UNIVERISIDADE FEDERAL DO RIO DE JANEIRO

PÓS-GRADUAÇÃO EM BIOTECNOLOGIA VEGETAL E BIOPROCESSOS

FRANCISCO PAIVA MACHADO

Preparação de nanoemulsão de óleo essencial de *Ocotea indecora* e *Myrciaria floribunda* para o controle de *Aedes aegypti* e *Biomphalaria glabrata*

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UFRJ
UNIVERSIDADE FEDERAL
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Tese apresentada ao Programa de Pós-graduação em Biotecnologia Vegetal e Bioprocessos da Universidade Federaldo Rio de Janeiro - UFRJ, como parte dos requisitos necessários à obtenção do título de grau Doutor em Biotecnologia Vegetal e Bioprocessos.

Orientadores: Prof. Dr. Leandro Machado Rocha
Prof. Dr. Eduardo Ricci Júnior

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RESUMO

Machado, Francisco Paiva. Preparação de nanoemulsão de óleo essencial de *Ocotea indecora* e *Myrciaria floribunda* para o controle de *Aedes aegypti* e *Biomphalaria glabrata*. Rio de Janeiro, 2023. Tese de doutoramento do Programa de Pós-Graduação em Biotecnologia, Vegetal e Bioprocessos, Decania do Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro.

As Doenças negligenciadas (DNs) são doenças transmitidas por agentes etiológicos de regiões climáticas tropicais e subtropicais. São endêmicas em diversos países nos continentes da Ásia, África e América Latina e afetam desproporcionalmente populações que vivem abaixo da linha da pobreza. Em 5 anos (2015-2019) afetaram mais de 1 bilhão de pessoas em 149 países implicando grandes perdas econômicas e sociais. Por este motivo, a Organização Mundial da Saúde (OMS) estimula pesquisa e desenvolvimento de estratégias e produtos para o controle e erradicação das DNs, dentre elas o controle do *Aedes aegypti,* vetor transmissor de diversas arboviroses, como Zika, Dengue e Chikungunya e do caramujo *Biomphalaria glabrata*, hospedeiro intermediário da esquistossomose. Entretanto, ambos mosquitos e caramujos estão se tornando resistentes aos pesticidas tradicionalmente utilizados no controle. Em 2022 foram registrados 1.450.270 casos prováveis de dengue resultando 1016 óbitos e 2.517 óbitos por esquistossomose no Brasil, sendo imperativo o controle das doenças no país. Desta forma, o presente estudo objetivou preparar e caracterizar nanoemulsões estáveis com óleo essencial das plantas de Mata Atlântica *Ocotea indecora* e *Myrciaria floribunda* para o controle de larvas de *Aedes aegypti* e caramujos *Biomphalaria glabrata,* respectivamente. Sesquirosefurano foi o metabólito majoritário do óleo essencial de *O. indecora* e para *M. floribunda* foram nerolidol, β-selineno e 1,8-cineol. A nanoemulsão de ambos óleos se apresentaram estáveis por 200-365 dias após o preparo com tamanho de gotícula reduzido de aproximadamente 100 nm e índice de polidispersão de ±0,26. Em relação ação larvicida em *Aedes aegypti* a nanoemulsão de *O. indecora* apresentou CL_{50} de 61.4 e 26.8 µg/mL, após 48 e 144 h, apresentando indução de alterações morfológicas e seletividade em *Apis mellifera*. Em relação a ação moluscicida em *Biomphalaria glabrata*, a nanoemulsão e óleo essencial de *O. indecora* apresentaram CL_{50} de 22.66 e 64.17 µg/mL após 24h, respectivamente. Além disso, uma docagem molecular sugeriu a interação dos constituintes do óleo essencial com a enzima acetilcolinesterase. Para a

nanoemulsão de *M. floribunda* foi observado CL_{50} de 233 µg/mL após 48h para larvas de *Aedes aegypti* e CL_{50} de 48.1, 29.6, 47.0 µg/mL para embriões, moluscos juvenis e adultos de *Biomphalaria glabrata* e 83.8 µg/mL para cercarias de *Schistosoma mansoni*. Ensaios de toxidez *in silico* para os majoritários do óleo essencial de *M. floribunda* sugerem uma menor toxidez em organismos aquáticos não alvo, do que o moluscicida de referência niclosamida. Além disso, não foi observado letalidade em peixes *Danio rerio* na concentração referente a CL_{50} para *B. glabrata*. Desta forma, este trabalho descreveu o método simples para obtenção de nanoemulsões para o controle do *Aedes aegypti* e *Biomphalaria glabrata* como uma alternativa ecologicamente amigável ao meio ambiente.
Palavras-chave: pesticidas naturais; produtos naturais; Myrtaceae; Lauraceae; nanodispersão; óleo essencial

ABSTRACT

Machado, Francisco Paiva. Nanoemulsion preparation from *Ocotea indecora* and *Myrciaria floribunda* essential oil for the control of *Aedes aegypti* and *Biomphalaria glabrata*. Rio de Janeiro, 2023. PhD thesis from the Graduate Program in Vegetal Biotechnology and Bioprocesses, Health Sciences Center, Federal University of Rio de Janeiro.

