membranes. However, it also induces oxidative stress through secondary mechanisms (da Costa et al., 2024; Giraldi et al., 2024).

ROS are highly reactive molecules generated as byproducts of normal cellular metabolism, particularly during mitochondrial respiration (Mittler, 2017). Under stress conditions induced by botanical or synthetic larvicides, as observed in our study with EO

from *P. cyrtopodon* and its main compounds, compared to control groups treated with  $\alpha$ -Cypermethrin and DMSO, cellular processes are disrupted, leading to excessive ROS production (Schieber & Chandel, 2014). Among these molecules,  $H_2O_2$  is particularly significant because, although less reactive than other ROS, it can be converted into hydroxyl radicals, which are highly toxic to cells (Wang et al., 2015).

$H_2O_2$  initiates oxidative damage by attacking the unsaturated lipids in cell membranes, resulting in the formation of free lipid radicals, which are unstable and reactive molecules, that further, react with molecular oxygen ( $O_2$ ) forming lipid peroxides, which are even more unstable and continue to react with other unsaturated lipids, propagating the chain reaction (Mittler, 2017; Mao et al., 2020).

Lipid peroxidation compromises the structure and function of cell membranes, ultimately affecting cellular integrity (Cutler et al., 2022). This process generates toxic byproducts, such as malondialdehyde, which can react with other biomolecules, including DNA, causing further damage (Ercan et al., 2022).

Additionally, proteins are also the targets of oxidative stress and can undergo oxidation to form protein carbonyls, which serve the markers of global protein oxidation (Weber et al., 2015). The accumulation of damage to membranes and other cellular structures can eventually lead to cell death, either through necrosis (uncontrolled cell death) or apoptosis (programmed cell death) (Sies, 2017; Zhang et al., 2019).

In order to maintain the ROS production under control, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) play a key role in cellular defense (Yang and Lee, 2015). Changes in the activity of these enzymes are reliable indicators of oxidative stress (Cutler et al., 2022). Furthermore, detoxification enzymes are crucial for maintaining physiological processes in insects when facing environmental challenges, such as exposure to toxic molecules (Ercan et al., 2022; Silva et al., 2025).

SOD catalyses the conversion of superoxide radical ( $O_2^-$ ) into  $H_2O_2$  and  $O_2$ , serving as the first line of defense against ROS in insects, as well as in other animals (Giraldi et al., 2024). CAT, primarily located in the peroxisome, converts  $H_2O_2$  into water and oxygen, neutralizing  $H_2O_2$  (Johnson et al., 2021b). GPx, on the other hand, uses reduced glutathione (GSH) to reduce  $H_2O_2$  and other peroxides, forming water or alcohols while regenerating GSH for use in subsequent protection cycles (Cutler et al., 2022b). These interconnected mechanisms provide defense in preserving cellular integrity against damage caused by ROS (Cutler et al., 2022b).

Even with the increased activity of the antioxidant enzymes SOD, CAT, and GPx, the toxic mechanism can be attributed to a redox imbalance (de Oliveira et al., 2024). This occurs when the generation of ROS exceeds the neutralizing capacity of the antioxidant defense system (da Costa et al., 2024). For instance, cadina-1(10),4-diene induced ROS production levels comparable to those observed for the EO of *P. cyrtopodon*. However, enzyme activities were even more elevated in the EO group, suggesting that other constituents in the EO may contribute to enhanced toxicity.

## Conclusion

The EO from *P. cyrtopodon* exhibited significant larvicidal activity against *Ae. aegypti*, with efficacy comparable to that of the synthetic insecticide  $\alpha$ -Cypermethrin after 24 and 48 h of exposure. This activity is associated with the induction of oxidative stress in larvae, as evidenced by increased production of ROS, lipid peroxidation, and protein oxidation, suggesting a mechanism of action based on the disruption of antioxidant defenses.

Among the major EO constituents, cadina-1(10),4-diene stood out due to its stronger oxidative effects and higher residual activity, outperforming  $\delta$ -Elemene and germacrene D the latter being the most abundant but less effective in larvicidal performance. These findings emphasize that larvicidal efficacy is more closely related to specific bioactivity than to compound abundance.

This study is the first to report the larvicidal potential of EO from *P. cyrtopodon* while also providing initial insights into its oxidative stress-mediated mechanism of action. Furthermore, it highlights the value of exploring secondary metabolites from Amazonian plants as promising natural alternatives for vector control, contributing to the development of sustainable and environmentally safe strategies against *Ae. aegypti*.

## Ethics Approval

The collection of *P. cyrtopodon* was authorized by the Sistema de Autorização e Informação sobre Biodiversidade (No. 96329-1). The Ethics Committee on the Use of Animals (No. 11/2022, SEI 01280.000601/2022–08) approved the blood meal of female mosquitoes performed in hamsters. The study was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado (No. A357D96).

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## CAPÍTULO 2

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Análises moleculares e eficácia repelente do óleo essencial e dos principais compostos de *Piper cyrtopodon* (Miq.) C.DC (Piperaceae) contra *Aedes aegypti* (Linnaeus, 1762) (Culicidae)

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(ANEXO 2)

Molecular insights and repellent efficacy of the essential oil and major compounds from *Piper cyrtopodon* (Miq.) C.DC (Piperaceae) against *Aedes aegypti* (Linnaeus, 1762) (Culicidae)

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## Abstract

Dengue, a mosquito-borne disease transmitted mainly by *Aedes aegypti*, remains a major global public health concern. In the Americas, over 13 million cases were reported in 2024 and more than 4 million in 2025, highlighting the urgent need for effective preventive strategies. Repellents are among the most practical tools to reduce mosquito–human contact. In this study, the essential oil (EO) from *Piper cyrtopodon* and cadina-1(10),4-diene showed the highest repellent efficacy ( $EC_{50} = 2.68$  and  $2.31$  mg/cm<sup>2</sup>), followed by germacrene D and  $\delta$ -Elemene ( $EC_{50} = 3.85$  and  $4.54$  mg/cm<sup>2</sup>). All treatments maintained high repellency levels for up to 270 min under laboratory conditions, showing

performance comparable to the reference compound DEET within the experimental conditions. Molecular docking with the mosquito odorant-binding proteins OBP1 and OBP22 suggested favorable binding interactions with the major compounds (−6.8 to −8.4 kcal/mol for OBP1 and −6.8 to −7.3 kcal/mol for OBP22), involving hydrophobic and aromatic contacts within the binding cavity. ADMET predictions indicated high lipophilicity, low aqueous solubility, and limited systemic absorption. No significant cytotoxicity was observed in Vero and PBMC cells up to 25 µg/mL for 72 h. These findings highlight the EO from *P. cyrtopodon* as a potential source of natural compounds with repellent activity, with emphasis on behavioral repellency under controlled laboratory conditions.

**Keywords:** cytotoxicity; DEET; mosquito repellent; *Piper*; sesquiterpenes

## Introduction

Dengue is one of the most prevalent arboviral diseases worldwide, caused by the dengue virus, which belongs to the genus *Flavivirus* and the family *Flaviviridae*, and is classified into four antigenically distinct serotypes: DENV-1, DENV-2, DENV-3, and DENV-4 [1,2].

Reflecting this alarming trend, the Pan American Health Organization [3] reported that the Americas recorded a historic record of dengue cases in 2024, exceeding 13 million notifications and 8,431 deaths, with Brazil accounting for more than 10 million cases and 6,321 deaths. In 2025, more than 4 million dengue cases and 2,247 deaths were registered across the Americas, including 1,826 deaths reported in the country.

This scenario is mainly driven by the transmission of the virus through the bite of the female mosquito *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae), a highly adapted urban vector characterized by strong hematophagous behavior and high vector competence [3]. The proliferation of this species is strongly influenced by uncontrolled urbanization and climate change, which create favorable conditions for its breeding and survival [4].

In this context, topical repellents represent one of the most practical and effective personal protection strategies against mosquito bites, acting as a chemical barrier that interferes with the insect's olfactory perception and prevents host–vector contact [5,6]. Among these, DEET (N,N-diethyl-meta-toluamide) is widely used as a reference compound in repellent studies due to its well-established efficacy [7,8]. However, their

mode of action is closely related to the mosquito's olfactory system, which governs the detection and recognition of potential hosts [9].

To explore possible mechanisms underlying the activity of natural repellents, it is important to consider how conventional compounds such as DEET interfere with mosquito olfactory perception. Mosquito host-seeking behavior relies on the detection of volatile organic compounds emitted from human skin, breath, and sweat [10]. These compounds are initially captured by odorant-binding proteins (OBPs) located within the sensillar lymph of the antennae and maxillary palps [11]. OBPs solubilize and transport the volatiles to odorant receptors (ORs) positioned on the membrane of olfactory sensory neurons, where receptor activation triggers neural signaling pathways that mediate host attraction [12].

Repellents such as DEET are thought to interfere with mosquito host-seeking behavior by blocking or altering the binding of OBPs and ORs, thereby disrupting odorant recognition and reducing the mosquito's ability to detect human hosts [13]. Despite its proven efficacy, the prolonged or excessive use of synthetic repellents like DEET has raised health and environmental concerns, including dermatological reactions, systemic toxicity, restrictions on pediatric application, and potential ecological risks [8,9]. These limitations have encouraged the search for safer and more sustainable alternatives based on natural products, particularly essential oils and their bioactive constituents [14].

Molecular modeling and *docking* approaches provide valuable tools to predict ligand–protein interactions and explore compounds with potential biological [15]. OBPs from different mosquito species exhibit a high degree of structural and functional conservation, particularly in the amino acid residues forming the hydrophobic binding site responsible for recognizing volatile compounds and semiochemicals [16].

Comparative sequence and modeling analyses show that the OBPs of *Anopheles* Meigen, *Aedes* Meigen, and *Culex* Linnaeus (Culicidae) share conserved cysteine motifs and display structurally similar binding cavities, whose conservation supports their ability to interact with a broad range of volatile ligands [17,18].

Accordingly, molecular *docking* studies involving compounds from essential oils (EOs) demonstrated high affinity for  $\beta$ -Pinene (–6.7 kcal/mol; OBP1, PDB ID: 3N7H), linalool (–5.4 kcal/mol; OBP4, PDB ID: 3Q8I), and  $\alpha$ -Terpinene (–7.1 kcal/mol; OBP7, PDB ID: 3R1O), all from *An. gambiae* Giles, 1902 [19]. In addition, citronellal (–7.2 kcal/mol) and  $\alpha$ -Humulene (–6.5 kcal/mol) showed strong interaction with OBP1 from *A. aegypti* (PDB ID: 3K1E) [10].

In parallel with such molecular investigations, evaluating the safety of potential repellent compounds is equally important to ensure their suitability for human use [20]. Cytotoxicity assays employing mammalian cell lines, such as peripheral blood mononuclear cells (PBMCs) and Vero cells, are therefore essential to assess the biocompatibility of formulations intended for topical application [21,22].

Building on this growing interest in natural products, the search for bioactive molecules with both repellent and larvicidal potential has increasingly focused on EOs from *Piper* (Piperaceae) species, which are known for their remarkable chemical diversity and broad biological activity [23,24].

In a previous investigation, we characterized for the first time the chemical composition and biological activity of the EO from the leaves of *P. cyrtopodon* (Miq.) C.DC, an underexplored Amazonian species. The EO showed a yield of 1.4% and was dominated by sesquiterpenes, mainly germacrene D (57.46%), cadina-1(10),4-diene (10.57%), and  $\delta$ -Elemene (8.0%). Remarkably, both the EO and its major constituents displayed unprecedented larvicidal activity against *A. aegypti*, with LC<sub>50</sub> values ranging from 23.38 to 124.52  $\mu$ g/mL, linked to the induction of oxidative stress and disruption of the larvae's antioxidant defense system [25].

These findings highlight the potential of *P. cyrtopodon* as a source of bioactive compounds, prompting further investigation of its repellent efficacy against *A. aegypti*. The present study employs an integrative approach based on *in vivo*, *in silico*, and *in vitro* assays, allowing for a comprehensive evaluation of the species with a focus on behavioral repellency and olfactory-mediated responses.

## Experimental

### Chemicals and reagents

All reagents used in this study were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), including acetone (HPLC), dimethyl sulfoxide (DMSO), MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Ficoll-Hypaque (GE Healthcare), RPMI (Roswell Park Memorial Institute medium), DMEM (Dulbecco's Modified Eagle Medium), and PBS (Phosphate Buffered Saline). Supplements included fetal bovine serum (FBS), penicillin–streptomycin, fungizone (amphotericin B). The commercial repellent N,N-diethyl-meta-toluamide (DEET, 7.125%, SC Johnson) was purchased from a local pharmacy. The Vero CCL-81 cell line derived from the kidney of the African green monkey *Chlorocebus sabaeus*

(Cercopithecidae) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

### **Plant material**

All procedures for the collection of *P. cyrtopodon*, deposition of the voucher specimen, as well as the methods for extraction, drying, chromatographic analyses of the EO by GC-MS and GC-FID, and the obtention of the major compounds, are described in previous study [25].

### **Mosquito rearing**

Female *A. aegypti* were obtained from a colony maintained at the Laboratory of Malaria and Dengue of the National Institute of Amazonian Research, under controlled conditions of temperature ( $26 \pm 3$  °C), relative humidity ( $70 \pm 10\%$ ), and photoperiod (12 h light/12 h dark), with small modifications [26].

In brief, eggs deposited on filter paper strips were hatched in plastic trays containing distilled water. Then, larvae were separated according to their developmental stage (L1 to L4) and fed cat food (Whiskas®) until reaching the fourth instar. Pupae were transferred to plastic cups containing 50 mL of distilled water and placed inside entomological cages ( $30 \times 30 \times 30$  cm).

After adult emergence, females were separated and placed in individual cages, receiving daily feeding with a 10% sucrose solution. For bioassays, females up to seven days old were selected, transferred using a mouth aspirator, and starved of sucrose for 24 h prior to experimentation.

### **Repellent activity**

The repellent activity was performed following the World Health Organization Guidelines for the Evaluation of Spatial Repellent Efficacy [27] with an adapted dual-choice olfactometer [28]. The apparatus consisted of a central cylinder for mosquito release, connected to two lateral cylinders: one containing the treatment and the other serving as control (Figure 1).

Filter papers ( $4 \text{ cm}^2$ ) were treated with 1, 2, 4, 6, 8, and  $10 \text{ mg/cm}^2$  of the EO from *P. cyrtopodon* and its major compounds germacrene D, cadina-1(10),4-diene, and  $\delta$ -Elemene, solubilized in 1 mL of acetone. After 10 min of solvent evaporation at room temperature ( $26 \pm 3$  °C), the treated papers were placed inside the exposure cylinder.

For each concentration, 10 replicates were performed using 20 unfed female *A. aegypti* (24 h deprivation;  $n = 200$  per treatment). After 1 min of acclimation in the central cylinder, the lateral lids were simultaneously opened, allowing mosquitoes to move freely for 10 min. At the end of the assay, insects were counted in each cylinder.

Acetone was used as the negative control, while DEET was included as a reference compound for comparative purposes, given its well-established efficacy in repellent studies. All assays were conducted under controlled environmental conditions of temperature ( $27 \pm 3$  °C) and relative humidity (70–80%).

The percentage of repellency was calculated using the equation: Percentage repellency =  $(M_c - M_t) / (M_c + M_t) \times 100$ , where  $M_c$  is the number of mosquitoes in the untreated cylinder and  $M_t$  is the number of mosquitoes in the cylinder treated with the test substance [29].

### **Repellency Persistence Assay**

Repellency persistence was evaluated as the time interval between application and the first mosquito entry into the treatment chamber under olfactometer conditions, using the  $EC_{90}$  values of the EO and its major compounds. The experimental setup consisted of 20 female *A. aegypti* per replicate, with three replicates per treatment ( $n = 60$ ).

The effective concentrations were applied to filter papers and placed in the treatment cylinder of the olfactometer. After a 1 min acclimation period in the central cylinder, the lids were opened for a 3 min exposure period, repeated every 30 min up to 270 min. Acetone was used as the negative control, while DEET was included as a reference compound for comparative purposes.

### **Molecular *docking* study**

#### **Protein structure preparation and validation**

The structures of OBP1 from *An. gambiae* (PDB ID: 3N7H) and OBP22 from *A. aegypti* (PDB ID: 6OGH) were retrieved from the RCSB Protein Data Bank (<http://www.rcsb.org>), with resolutions of 1.60 Å and 1.85 Å, respectively. Structures were prepared by removing water molecules, ions, and co-crystallized ligands, followed by the addition of polar hydrogens and Kollman charges using AutoDockTools v1.5.6 (The Scripps Research Institute, La Jolla, CA, USA) [30].

Validation of the *docking* protocol was carried out through re-docking of the native ligands (DEET for OBP1 and linoleic acid for OBP22). For each ligand, 20 *docking* poses were generated using an exhaustiveness parameter of 20 and an energy range of 20, with the grid box dimensions and coordinates specific. Root Mean Square Deviation (RMSD) values  $\leq 2.0$  Å were considered indicative of reliable reproduction of experimental binding poses.

### **Selection of 3D ligand structures**

The major compounds of EO from *P. cyrtopodon*, namely germacrene D (CID 5317570), cadina-1(10),4-diene (CID 10223), and  $\delta$ -Elemene (CID 89316), together with the synthetic repellent DEET (CID 4284) were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>) in SDF format. The chemical structures of these compounds are shown in Figure 2. Ligands structures were energy-minimized and converted to PDBQT format using AutoDockTools v1.5.6 [31].

### **Docking simulations**

Molecular *docking* studies were performed using AutoDock Vina (v1.2.0), with a grid box adjusted to fully cover the active site, as detailed in Table 1. For each ligand, 20 *docking* poses were generated, with an exhaustiveness parameter set to 20 and an energy range of 20. The best-ranked pose was selected based on the lowest binding free energy ( $\Delta G$ , kcal/mol), and analyzed in BIOVIA Discovery Studio Visualizer (Dassault Systems, Waltham, MA, USA) to identify interacting residues and binding modes.

Inhibition constants ( $K_i$ ,  $\mu\text{M}$ ) were calculated from the binding free energy ( $\Delta G$ , kcal/mol) using the equation:  $K_i = e^{(\Delta G \times 1000) / (R \times T)}$ , where  $R$  is the universal gas constant ( $1.987 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ) and  $T$  is the absolute temperature (298.15 K).

### **Prediction of properties of ADMET**

Germacrene D, cadina-1(10),4-diene, and  $\delta$ -Elemene, and DEET selected for molecular *docking* studies, were also evaluated for their absorption, distribution, metabolism, excretion, and toxicity profiles using the ADMETlab 3.0 web server (<https://admetlab3.scbdd.com/>). Their canonical SMILES were retrieved from the PubChem database and analyzed in the platform's default mode to estimate physicochemical and toxicological parameters, aiming to correlate the predicted profiles with mosquito behavioral activity [19].

## Cytotoxicity assay

*In vitro* cell viability assays were performed to confirm the toxicity levels of the EO and its major compounds, which were evaluated using the colorimetric MTT assay, following the methodology of [32] with modifications described by [33]. Peripheral blood mononuclear cells (PBMCs) were obtained from ten healthy donors after written informed consent, isolated by density gradient centrifugation using Ficoll-Hypaque, washed three times with RPMI, and maintained in culture under standard conditions.

For the assays, PBMCs ( $5 \times 10^5$  cells/well) and Vero cells lines ( $1 \times 10^5$  cells/well) were seeded in 96-well plates containing 200  $\mu$ L of RPMI or DMEM, respectively, supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and fungizone, and incubated at 37 °C with 5% CO<sub>2</sub> for 24 h to allow cell adhesion and formation of subconfluent monolayers.

Cells were then exposed to the EO and constituents at concentrations of 3, 6, 12, 25, 50, and 100  $\mu$ g/mL, and incubated for 24, 48, and 72 h. Untreated cells served as the negative control, and DMSO at 100% as the positive control. At the end of each time point, the medium was replaced with 10  $\mu$ L of MTT solution (5 mg/mL in PBS) diluted in 100  $\mu$ L of phenol red-free DMEM, and incubated for 4 h at 37 °C. The supernatant was removed, 50  $\mu$ L of lysis buffer was added, and the plates were gently shaken to dissolve the formazan crystals, followed by an additional 10 min incubation at 37 °C.

Absorbance was measured at 492 nm using a microplate reader. Cell viability was expressed as the percentage of absorbance in treated samples relative to untreated controls, according to the equation:  $(A_{492} \text{ treated} / A_{492} \text{ untreated}) \times 100$  [34].

## Statistical analysis

The effective concentrations required to achieve 50% and 90% repellency (EC<sub>50</sub> and EC<sub>90</sub>) were estimated using Probit regression analysis [35]. For the MTT assay performed on PBMCs and VERO cells, cell viability was calculated as the absorbance ratio between treated and control groups [36].

The mean repellency persistence time was used as an indicator of sustained behavioral repellency under experimental conditions, and differences among treatments were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons ( $p < 0.05$ ). Statistical analyses were conducted using GraphPad Prism v.9 and IBM® SPSS® Statistics, with 95% confidence limits [37].

## Results

### Spatial repellency

The EO from *P. cyrtopodon* exhibited a clear concentration-dependent repellent effect against *A. aegypti* (Figure 3), with repellency increasing from 13% to 100% as the concentration rose. Two-way ANOVA revealed significant effects of both concentration and treatment ( $p < 0.0001$ ), as well as their interaction, indicating that repellency varied according to the compound and its concentration.

Similarly, the major constituents of the EO also displayed concentration-dependent repellency. Cadina-1(10),4-diene showed the highest activity, with repellency ranging from 21% to 100%, followed by germacrene D, which increased from 9% to 91%.  $\delta$ -Elemene was the least effective, showing a repellency range from 2% to 86%. At intermediate concentrations, no significant difference was observed between the EO and cadina-1(10),4-diene ( $p > 0.05$ ), suggesting that this sesquiterpene is a major contributor to the EO's repellent effect. In contrast,  $\delta$ -Elemene consistently exhibited the lowest repellency across all concentrations ( $p < 0.0001$ ).

To further quantify the potency of these effects, Probit analysis was performed to estimate the effective concentrations required for 50% and 90% repellency. The results (Table 2) corroborated the concentration-dependent pattern observed in the behavioral assays, with the EO showing  $EC_{50} = 2.68$  and  $EC_{90} = 7.55$  mg/cm<sup>2</sup>, values close to those of cadina-1(10),4-diene ( $EC_{50} = 2.31$  mg/cm<sup>2</sup> and  $EC_{90} = 7.11$  mg/cm<sup>2</sup>), confirming this compound as the main contributor to the EO activity.

Germacrene D presented intermediate values ( $EC_{50} = 3.85$  mg/cm<sup>2</sup> and  $EC_{90} = 9.81$  mg/cm<sup>2</sup>), while  $\delta$ -Elemene showed the highest values ( $EC_{50} = 4.54$  mg/cm<sup>2</sup> and  $EC_{90} = 11.85$  mg/cm<sup>2</sup>), consistent with its lower efficacy. As expected, DEET exhibited 100% repellency at the tested concentration. These data demonstrate that both the EO and its compounds constituents exhibit potent repellency even at low concentrations.

### Repellency Persistence Assay

At the  $EC_{90}$  concentration, the EO from *P. cyrtopodon* and its major constituents cadina-1(10),4-diene, germacrene D, and  $\delta$ -Elemene exhibited strong repellent activity against *A. aegypti* females throughout the entire observation period. During the first 210 min of evaluation, all treatments, including DEET, maintained 100% repellency, with no statistically significant differences among them ( $p > 0.05$ ) (Table 3). This finding

indicates that the EO and its isolated compounds were able to maintain complete repellency, with performance comparable to the reference compound DEET, demonstrating high initial efficacy and persistence under laboratory conditions.