Neglected diseases (NDs) are diseases transmitted by etiological agents from tropical and subtropical regions. They are endemic in several countries on the continents of Asia, Africa and Latin America and disproportionately affect populations living below the poverty line. In 5 years (2015-2019) they affected more than 1 billion people in 149 countries resulting in great economic and social losses. For this reason, the World Health Organization (WHO) encourages research and development of strategies and products for the control and eradication of NDs, among them the control of *Aedes aegypti*, a vector that transmits several arboviruses, such as Zika, Dengue and Chikungunya, and the snail *Biomphalaria glabrata*, intermediate host of schistosomiasis. However, both mosquitoes and snails are becoming resistant to the pesticides traditionally used for control. In 2022, 1,450,270 probable cases of dengue resulted in 1016 deaths, and 2,517 deaths from schistosomiasis were reported in Brazil, making it imperative to control the diseases in Brazil. Thus, the present study aimed to prepare and characterize stable nanoemulsions with essential oil from the Atlantic Forest plants *Ocotea indecora* and *Myrciaria floribunda* for the control of *Aedes aegypti* larvae and *Biomphalaria glabrata* snails, respectively. Sequirosefuran was the major metabolite in the essential oil of *O. indecora* and for *M. floribunda* were nerolidol, β-selinene, and 1,8-cineole. The nanoemulsions of both oils were stable for 200-365 days after preparation with a reduced droplet size of approximately 100 nm and polydispersity index of ±0.26. Regarding the larvicidal action on *Aedes aegypti*, the nanoemulsion of *O. indecora* showed LC_{50} of 61.4 and 26.8 µg/mL, after 48 and 144 h, with induction of morphological alterations in body and selectivity on *Apis mellifera*. In relation to the molluscicidal action on *Biomphalaria glabrata*, the nanoemulsion and essential oil of *O. indecora* showed a CL_{50} of 22.66 and 64.17 µg/mL after 24h, respectively. Furthermore, molecular docking suggested the interaction of the essential oil constituents with the enzyme acetylcholinesterase. For the nanoemulsion of *M. floribunda* the LC_{50} of 233 µg/mL was observed after 48h for *Aedes aegypti* larvae and LC_{50} of 48.1, 29.6, 47.0 µg/mL for embryos,

juvenile and adult mollusks of *Biomphalaria glabrata* and 83.8 µg/mL for cercariae of *Schistosoma mansoni*. In silico toxicity assays for the major compounds of *M. floribunda* essential oil suggest lower toxicity in aquatic non-target organisms than the reference molluscicide niclosamide. Furthermore, no lethality was observed in *Danio rerio* fish at the concentration concerning to the LC_{50} for *B. glabrata*. Thus, this study describes a simple method for obtaining nanoemulsions for the control of *Aedes aegypti* and *Biomphalaria glabrata* as an environmentally friendly alternative.
Keywords: natural pesticides; natural products; Myrtaceae; Lauraceae; nanodispersion; essential oil