After 240 min, a slight decline in repellency was observed only for the EO (95.00%), whereas cadina-1(10),4-diene, germacrene D,  $\delta$ -Elemene, and DEET continued to confer complete protection (100%). At the final assessment (270 min), the repellency percentages decreased slightly for some treatments, reaching 93.33% for the EO, 95% for germacrene D, 96.66% for  $\delta$ -Elemene, and 95.66% for DEET, while cadina-1(10),4-diene remained fully effective (100%). These results suggest that the EO experiences a marginal reduction in volatility or stability after prolonged exposure, whereas cadina-1(10),4-diene, which may be related to differences in volatility or chemical stability.

Two-way ANOVA with repeated measures revealed that both the time and compound factors had significant effects ( $p < 0.0001$ ), as well as a highly significant interaction between these factors, indicating that repellency varied across treatments depending on the exposure time. In practical terms, this means that although all compounds showed similar repellency at the beginning of the test, subtle differences became evident only after longer exposure intervals ( $\geq 240$  min). Post hoc comparisons using Tukey's test confirmed that these late differences were responsible for the significant interaction detected by the ANOVA model.

Overall, these findings highlight the prolonged and stable repellent action of the EO from *P. cyrtopodon*, particularly emphasizing cadina-1(10),4-diene as the most persistent constituent. Its performance, maintaining high repellency levels even after 270 min under laboratory conditions. Germacrene D and  $\delta$ -Elemene also contributed to the overall repellency of the EO, suggesting a possible contribution of combined effects among these sesquiterpenes. These findings highlight the potential of EO from *P. cyrtopodon* as a natural bioactive agent against *A. aegypti*, with sustained repellent activity under laboratory olfactometer conditions.

### **Molecular docking study**

RMSD values  $\leq 2.0$  Å indicated that the docking protocol was able to reproduce the crystallographic binding poses with acceptable accuracy. The OBP1–DEET complex (3N7H) showed an RMSD of 0.29 Å, while the OBP22–linoleic acid complex (6OGH) yielded 1.23 Å (Figure 4), supporting the suitability of the protocol for the simulations

performed. The docking results suggested differences in binding affinity and interaction patterns between the EO compounds and the reference ligands (Tables 4 and 5).

For OBP1, the co-crystallized ligand DEET displayed strong affinity (−7.6 kcal/mol;  $K_i$  = 2.69  $\mu$ M), interacting mainly through hydrophobic and aromatic contacts (Figure 5). Cadina-1(10),4-diene presented a more favorable predicted affinity (−8.4 kcal/mol;  $K_i$  = 0.69  $\mu$ M), forming alkyl,  $\pi$ -alkyl, and  $\pi$ -sigma (Figure 6). Germacrene D (−7.4 kcal/mol;  $K_i$  = 3.74  $\mu$ M) and  $\delta$ -Elemene (−6.8 kcal/mol;  $K_i$  = 10.30  $\mu$ M) showed comparatively lower predicted affinities (Figure 7 and 8).

In OBP22, the native ligand linoleic acid exhibited an affinity (−7.7 kcal/mol;  $K_i$  = 2.22  $\mu$ M), stabilized by extensive alkyl,  $\pi$ -alkyl, and hydrogen-bond interactions (Figure 9). Among the EO compounds, cadina-1(10),4-diene (−7.3 kcal/mol;  $K_i$  = 3.9  $\mu$ M), germacrene D (−7.2 kcal/mol;  $K_i$  = 5.2  $\mu$ M), and  $\delta$ -Elemene (−6.8 kcal/mol;  $K_i$  = 10.2  $\mu$ M) showed similar interaction profiles, mainly involving hydrophobic and  $\pi$ -type contacts with residues in the binding cavity (Figures 10–12). DEET also showed favorable binding (−7.9 kcal/mol;  $K_i$  = 1.6  $\mu$ M) (Figure 13).

Overall, these results suggest that cadina-1(10),4-diene may interact favorably with OBP1 and OBP22, which could be associated with the repellency observed in the behavioral assays. However, these findings should be interpreted as a hypothesis, since no experimental validation of these molecular interactions was performed.

### **Prediction of properties of ADMET**

The EO constituents exhibited physicochemical properties typical of lipophilic sesquiterpenes, including high logP values (4.53–4.96) and low aqueous solubility (logS −4.46 to −5.01), compared to DEET (logP 2.13; logS −1.44) (Table 6).

Permeability estimates based on Caco-2 and MDCK models suggested limited passive diffusion across epithelial barriers for all compounds. Plasma protein binding was higher for the natural compounds (94.40–98.40%) than for DEET (78.90%), which may influence their distribution profiles. Variations in blood–brain barrier (BBB) penetration were observed among the compounds, with lower values for germacrene D and higher values for cadina-1(10),4-diene and DEET.

In terms of toxicity, all natural compounds were predicted to cause high eye irritation (0.9–1.0). Germacrene D showed intermediate mutagenicity (0.5–0.7) and carcinogenicity (0.3–0.5), whereas cadina-1(10),4-diene and  $\delta$ -Elemene exhibited low mutagenic potential (0.1–0.3) but intermediate carcinogenicity (0.5–0.7). Skin

sensitization was high for germacrene D (0.9–1.0) and  $\delta$ -Elemene (0.7–0.9), moderate for DEET (0.5–0.7), and low for cadin-1(10),4-diene (0.3–0.5). All compounds presented low hematotoxicity (0.1–0.3). Overall, these results suggest differences in the toxicological profiles of the compounds.

### Cytotoxicity assay

At the lowest concentration (3  $\mu$ g/mL), all treatments maintained  $100 \pm 0\%$  cell viability, indicating no cytotoxic effect. In contrast, exposure to 100  $\mu$ g/mL caused marked reductions in cell viability, particularly for the EO ( $19.0 \pm 6.08\%$ ,  $19.0 \pm 1.00\%$ , and  $17.6 \pm 0.58\%$ ) and cadin-1(10),4-diene ( $19.7 \pm 0.58\%$ ,  $18.7 \pm 1.15\%$ , and  $14.3 \pm 0.58\%$ ) after 24, 48, and 72 h, respectively (Figure 14).

Conversely, germacrene D and  $\delta$ -Elemene exhibited low cytotoxicity, maintaining high cell viability even at the highest concentration tested. For germacrene D, mean values were  $90.7 \pm 1.53\%$ ,  $85.0 \pm 4.58\%$ , and  $86.7 \pm 2.08\%$ , while for  $\delta$ -Elemene, viability remained at  $89.0 \pm 1.73\%$ ,  $88.7 \pm 3.21\%$ , and  $86.0 \pm 2.00\%$  at the same exposure periods (Figure 15).

Two-way ANOVA revealed highly significant effects of both concentration and exposure time ( $p < 0.0001$ ) for the EO and cadin-1(10),4-diene, with a significant interaction between the factors and the concentration accounting for over 88% of total variation. For germacrene D, the ANOVA also showed significance for concentration and interaction, whereas for  $\delta$ -Elemene, only concentration had a significant effect, with no isolated influence of time.

To further confirm these findings, cytotoxicity was also assessed in human PBMCs after 24 h of exposure to the same compounds and concentrations. As observed on Vero cells, all treatments maintained  $100 \pm 0\%$  viability at 3  $\mu$ g/mL. However, at 100  $\mu$ g/mL, the EO ( $66.3 \pm 0.58\%$ ) and cadin-1(10),4-diene ( $51.7 \pm 3.79\%$ ) induced significant reductions in PBMC viability, while germacrene D ( $92.7 \pm 0.58\%$ ) and  $\delta$ -Elemene ( $91 \pm 1\%$ ) showed minimal cytotoxic effects, maintaining viability above 90% (Figure 16). Two-way ANOVA confirmed highly significant effects of concentration and compound ( $p < 0.0001$ ), with concentration explaining over 90% of total variation.

The DMSO control showed low viability ( $<10\%$ ), confirming the sensitivity and reliability of the assays, whereas untreated cells retained 100% viability throughout all exposure periods. Altogether, these results demonstrate that the EO from *P. cyrtopodon* and cadin-1(10),4-diene exert cytotoxic effects only at higher concentrations, while

germacrene D and  $\delta$ -Elemene remain largely non-cytotoxic, supporting their potential use as biocompatible and selective agents in future pharmacological or toxicological applications.

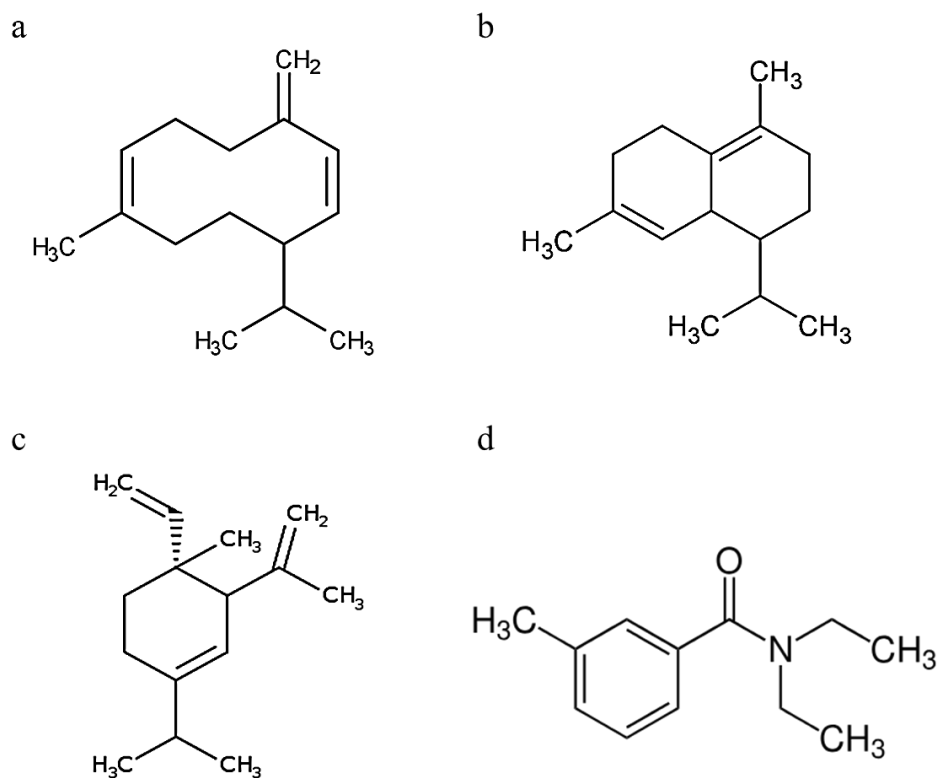


**Figure 1.** Chemical structures of the compounds investigated in this study (a) germacrene D, (b) cadin-1(10),4-diene, (c)  $\delta$ -Elemene, and (d) N,N-diethyl-m-tolamide (DEET).

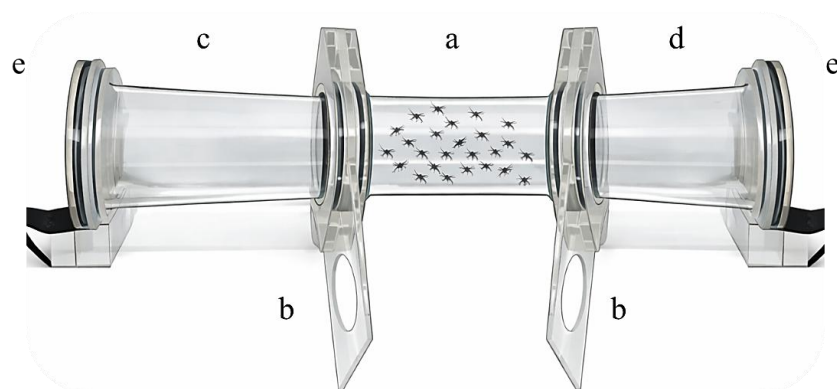


**Figure 2.** Olfactometer used in the repellency assays: (a) central chamber for mosquito acclimatization; (b) connectors with sliding gates; (c) treatment cylinder; (d) control cylinder; and (e) terminal compartments of the lateral cylinders for the placement of filter papers containing the test solutions and their respective covers.

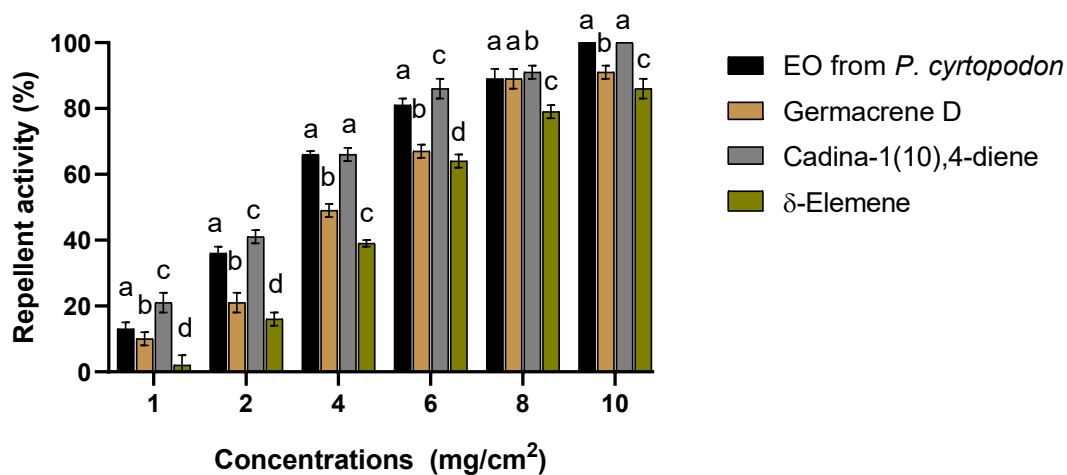


**Figure 3.** Repellent activity of the essential oil from *P. cyrtopodon*, major compounds, and DEET against female *A. aegypti*. Bars with different letters (a – d) indicate statistically significant differences ( $F(5, 2376) = 89724, p < 0.0001$ ). Data are expressed as mean  $\pm$  standard deviation.

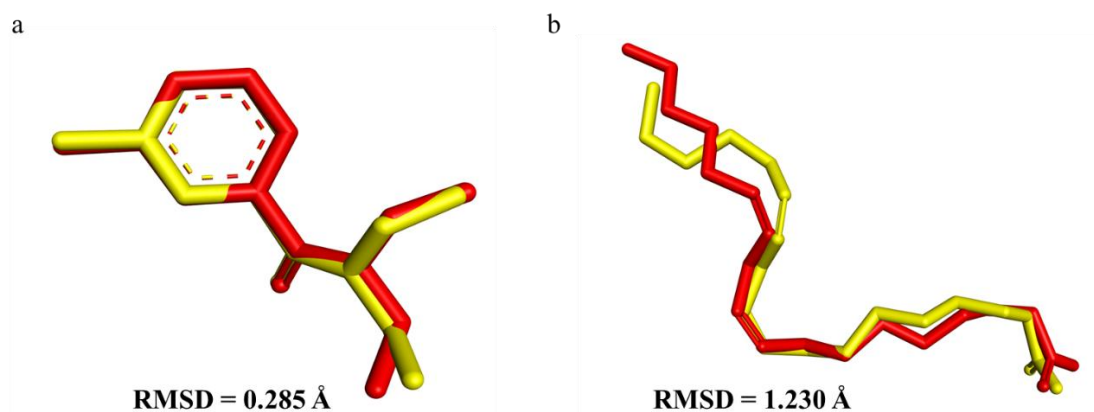


**Figure 4.** Superposition of crystallographic ligand conformations (red) and docking poses (yellow). (a) DEET bound to OBP1 (PDB ID: 3N7H); (b) linoleic acid bound to OBP22 (PDB ID: 6OGH). The high degree of overlap between the structures indicates good agreement between the experimental and computational binding conformations.

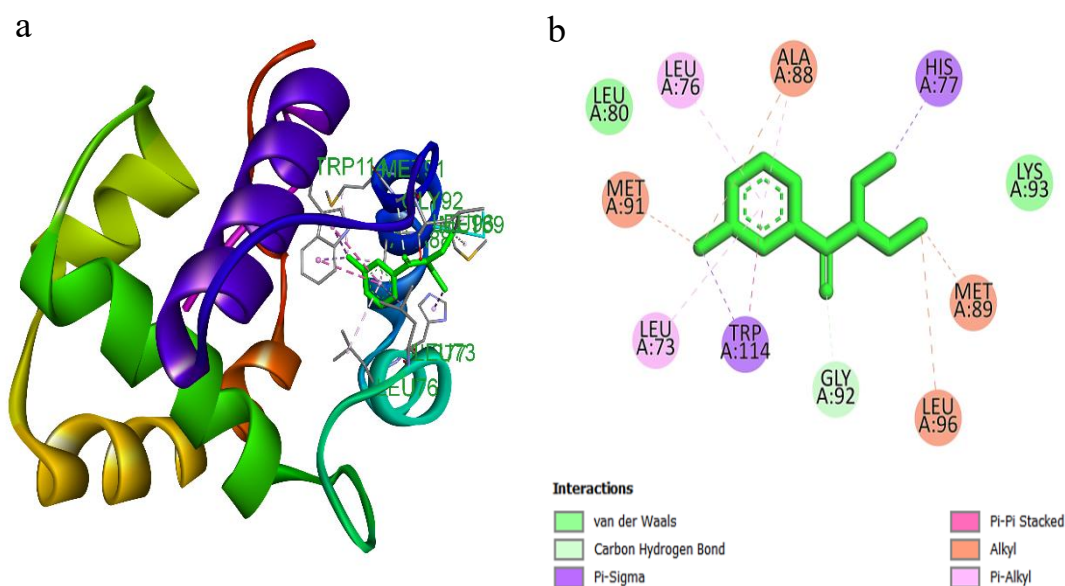


**Figure 5.** 3D (a) and 2D (b) representations of the interactions between DEET and OBP1 (PDB ID: 3N7H).

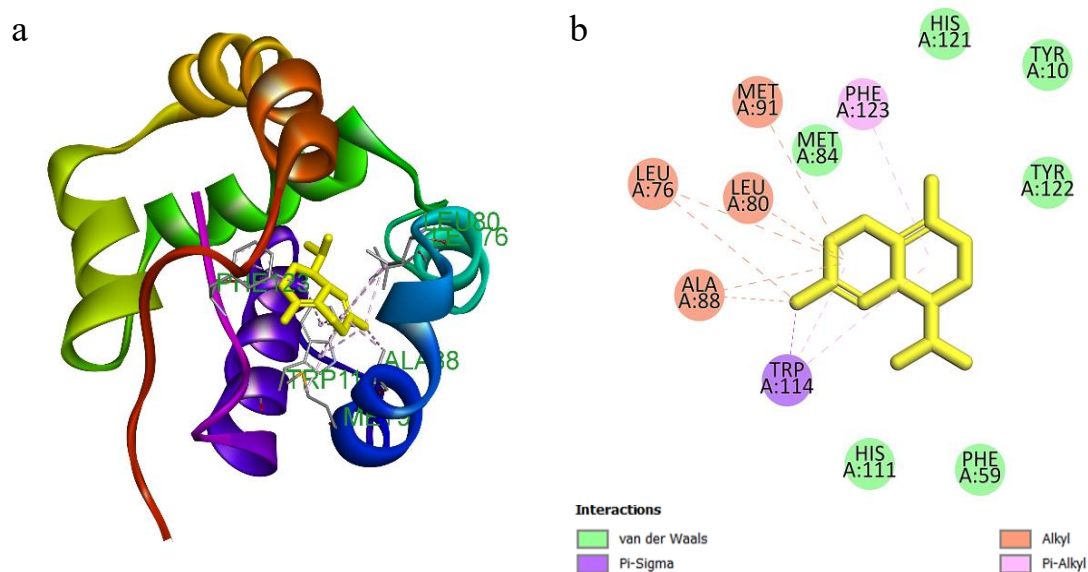


**Figure 6.** 3D (a) and 2D (b) representations of the interactions between cadina-1(10),4-diene and OBP1 (PDB ID: 3N7H).

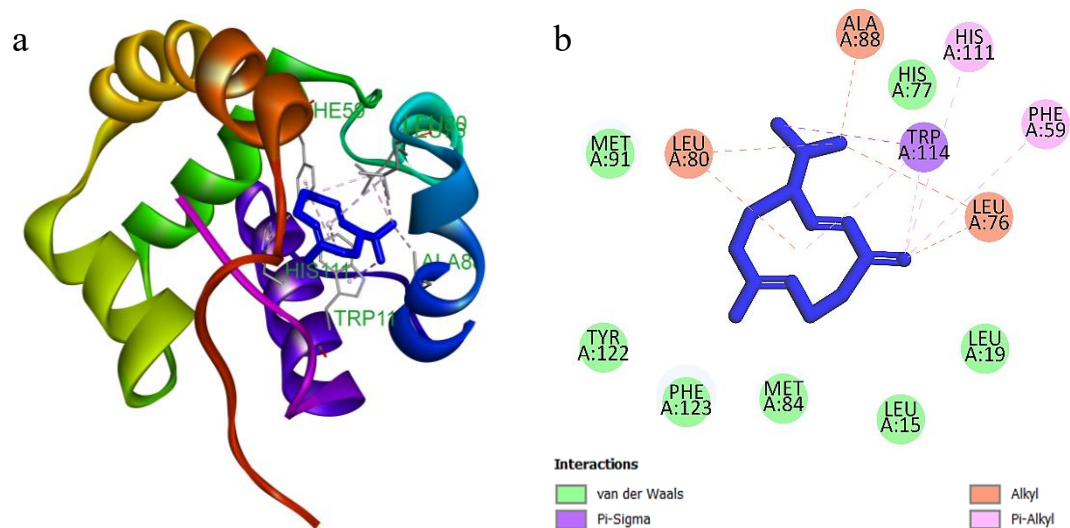


**Figure 7.** 3D (a) and 2D (b) representations of the interactions between germacrene D and OBP1 (PDB ID: 3N7H).



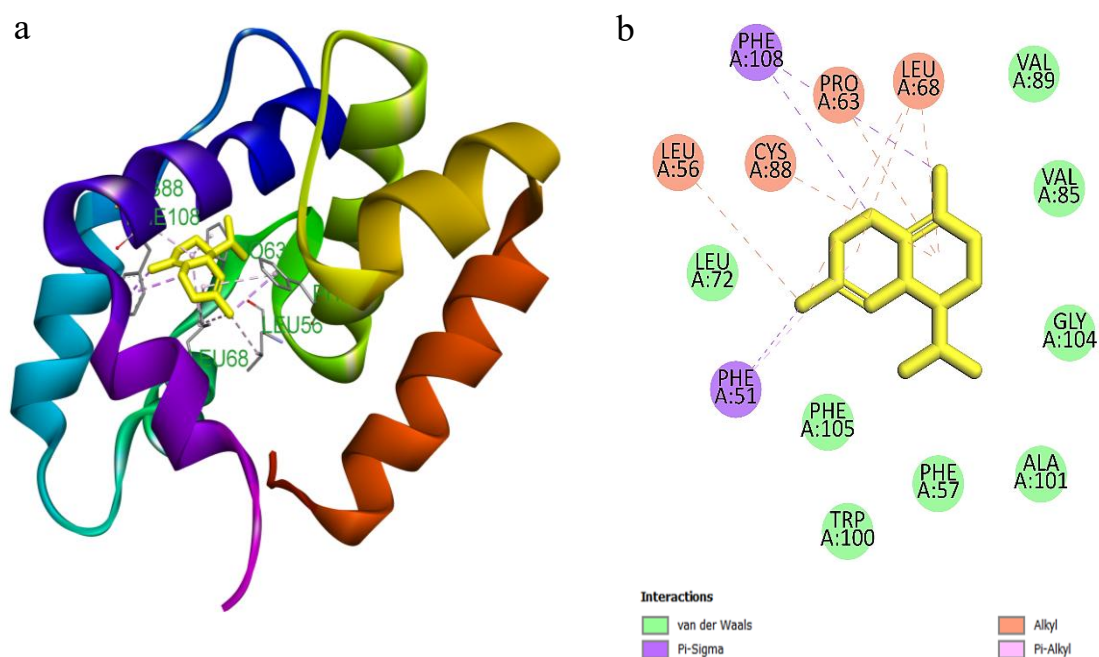


**Figure 10.** 3D (a) and 2D (b) representations of the interactions between cadina-1(10),4-diene and OBP22 (PDB ID: 6OGH).