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LISTA DE ABREVIATURAS

ABDI- Agência Brasileira de Desenvolvimento Industrial

AchE - Acetilcolinesterase

AHJ – Análogos de Hormônio Juvenil

AI – Índice aritmético

ANVISA – Agência Nacional de Vigilância Sanitária

A/O – Água em óleo

DDT – diclorodifeniltricloroetano

DNs - Doenças negligenciadas

DTV - Doenças Transmitidoas por Vetores

EHL - Equilíbrio Hidrófilo-Lipófilo

EM – Espectroscopia de Massas

FFP – Faculdade de Formação de Professores

GABA – Ácido Aminobutil

HCH - Hexaclorociclohexanos

ICMBio - Instituto Chico Mendes de Conservação da Biodiversidade

NE – Nanoemulsão

NEOI – Nanoemulsão de *Ocotea indecora*

NEMF – Nanoemulsão de *Myrciaria floribunda*

NIST - National Institute of Standards and Technology

RFFP – Registro da Faculdade de Formação de Professores

SNC – Sistema Nervoso Central

OMS – Organização Mundial da Saúde

ONU– Organização das Nações Unidas

OF - Organofosforado

O/A – Óleo em Água

OR – Ostwald *ripening*

PAHO – *Pan American Health Organization*

PMD - p-menthane-3,8-diol

PNRJ – Parque Nacional da Restinga de Jurubatiba

RC – Regulador de Crescimento

UC – Unidade de Conservação

UERJ – Universidade Estadual do Rio de Janeiro

UFRJ – Universidade Federal do Rio de Janeiro

WHO – *World Health Organization*

SUMÁRIO

CAPÍTULO I

INTRODUÇÃO, JUSTIFICATIVA E OBJETIVOS

1. INTRODUÇÃO

1.1 Doenças negligenciadas

As doenças negligenciadas (DNs) são doenças transmissíveis provocadas por agentes infecciosos e parasitológicos em populações vulneráveis com ocorrência em regiões tropicais e subtropicais. São endêmicas em países da Ásia, África e América Latina. Deste modo, afetam desproporcionalmente as comunidades de baixo poder econômico e socialmente marginalizadas (Meurer e Coimbra, 2022). De 2015 a 2019 mais de 1 bilhão de pessoas em 149 países foram acometidas por doenças negligenciadas promovendo consequências duradouras para saúde pública da sociedade. As DNs afetam o nível de escolarização de crianças em idade escolar, e incapacita adultos trabalhadores e, desta forma, podem ser diretamente relacionadas com a permeação do ciclo de pobreza e desigualdade na sociedade (OPAS, 2021).

As DNs influem um fardo pesado sobre os países em desenvolvimento, a falta de atenção por parte de gestões públicas e fabricantes de medicamentos, geram grandes perdas sociais e econômicas. Estima-se que 35.000 pessoas venham à óbito diariamente em decorrência destas doenças (Weng, Chen e Wang, 2018). As indústrias farmacêuticas não estimulam o desenvolvimento e comercialização de novas opções terapêuticas devido ao baixo retorno financeiro, dado que grande parte das pessoas acometidas por estas doenças não apresentarem condições financeiras para arcar com o tratamento, sendo este o fator limitante na erradicação destas doenças (Neto *et al.*, 2022).

Desta forma, iniciativas fomentando a pesquisa e inovação de produtos para o controle de DNs são estimuladas pela Organização Mundial da Saúde (OMS). O Programa Especial de Pesquisa e Treinamento em Doenças Tropicais (TDR) foi criado em 1975 com objetivos de apoiar a saúde global por meio da facilitação de pesquisas com DNs de forma a reduzir o fardo e construir resiliência de populações negligenciadas em países endêmicos (WHO, 2017).

As DNs mais importantes listadas pela OMS são leishmanioses, doença de chagas, ascaridíase, ancilostomíase, hanseníase, tracoma, trematodioses, malária, teníase/cisticercose, dengue, raiva, micoses profundas, escabiose e esquistossomose (Meurer e Coimbra, 2022).

1.2 O mosquito *Aedes aegypti*

O *Aedes aegypti* é um inseto da ordem Diptera, pertencente à família Culicidae e é conhecido popularmente no Brasil como "pernilongo-rajado" ou "Mosquito da Dengue". Resumidamente, a morfologia externa do *A. aegypti* é dividida em três partes: cabeça, onde possui um par de olhos; antenas (estruturas sensoriais) e peça bucal picador-sugador; tórax, formado por três segmentos: protórax; mesotórax; matatórax e abdome (Hodapp e Jones, 1961). O mosquito foi descrito originalmente no Egito, onde pode-se encontrar outras subespécies do mesmo gênero, como o *Ae. aegypti formosus,* uma subespécie que ocorre em ambientes vegetados da África sub-sahariana e possui preferência por sangue animal (Figura 1) e *Aedes albopictus*. Este fato, dentre outros, indica a origem do *Ae. aegypti* sendo proveniente do continente Africano. Foi provavelmente introduzido no Brasil em barris de água de navios europeus durante a colonização da América do Sul. Atualmente essa espécie possui distribuição mundial como consequência da constante migração do homem no planeta (Chadee, Martinez e Sutherland, 2017; Zara *et al.*, 2016)