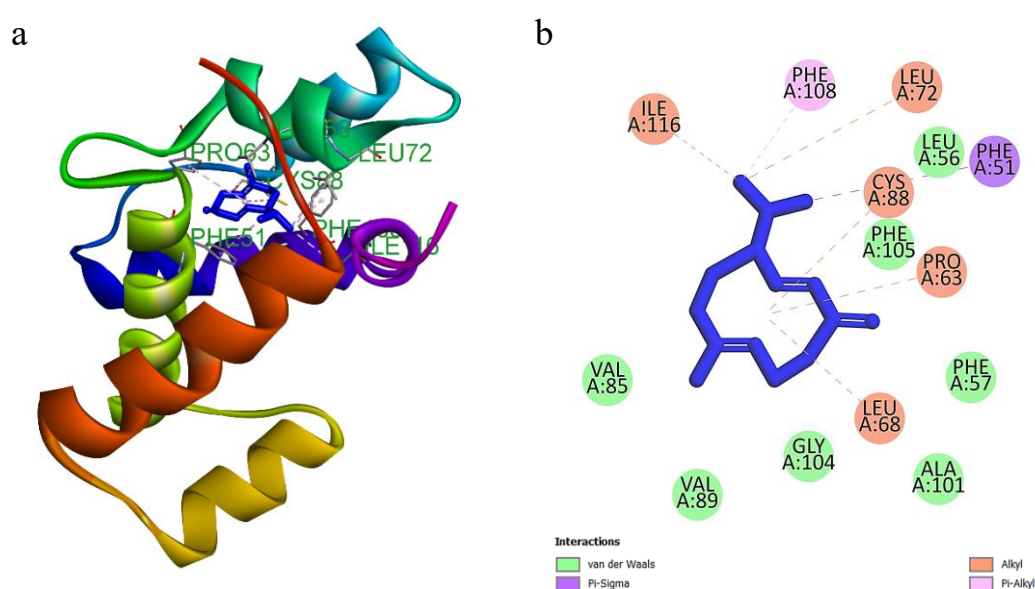


**Figure 11.** 3D (a) and 2D (b) representations of the interactions between germacrene D and OBP22 (PDB ID: 6OGH).

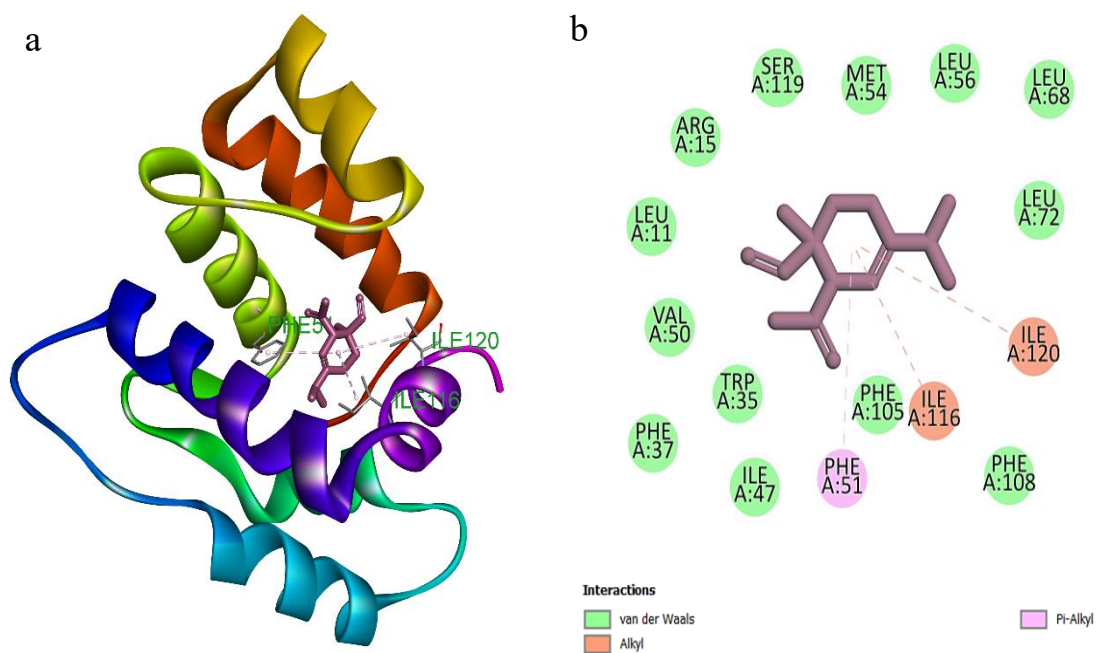


**Figure 12.** 3D (a) and 2D (b) representations of the interactions between  $\delta$ -Elemene binding to OBP22 (PDB ID: 6OGH).

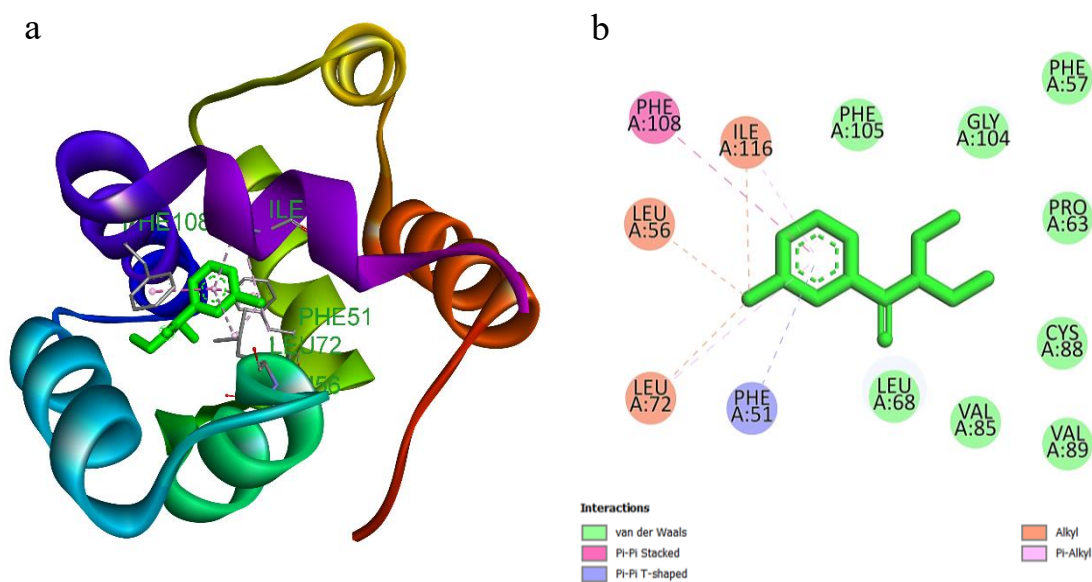


**Figure 13.** 3D (a) and 2D (b) representations of DEET binding to OBP22 (PDB ID: 6OGH).

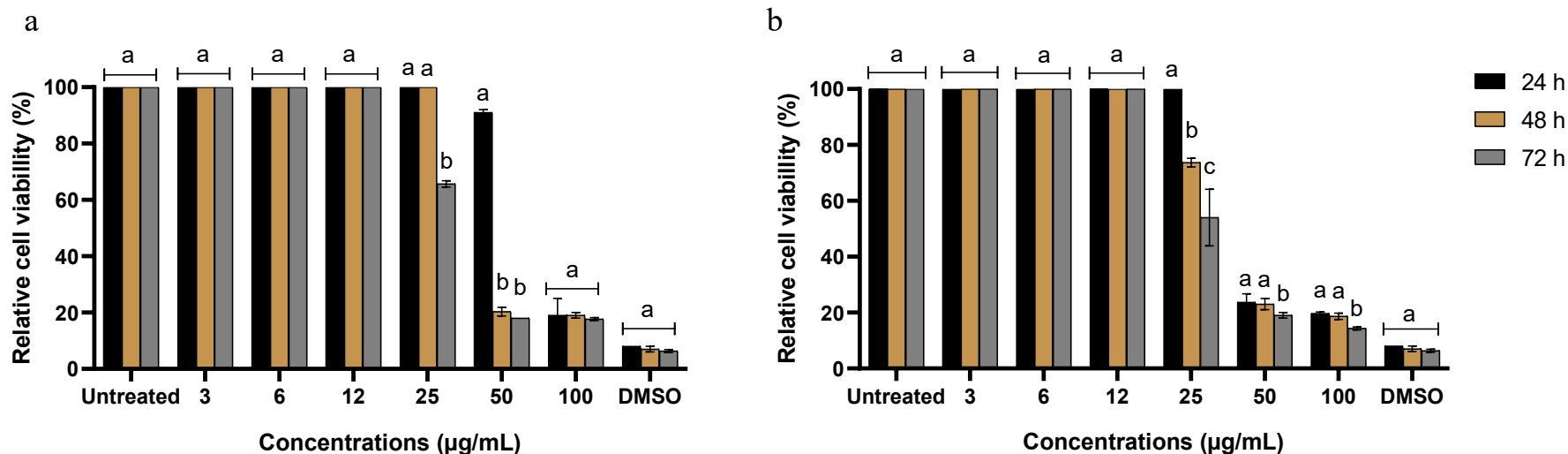


**Figure 14.** Cytotoxic effect of the essential oil from *P. cyrtopodon* (a) and cadina-1(10),4-diene (b) on Vero cells after 24, 48, and 72 h of exposure at different concentrations (0 – 100 µg/mL), compared with the positive control (DMSO at 100%). Results are expressed as mean ± standard deviation ( $n = 3$ ). Different letters (a – c) indicate statistically significant differences among time of exposure ( $F(7, 48) = 7843$ ,  $p < 0.0001$  for essential oil;  $F(7, 48) = 3130$ ,  $p < 0.0001$  for cadina-1(10),4-diene).