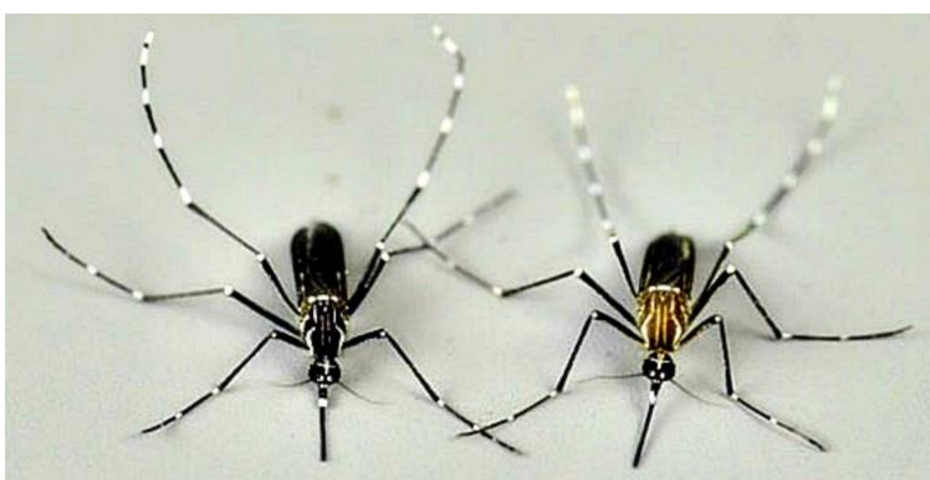


Figura 1: A esquerda *Ae. aegypti aegypti* e na direita *Ae. aegypti formosus* (Foto: Carolyn McBride).

As fêmeas de *Aedes aegypti* são fertilizadas pelos machos durante voo, após a cópula, as fêmeas realizam a hematofagia, que é essencial para a produção dos ovos. Uma fêmea pode colocar até 300 ovos nas superfícies de objetos que acumulam água, podendo gerar até 1500 mosquitos durante toda sua vida (Chadee, Martinez e Sutherland, 2017).

O ciclo de vida do *A. aegypti* é holometabólico, ou seja, possui metamorfose completa que consiste em 4 etapas: ovo, larva, pupa e adulta (Figura 2). O primeiro estágio é o ovo, que possui um mecanismo de resistência à dessecação, permitindo viabilidade dos ovos durante 6 meses. Os ovos eclodem em contato com a água iniciando a segunda etapa que é o período aquático, com quatro formas do estágio larvar (L1-L4). Essas larvas possuem capacidade de locomoção na água, permitindo alimentação a partir de matérias orgânicas presentes no ambiente e respiração, que é realizada na superfície da água por meio de uma estrutura anatômica chamada sifão respiratório. Ao final do estágio larvar ocorre a metamorfose e se inicia o estágio de pupa, que realiza a captação de oxigênio somente na superfície da água. Todo o ciclo aquático tem duração em torno de 10 a 15 dias de acordo com as condições ambientais. Quanto maior a temperatura ambiental, mais rápido ocorrerá o ciclo de vida do *Aedes aegypti* (Marinho *et al.*, 2016). Por fim, a fase adulta do mosquito emerge da pupa possuindo expectativa de vida entre 2 a 3 semanas (Beserra *et al.*, 2009).