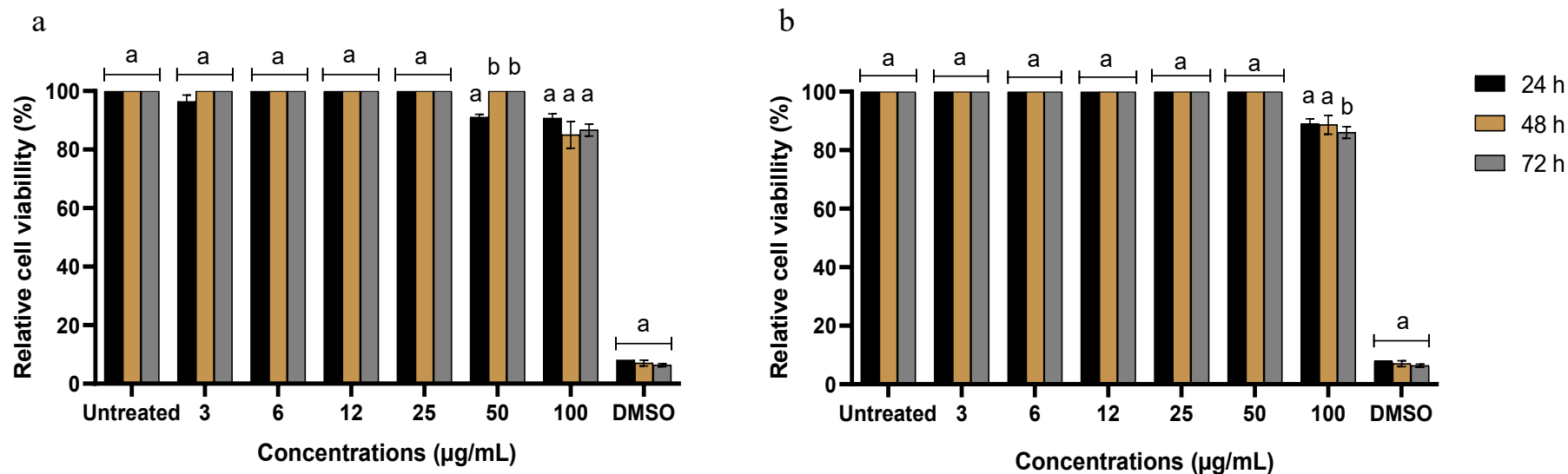


**Figure 15.** Cytotoxic effect of the germacrene D (a) and  $\delta$ -Elementene (b) on Vero cells after 24, 48, and 72 h of exposure at different concentrations (0 – 100  $\mu\text{g/mL}$ ), compared with the positive control (DMSO at 100%). Results are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Different letters (a – b) indicate statistically significant differences among time of exposure ( $F(7, 48) = 6369, p < 0.0001$  for germacrene D;  $F(7, 48) = 12228, p < 0.0001$  for  $\delta$ -elementene).

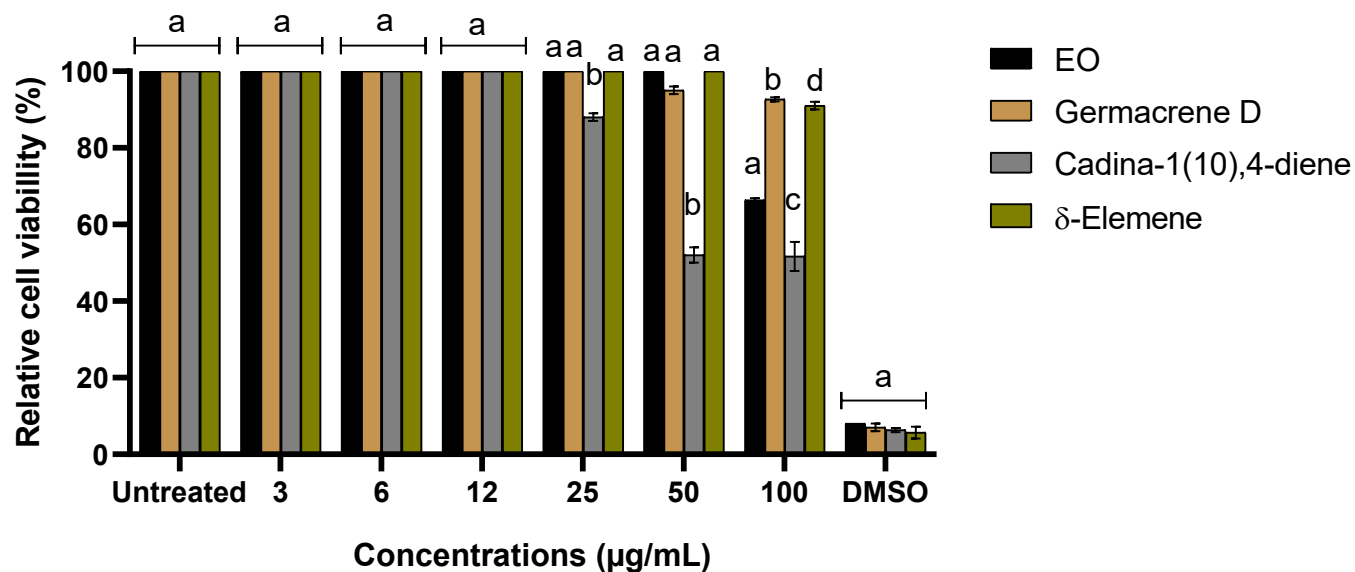


**Figure 16.** Cytotoxic effect of the essential oil from *P. cyrtopodon*, germacrene D, cadina-1(10),4-diene, and  $\delta$ -Elemene on PBMCs cells after 24 h of exposure at different concentrations (0 – 100  $\mu\text{g/mL}$ ), compared with the positive control (DMSO). Results are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Different letters (a – d) indicate statistically significant differences among time of exposure ( $F(7, 64) = 15468, p < 0.0001$ ).

**Table 1.** Grid box information for the selection of the active pockets of the four odorant binding proteins.

| Structure | Compoud              | Center   |          |          | Dimension |        |        |
|-----------|----------------------|----------|----------|----------|-----------|--------|--------|
|           |                      | Center_x | Center_y | Center_z | Size_x    | Size_y | Size_z |
| 3N7H      | DEET*                | 12.289   | 3.068    | 18.372   | 40        | 40     | 40     |
|           | Germacrene D         | 0.657    | -2.090   | 5.656    | 40        | 40     | 40     |
|           | Cadina-1(10),4-diene | 0.600    | -2.125   | 5.724    | 40        | 40     | 40     |
|           | $\delta$ -Elemene    | 0.692    | -2.106   | 5.675    | 40        | 40     | 40     |
| 6OGH      | EIC**                | 7.526    | 18.695   | 12.732   | 40        | 40     | 40     |
|           | DEET                 | 9.794    | 4.275    | 12.298   | 40        | 40     | 40     |
|           | Germacrene D         | 2.392    | 2.806    | 6.837    | 40        | 40     | 40     |
|           | Cadina-1(10),4-diene | 2.811    | 4.667    | 6.558    | 40        | 40     | 40     |
|           | $\delta$ -Elemene    | 2.727    | 2.963    | 7.164    | 40        | 40     | 40     |

\*N,N-diethyl-meta-toluamide. \*\*linoleic acid.

**Table 2.** EC<sub>50</sub> and EC<sub>90</sub> of essential oil from *P. cyrtopodon* and main compounds against *A. aegypti* in comparison with DEET.

| Sample               | EC <sub>50</sub> (mg/cm <sup>2</sup> ) (LCL-UCL) | EC <sub>90</sub> (mg/cm <sup>2</sup> ) (LCL-UCL) | $\chi^2$ (Df) | <i>p</i> -value | Linear equation   |
|----------------------|--------------------------------------------------|--------------------------------------------------|---------------|-----------------|-------------------|
| EO                   | 2.68 (2.400 – 2.971) <sup>a</sup>                | 7.55 (6.620 – 8.870) <sup>a</sup>                | 7.473 (4)     | 0.113           | Y = -1.223+2.852x |
| Germacrene D         | 3.85 (3.458 – 4.239) <sup>b</sup>                | 9.81 (8.598 – 11.689) <sup>ab</sup>              | 4.299 (3)     | 0.231           | Y = -1.850+3.157x |
| Cadina-1(10),4-diene | 2.31 (2.045 – 2.592) <sup>a</sup>                | 7.11 (6.182 – 8.467) <sup>a</sup>                | 9.062 (4)     | 0.060           | Y = -0.961+2.630x |
| $\delta$ -Elemene    | 4.54 (4.143 – 4.967) <sup>b</sup>                | 11.85 (10.304 – 14.178) <sup>b</sup>             | 1.379 (4)     | 0.848           | Y = -2.021+3.075x |
| DEET                 | 1                                                | -                                                | -             | -               | -                 |

EC<sub>50</sub> and EC<sub>90</sub> refer to the concentrations required to repel 50% and 90% of mosquitoes, respectively. LCL and UCL indicate the lower and upper 95% confidence limits. Different letters (a – b) within the same column indicate statistically significant differences among the EC<sub>50</sub> values ( $F(3, 8) = 26.75$ ,  $p = 0.0002$ ), as well as among the EC<sub>90</sub> values ( $F(3, 8) = 6.917$ ,  $p = 0.0130$ ). The *p*-value obtained for  $\chi^2$  was not significant and corresponds to Pearson's goodness-of-fit test, with Df representing the degrees of freedom. The negative control acetone (1 to 10 mg/cm<sup>2</sup>), did not show repellent activity, while the positive control, DEET (1 mg/cm<sup>2</sup>), containing 7.125% active ingredient according to the manufacturer's information, resulted in 100% repellency.

**Table 3.** Cumulative repellency percentage of *A. aegypti* females over the protection period, according to the effective repellent concentration of the essential oil and major compounds from *P. cyrtopodon* leaves, in comparison with DEET.

| Time      | Treatments. Cumulative repellency (%) $\pm$ SE. |                                |                              |                                |                                | Value F (DFn, DFd) | p-value |
|-----------|-------------------------------------------------|--------------------------------|------------------------------|--------------------------------|--------------------------------|--------------------|---------|
| (Minutes) | EO                                              | Germacrene D                   | Cadina-1(10),4-diene         | $\delta$ -Elemene              | DEET                           |                    |         |
| 30        | 100% $\pm$ 0.00 <sup>a</sup>                    | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup> | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup>   | 25.90 (32, 2655)*  | 0.0001  |
| 60        | 100% $\pm$ 0.00 <sup>a</sup>                    | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup> | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup>   | 189.20 (8, 2655)** | 0.0001  |
| 90        | 100% $\pm$ 0.00 <sup>a</sup>                    | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup> | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup>   | 45.75 (4, 2655)*** | 0.0001  |
| 120       | 100% $\pm$ 0.00 <sup>a</sup>                    | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup> | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup>   |                    |         |
| 150       | 100% $\pm$ 0.00 <sup>a</sup>                    | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup> | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup>   |                    |         |
| 180       | 100% $\pm$ 0.00 <sup>a</sup>                    | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup> | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup>   |                    |         |
| 210       | 100% $\pm$ 0.00 <sup>a</sup>                    | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup> | 100% $\pm$ 0.00 <sup>a</sup>   | 100% $\pm$ 0.00 <sup>a</sup>   |                    |         |
| 240       | 95.00% $\pm$ 2.89 <sup>a</sup>                  | 100% $\pm$ 0.00 <sup>b</sup>   | 100% $\pm$ 0.00 <sup>b</sup> | 100% $\pm$ 0.00 <sup>b</sup>   | 100% $\pm$ 0.00 <sup>b</sup>   |                    |         |
| 270       | 93.33% $\pm$ 4.41 <sup>a</sup>                  | 95.00% $\pm$ 2.89 <sup>b</sup> | 100% $\pm$ 0.00 <sup>c</sup> | 96.66% $\pm$ 1.67 <sup>d</sup> | 95.66% $\pm$ 0.00 <sup>b</sup> |                    |         |

Values are expressed as mean  $\pm$  standard deviation. Identical letters in the same row indicate no significant difference according to Tukey's post hoc test ( $p > 0.05$ ). DF = degrees of freedom. F value = ANOVA F statistic, with numerator (DFn) and denominator (DFd) degrees of freedom. \*Time factor = effect of the row variable; \*\*Sample factor = effect of the column variable; \*\*\*Interaction = combined effect of both factors. In this analysis, the interaction between time and sample factors was highly significant ( $p < 0.0001$ ), indicating that the effect of one factor depends on the level of the other. Both main effects (time and sample factors) were also statistically significant ( $p < 0.0001$ ).

**Table 4.** Comparative inhibitory potential of the major compounds of the essential oil from *P. cyrtopodon* against OBP1 (PDB ID: 3N7H) in comparison with DEET.

| Compound             | Interacting residues (distance Å)                                                                                                   | Type of Interaction                                                 | Binding energy (kcal/mol) | Inhibition constant (Ki $\mu$ M) |
|----------------------|-------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------|---------------------------|----------------------------------|
| DEET*                | ALA88 (3.99; 4.05), LEU96 (5.25), MET91 (4.31), LEU73 (5.40), LEU76 (4.66), ALA88 (4.05), HIS77 (3.67), TRP114 (4.09), GLY92 (3.52) | Alkyl (4x), Pi-Alkyl (3x), Pi-Sigma (2x), Carbon Hydrogen Bond (1x) | −7.6                      | 2.69                             |
| Germacrene D         | LEU80 (4.84; 4.92), ALA88 (3.77), LEU76 (4.01; 4.54), TRP114 (3.71; 4.98; 5.12), PHE59 (4.72), HIS111 (5.09)                        | Alkyl (5x), Pi-alkyl (4x), Pi-Sigma (1x)                            | −7.4                      | 3.74                             |
| Cadina-1(10),4-diene | LEU80 (4.80), MET91 (5.16), LEU76 (4.39; 5.30), ALA88 (3.97; 4.30), TRP114 (3.87; 5.24, 6.06), PHE123 (5.12)                        | Alkyl (6x), Pi-alkyl (3x), Pi-sigma (1x)                            | −8.4                      | 0.69                             |
| $\delta$ -Elemene    | LEU96 (5.32), MET89 (4.78), LEU73 (4.34), LEU76 (4.97), HIS77 (5.04)                                                                | Alkyl (4x), Pi-alkyl (1x)                                           | −6.8                      | 10.30                            |

\*N,N-diethyl-meta-toluamide.

**Table 5.** Comparative inhibitory potential of the major compounds of the essential oil from *P. cyrtopodon* against OBP22 (PDB ID: 6OGH), in comparison with DEET.

| Compound             | Interacting residues (distance Å)                                                                                                                                                                  | Type of Interaction                                             | Binding energy (kcal/mol) | Inhibition constant (Ki $\mu$ M) |
|----------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|---------------------------|----------------------------------|
| EIC*                 | VAL50 (5.10), ILE47 (5.37), MET54 (4.83), ILE126 (4.06), LEU72 (5.12), LEU68 (4.94), PHE37 (5.06), PHE108 (5.13), PHE105 (5.01), PHE51 (4.99; 5.07; 5.15; 5.49), TRP35 (5.18; 5.24), GLY104 (3.32) | Alkyl (6x), Pi-Alkyl (9x), Conventional Hydrogen Bond (1x)      | -7.7                      | 2.22                             |
| Germacrene D         | ILE116 (4.08), LEU72 (4.87), CYS88 (5.33), PRO63 (5.08), LEU68 (4.77), PHE108 (4.45), PHE51 (3.93)                                                                                                 | Alkyl (5x), Pi-Alkyl (1x), Pi-Sigma (1x)                        | -7.2                      | 5.24                             |
| Cadina-1(10),4-diene | LEU56 (4.47), CYS88 (5.39), PRO63 (5.10), LEU68 (4.56; 4.58; 4.90), PHE51 (3.90; 4.96), PHE108 (3.77, 3.78)                                                                                        | Alkyl (3x), Pi-Alkyl (1x), Pi-Sigma (3x)                        | -7.3                      | 3.90                             |
| $\delta$ -Elemene    | ILE116 (4.79), ILE120 (5.25), PHE51 (5.34)                                                                                                                                                         | Alkyl (2x), Pi-Alkyl (1x)                                       | -6.8                      | 10.20                            |
| DEET**               | LEU56 (5.29), LEU72 (4.17; 5.36), ILE116 (4.71; 4.67), PHE51 (5.15), PHE108 (4.87)                                                                                                                 | Alkyl (3x), Pi-Alkyl (2x), Pi-Pi T-shaped (1x), Pi-Stacked (1x) | -7.9                      | 1.59                             |

\*linoleic acid. \*\*N,N-diethyl-meta-toluamide.

**Table 6.** Predicted ADMET parameters of the major compounds of the essential oil from *P. cyrtopodon* and the synthetic repellent DEET.

| Properties                         | Compound         |                      |                   |                  |
|------------------------------------|------------------|----------------------|-------------------|------------------|
|                                    | Germacrene D     | Cadina-1(10),4-diene | $\delta$ -Elemene | DEET             |
| <b>Physicochemical</b>             |                  |                      |                   |                  |
| Molecular weight (g/mol)           | 204.19           | 204.19               | 204.19            | 191.13           |
| logP                               | 4.533            | 4.957                | 4.589             | 2.128            |
| logS                               | -4.465           | -5.013               | -4.51             | -1.441           |
| TPSA ( $\text{\AA}^2$ )            | 0.0              | 0.0                  | 0.0               | 20.31            |
| <b>Absorption and Distribution</b> |                  |                      |                   |                  |
| Caco-2 Permeability                | -4.626           | -4.816               | -4.791            | -4.38            |
| MDCK Permeability                  | -4.655           | -4.443               | -4.766            | -4.693           |
| PPB (%)                            | 98.428           | 94.373               | 97.762            | 78.875           |
| BBB penetration                    | 0.001            | 0.799                | 0.816             | 0.997            |
| <b>Toxicity</b>                    |                  |                      |                   |                  |
| AMES mutagenicity                  | Intermediary (+) | Low (--)             | Low (--)          | Low (--)         |
| Carcinogenicity                    | Intermediary (-) | Intermediary (+)     | Intermediary (+)  | Intermediary (-) |
| Skin sensitization                 | High (+++)       | Intermediary (-)     | High (++)         | Intermediary (+) |
| Eye irritation                     | High (+++)       | High (+++)           | High (+++)        | High (+++)       |
| Hematotoxicity                     | Low (--)         | Intermediary (-)     | Low (--)          | Low (--)         |

For classification endpoints, prediction probability values were converted into six symbols: 0–0.1 (---), 0.1–0.3 (--), 0.3–0.5 (-), 0.5–0.7 (+), 0.7–0.9 (++), and 0.9–1.0 (+++).

## Discussion

In the present study, the EO from *P. cyrtopodon* and its major compounds cadina-1(10),4-diene, germacrene D, and  $\delta$ -Elemene exhibited dose-dependent repellency against *A. aegypti* under laboratory conditions. The overall repellent efficacy of the EO is likely the result of synergistic interactions among volatile sesquiterpenes that modulate mosquito olfactory perception [38,39].

From a chemical standpoint, these sesquiterpenes contribute to repellency through distinct structural and physicochemical features [40]. Germacrene D, a monocyclic sesquiterpene hydrocarbon, exhibits moderate volatility and high lipophilicity, favoring its diffusion and interaction with mosquito odorant receptors, potentially masking host odors [41]. Cadina-1(10),4-diene, with its cadinene skeleton and conjugated double bonds, is both hydrophobic and volatile, facilitating the formation of a persistent olfactory barrier that interferes with receptor activation [42].  $\delta$ -Elemene, a highly lipophilic hydrocarbon, combines rapid volatilization with prolonged surface adherence, enhancing repellent persistence on skin or clothing [43].

Together, these sesquiterpenes create a multifaceted defense mechanism. Volatility enables formation of a vapor-phase protective barrier, while lipophilicity enhances adherence and sustained release [44,45]. This integrated chemical behavior mirrors, in part, the mechanism of DEET, which interferes with the binding of human volatiles to mosquito OBPs [11]. However, unlike DEET, known for potential dermal absorption, environmental persistence, and neurotoxic concerns of the EO from *P. cyrtopodon* offers a natural alternative with lower toxicological risk and improved environmental compatibility [46,47].

Comparatively, other plant-derived EOs have shown repellent activity against *A. aegypti*, although with different potency and persistence profiles. For instance, [28] reported that the EO from *Croton tetradenius* Baill. (Euphorbiaceae) exhibited EC<sub>50</sub> (14.1 mg/cm<sup>2</sup>) and EC<sub>90</sub> (163.4 mg/cm<sup>2</sup>), indicating lower potency than observed for *P. cyrtopodon*.

Similarly, EOs from *Erigeron canadensis* L. (Asteraceae), *Mentha spicata* L. (Lamiaceae), and *Parthenium hysterophorus* L. (Asteraceae) reached repellency levels above 50% at 33  $\mu$ g/cm<sup>2</sup>, but protection dropped significantly after only 45 min [48]. In contrast, the EO from *P. cyrtopodon* maintained high repellency levels for up to 270 min

under laboratory conditions, with performance comparable to the reference compound DEET within the experimental setup.

Considering these findings, the repellency observed for the EO from *P. cyrtopodon* may be associated with its chemical composition and possible interactions with mosquito olfactory mechanisms [49]. In this context, molecular docking analyses suggested that cadina-1(10),4-diene, germacrene D, and  $\delta$ -Elemene may interact with OBP1 and OBP22 of *A. aegypti*, likely interfering with odor recognition [50]. By occupying hydrophobic pockets within these proteins, the sesquiterpenes may hinder the recognition of human volatiles and impair olfactory signal transduction essential for mosquito orientation [11,12].

ADMET predictions additional *in silico* information on physicochemical and toxicological properties. The sesquiterpenes exhibited high lipophilicity, low aqueous solubility, and limited hydrogen-bonding capacity, characteristics that favor skin adherence, controlled volatilization, and reduced systemic absorption [10].

Such properties enable slow, sustained release of active volatiles, maintaining an effective vapor barrier over time [19]. Additionally, their predicted low central nervous system permeability minimizes systemic toxicity concerns, reinforcing their suitability for topical application. Achieving this balance between persistence and safety is particularly challenging in repellent formulation design [47].

Cytotoxicity evaluations further support the favorable safety profile of the EO from *P. cyrtopodon*. At concentrations relevant to practical application, the EO and its major constituents maintained high cell viability in both Vero and PBMC assays. The mild cytotoxicity observed only at the highest doses suggests that these compounds act selectively on mosquito olfactory targets rather than through general cytotoxic mechanisms [51, 52].

$\delta$ -Elemene and germacrene D, in particular, may indicate excellent biocompatibility, reinforcing their potential as safe active ingredients. These findings align with reports on other *Piper* species, such as *P. corcovadense* (Miq.) C.DC., whose sesquiterpene-rich EO did not significantly reduce PBMC viability [53], and *P. callosum* Ruiz & Pav., whose EO also exhibited no cytotoxicity in VERO cells [54, 55].

Collectively, these findings highlight the potential of EO from *P. cyrtopodon* as a source of natural compounds with repellent activity, particularly within the context of behavioral assays under laboratory conditions. These results may contribute to the

development of alternative strategies for mosquito control, especially in dengue-endemic regions.

## Conclusion

This study shows that the EO from *P. cyrtopodon* and its major constituents exhibit repellency against *A. aegypti* under laboratory conditions. The integration of chemical characterization, behavioral assays, and *in silico* analyses provides preliminary insights into the potential mechanisms involved.

These findings highlight the relevance of Amazonian plant species as sources of bioactive compounds with repellent activity. Further studies are required to validate these effects under field conditions and to support the development of formulations suitable for application to human skin.

## Ethics Approval

Peripheral blood samples were collected from ten healthy donors after written informed consent was obtained, in accordance with the guidelines of the local Ethics Committee (approval number: 4.982.395; approval date: 17 September 2021).

## Acknowledgements

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## 5. CONSIDERAÇÕES FINAIS

A composição química do óleo evidenciou a predominância de sesquiterpenos, com destaque para germacreno D (57,46%), cadina-1(10),4-dieno (10,57%) e  $\delta$ -Elemeno (8,0%), associados às atividades biológicas observadas. O óleo e seus constituintes majoritários apresentaram significativa atividade larvica, com  $CL_{50}$  entre 20,78 e 146,61  $\mu\text{g/mL}$ . O cadina-1(10),4-dieno foi o constituinte mais ativo, com efeito residual de 100% por até dois dias, comparável ao inseticida  $\alpha$ -Cipermetrina.

Além disso, foi observada expressiva atividade repelente, com valores de  $CE_{50}$  entre 2,31 e 4,54  $\text{mg/cm}^2$  e proteção de 100% por até 210 minutos, comparável ao repelente comercial DEET, evidenciando a eficácia do óleo e de seus constituintes também sobre fêmeas adultas do vetor.

No que se refere aos mecanismos de ação, os resultados indicam que a atividade larvica está associada à indução de estresse oxidativo e à alteração na atividade das enzimas antioxidantes (SOD, CAT e GPx), enquanto o efeito repelente está relacionado à interação dos constituintes com proteínas do sistema olfatório do mosquito, como OBP1 e OBP22. Os estudos de *docking* molecular demonstraram afinidade de ligação compatível com a atividade repelente observada experimentalmente, evidenciando uma correlação entre as abordagens *in silico* e experimental.

Quanto ao perfil de segurança, o óleo e seus constituintes apresentaram baixa citotoxicidade, com manutenção da viabilidade celular até 25  $\mu\text{g/mL}$ , sendo observadas reduções apenas nas maiores concentrações, especialmente para o óleo essencial e o cadina-1(10),4-dieno. Adicionalmente, as predições de ADMET indicaram propriedades farmacocinéticas e toxicológicas compatíveis com o uso tópico, com destaque para a alta lipofilicidade e baixa polaridade dos constituintes, características que favorecem a retenção cutânea e reforçam seu potencial como agentes repelentes.

De forma integrada, os achados demonstram que o óleo essencial de *P. cyrtopodon* apresenta ação multifacetada sobre o vetor, associando toxicidade larval e efeito repelente. A concordância entre os resultados experimentais e *in silico* reforça a robustez dos mecanismos propostos e evidencia o potencial do óleo e de seus constituintes como agentes promissores para o controle de *A. aegypti*. Além disso, os resultados destacam o valor da biodiversidade amazônica como fonte de compostos bioativos e indicam que o desenvolvimento de formulações e a validação em condições de campo são etapas essenciais para sua aplicação prática.

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# ANEXO 1 – Artigo publicado (Capítulo 1)

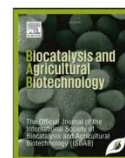
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## Larvicidal activity, oxidative stress, and enzymatic alterations induced by essential oil from *Piper cyrtopodon* (Miq.) C.DC (Piperaceae) on *Aedes aegypti* (Culicidae)

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### ARTICLE INFO

#### Keywords:

*Piper*  
Sesquiterpenes  
Oxidative stress  
Detoxification enzymes

### ABSTRACT

Dengue, Zika, and chikungunya are mosquito-borne diseases transmitted by *Aedes aegypti*, with over 13 million dengue cases, 431,223 chikungunya cases, and 44,242 Zika cases reported in the Americas in 2024. The resistance of *Ae. aegypti* to synthetic insecticides has driven the search for botanical alternatives, such as essential oils (EOs) from *Piper* species. This study evaluated the EO from *Piper cyrtopodon* and its main compounds as larvicides, investigating their mechanism of action. The EO, extracted by hydrodistillation (yield:  $1.4 \pm 0.2$  %), contained germacrene D (57.46 %), cadina-1(10),4-diene (10.57 %), and  $\delta$ -elemene (8.0 %). It showed significant larvicidal activity against *Ae. aegypti*, with  $LC_{50}$  values ranging from 20.78 to 146.61  $\mu\text{g/mL}$  over 24 and 48 h. Cadina-1(10),4-diene was the most active compound ( $p < 0.0001$ ). Biochemical analyses revealed oxidative stress in larvae, with increased reactive oxygen species production ( $11 \pm 2$  to  $26 \pm 2$   $\mu\text{mol H}_2\text{O}_2/\text{g}$ ), lipid peroxidation ( $0.54 \pm 0.06$  to  $2.8 \pm 0.22$   $\eta\text{mol MDA/g}$ ), and protein oxidation ( $1.62 \pm 0.04$  to  $4.54 \pm 0.51$   $\text{nmol/mg protein}$ ) compared to controls ( $p < 0.0001$ ). Antioxidant enzymes superoxide dismutase ( $10.33 \pm 3$  to  $28.33 \pm 6$   $\text{mU/mg protein}$ ), catalase ( $1.04 \pm 0.09$  to  $7.08 \pm 2.42$   $\text{mmol H}_2\text{O}_2/\text{min/mg protein}$ ), and glutathione peroxidase ( $5.40 \pm 1.37$  to  $14.67 \pm 1.52$   $\text{mmol NADPH/min/mL}$ ) were altered, suggesting redox imbalance as a toxicity mechanism. These findings highlight the potential of the EO from *P. cyrtopodon* as a natural alternative for *Ae. aegypti* control.

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## FULL PAPER OPEN ACCESS

## Molecular Insights and Repellent Efficacy of the Essential Oil and Major Compounds From *Piper cyrtopodon* (Miq.) C.DC (Piperaceae) Against *Aedes aegypti* (Linnaeus, 1762) (Culicidae)

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Keywords: cytotoxicity | DEET | mosquito repellent | *Piper* | sesquiterpenes

### ABSTRACT

Dengue, a mosquito-borne disease transmitted mainly by *Aedes aegypti*, remains a major global public health concern. In the Americas, over 13 million cases were reported in 2024 and more than 4 million in 2025, highlighting the urgent need for effective preventive strategies. Repellents are among the most practical tools to reduce mosquito-human contact. In this study, the essential oil (EO) from *Piper cyrtopodon* and cadina-1(10),4-diene showed the highest repellent efficacy ( $EC_{50} = 2.68$  and  $2.31$  mg/cm<sup>2</sup>), followed by germacrene D and  $\delta$ -Elemene ( $EC_{50} = 3.85$  and  $4.54$  mg/cm<sup>2</sup>). All treatments maintained high repellency levels for up to 270 min under laboratory conditions, showing performance comparable to the reference compound DEET within the experimental conditions. Molecular docking with the mosquito odorant-binding proteins OBP1 and OBP22 suggested favorable binding interactions with the major compounds ( $-6.8$  to  $-8.4$  kcal/mol for OBP1 and  $-6.8$  to  $-7.3$  kcal/mol for OBP22), involving hydrophobic and aromatic contacts within the binding cavity. ADMET predictions indicated high lipophilicity, low aqueous solubility, and limited systemic absorption. No significant cytotoxicity was observed in Vero and PBMC cells up to  $25$   $\mu$ g/mL for 72 h. These findings highlight the EO from *P. cyrtopodon* as a potential source of natural compounds with repellent activity, with emphasis on behavioral repellency under controlled laboratory conditions.

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## ANEXO 3 – Registro SISBIO



Ministério do Meio Ambiente - MMA  
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio  
Sistema de Autorização e Informação em Biodiversidade - SISBIO

### Registro voluntário para a coleta de material botânico, fúngico e microbiológico

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                      |                       |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------|-----------------------|
| Número: 96329-1                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Data da Emissão: 01/10/2024 12:58:53 | Validade*: 01/10/2025 |
| De acordo com o artigo 22 da Portaria ICMBio nº 748/2022, a coleta e transporte de material botânico, fúngico e microbiológico, exceto vegetais hidróbios e espécies que constem nas listas oficiais de espécies ameaçadas de extinção, dispensa autorização do Sisbio; sendo facultativa, exceto em unidade de conservação ou em caverna, a obtenção de registro voluntário para a coleta de material botânico, fúngico e microbiológico, não classificado como oficialmente ameaçado. |                                      |                       |

#### Dados do titular

|                                 |                 |
|---------------------------------|-----------------|
| Nome: MARIA LUIZA LIMA DA COSTA | CPF: [REDACTED] |
|---------------------------------|-----------------|

#### Observações e ressalvas

|   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
|---|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 | Todos os membros da equipe de pesquisa devem estar cientes das recomendações e boas práticas a serem seguidas neste momento de emergência zoonossanitária no Brasil devido à gripe aviária. Informe-se na página do CEMAVE na Internet: <a href="https://www.gov.br/icmbio/pt-br/assuntos/centros-de-pesquisa/cemave/destaques/gripe-aviaria/gripe-aviaria-1">https://www.gov.br/icmbio/pt-br/assuntos/centros-de-pesquisa/cemave/destaques/gripe-aviaria/gripe-aviaria-1</a> .                                                                                                                                                                                                                                                                 |
| 2 | Esse documento não eximirá o pesquisador da necessidade de obter outras anuências, como: I) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; II) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; III) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; IV) do Departamento Nacional da Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; V) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras. |
| 3 | Este documento não é válido para: a) coleta ou transporte de espécies que constem nas listas oficiais de espécies ameaçadas de extinção; b) recebimento ou envio de material biológico ao exterior; e c) realização de pesquisa em unidade de conservação federal ou em caverna.                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| 4 | O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| 5 | Este documento não dispensa o cumprimento da Lei nº 13.123/2015, que dispõe sobre o acesso ao patrimônio genético, sobre a proteção e o acesso ao conhecimento tradicional associado e sobre a repartição de benefícios para conservação e uso sustentável da biodiversidade.                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 6 | As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia (Decreto nº 98.830, de 15/01/90).                                                                                                                                                                                                               |
| 7 | Caso seja identificada a ocorrência de espécie exótica dentro ou no entorno de UNIDADE DE CONSERVAÇÃO FEDERAL, além de descrever no relatório de atividades, o pesquisador deve informar à equipe gestora com maior brevidade possível.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| 8 | Este documento não abrange a coleta de vegetais hidróbios, tendo em vista que o Decreto-Lei nº 221/1967 e o Art. 36 da Lei nº 9.605/1998 estabelecem a necessidade de obtenção de autorização para coleta de vegetais hidróbios para fins científicos.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |

#### Atividades

| # | Atividade                                                         | Grupo de Atividade |
|---|-------------------------------------------------------------------|--------------------|
| 1 | Coleta/transporte de material botânico, fúngico ou microbiológico | Fora de UC Federal |

#### Táxons autorizados

| # | Nível taxonômico | Táxon(s)                                                                              |
|---|------------------|---------------------------------------------------------------------------------------|
| 1 | Espécie          | Plantae > Magnoliophyta > Magnoliopsida > Piperales > Piperaceae > Piper > Cyrtopodon |

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## ANEXO 4 – Registro SisGen



**Ministério do Meio Ambiente**  
**CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO**  
**SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO**  
**Comprovante de Cadastro de Acesso**  
**Cadastro nº A357D96**

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro: **A357D96**  
Usuário: **Maria Luiza Lima da Costa**  
CPF/CNPJ: **[REDACTED]**  
Objeto do Acesso: **Patrimônio Genético**  
Finalidade do Acesso: **Pesquisa**

### **Espécie**

**Piper cyrtopodon**

Título da Atividade: **Nanoemulsão a base do óleo essencial de Piper cyrtopodon (Miq.) C.DC (Piperaceae) com ação repelente contra Aedes aegypti (Diptera: Culicidae)**

### **Equipe**

|                                          |                                                    |
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| <b>Maria Luiza Lima da Costa</b>         | <b>Universidade Federal do Amazonas</b>            |
| <b>Cláudia Patrícia da Silva Tavares</b> | <b>Instituto Nacional de Pesquisas da Amazônia</b> |
| <b>Tatiane Pereira de Souza</b>          | <b>Universidade Federal do Amazonas</b>            |
| <b>Rosemary Aparecida Roque</b>          | <b>Instituto Nacional de Pesquisas da Amazônia</b> |

Data do Cadastro: **01/10/2024 15:48:12**  
Situação do Cadastro: **Concluído**

Conselho de Gestão do Patrimônio Genético  
Situação cadastral conforme consulta ao SisGen em **15:08** de **03/07/2025**.



SISTEMA NACIONAL DE GESTÃO  
DO PATRIMÔNIO GENÉTICO  
E DO CONHECIMENTO TRADICIONAL  
ASSOCIADO - **SISGEN**