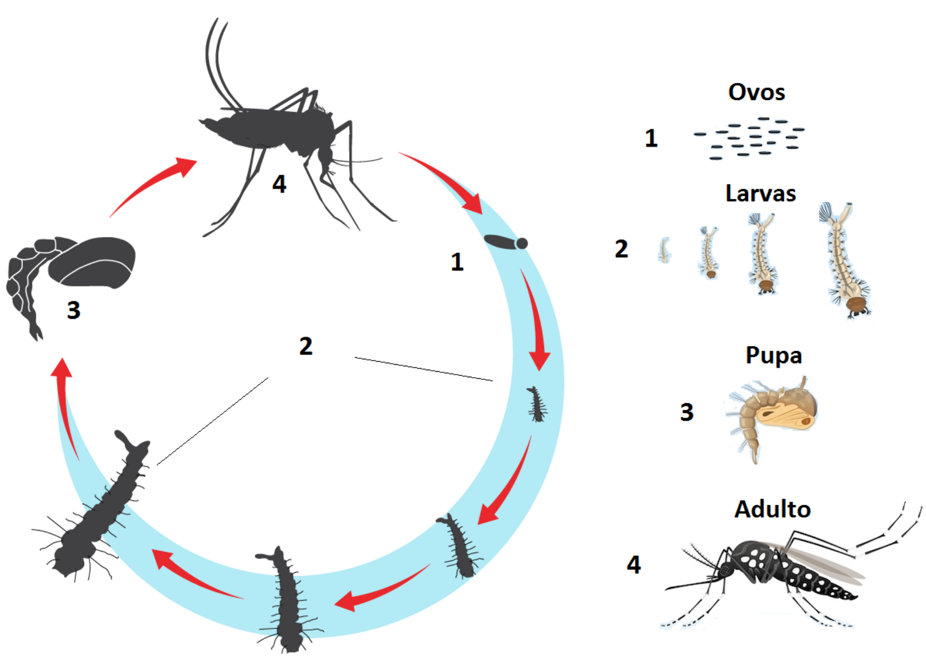


Figura 2: Ciclo de vida do *Aedes aegypti* (Fonte: <http://www.casadaciencia.com.br/o-fim-da-picada> Acessado em 03 de janeiro de 2020).

O mosquito é um vetor com ciclo de transmissão: homem – *A aegypti* – homem. É considerado de importância médica, por causar problemas à saúde púbica devido à capacidade de transmitir através de sua picada diversos arbovírus, ou seja, vírus transmitidos por artrópodes, como: Dengue, Zika, Chikungunya e mais recentemente o Mayaro (Tabela 1) (Gómez *et al.*, 2022).

Tabela 1: Família e gêneros das arboviroses mais comuns.

Arbovírus	**Família**	**Gênero**
Dengue	Flaviviridae	*Flavivirus*
Zika	Flaviviridae	*Flavivirus*
Chikungunya	Togaviridae	*Alphavirus*e
Mayaro	Togaviridae	*Alphavirus*e

As arboviroses possuem sinais e sintomas semelhantes que dificultam o diagnóstico imediato das doenças (Tabela 2). A dengue na forma grave inclui entre os sintomas, dor abdominal intensa e contínua, vômito persistente e sangramento de mucosas, podendo levar a óbito. A Zika, em especial, possui transmissão vertical, podendo gerar complicações cerebrais na formação do feto, como microcefalia e outras doenças neurológicas. Em relação a Chikungunya, a artralgia pode se tornar crônica, mantendo o quadro clínico por anos. Em casos mais raros pode ocorrer acometimentos neurológicos, como: encefalite, síndrome de Guillain-Barré e mielite (BRASIL, 2021).

Tabela 2: Sintomas generalistas da Dengue, Zika e Chikungunya.

Sintomas	**Dengue**	**Zika**	**Chikungunya**
Febre	>38°C (4-7 dias)	baixa ou ausente	38°C (2-3 dias)
Dores nas articulações	moderadas	Leves	Intensas (ou crônico)
Machas vermelhas	a partir do 4° dia	Em 24 h	24 ou 48 h
Coceira	Leve	Leve a intensa	leve
Vermelhidão nos olhos	ausente	Presente	presente
Microcefalia em bebês	ausente	Possível	ausente

No Brasil no ano de 2022 até a semana 52 ocorreram 1.450.270 casos prováveis de dengue (Figura 3A), 1.016 óbitos em território nacional e predominância no estado de São Paulo com 27,7% dos óbitos. Em relação a Chikungunya, ocorreram 174.517 casos prováveis em 2022, aumentando 32,4% em relação a 2019 (Figura 3B), a região Nordeste apresentou maior incidência com 257,4 casos/100 mil habitantes e 94 óbitos, sendo o estado do Ceará detetor de 41,5% dos óbitos. No que se refere a Zika, o país apresentou 9.204 casos prováveis em 2022 (Figura 3C), com o apenas um óbito no estado de Goiás. Entretanto, foram registrados 591 prováveis casos de Zika em gestantes, grupo de risco devido a microcefalia em neonatos (BRASIL, 2023).

Em 2016 o investimento para o controle e combate do vetor *Aedes aegypti* custou ao Brasil aproximadamente 1,5 bilhão de reais, sendo 78,6 milhões para aquisição de inseticidas e larvicidas. Os custos médicos diretos decorrentes das arboviroses foram de 374 milhões e os custos indiretos totalizam 431 milhões de reais com perda de produtividade por afastamento, especialmente sobre o quadro clínico da Chikungunya. O somatório dos custos gerais decorrentes das arboviroses, combate e controle de vetores somam aproximadamente 2,3 bilhões (Teich, Arinelli e Fahham, 2017).

Desta forma, é imperioso a existência de programas de controle do mosquito *Aedes aegypti* de maneira a reduzir a mortalidade e morbidade associada as arboviroses transmitidas no país (Zara *et al.*, 2016).

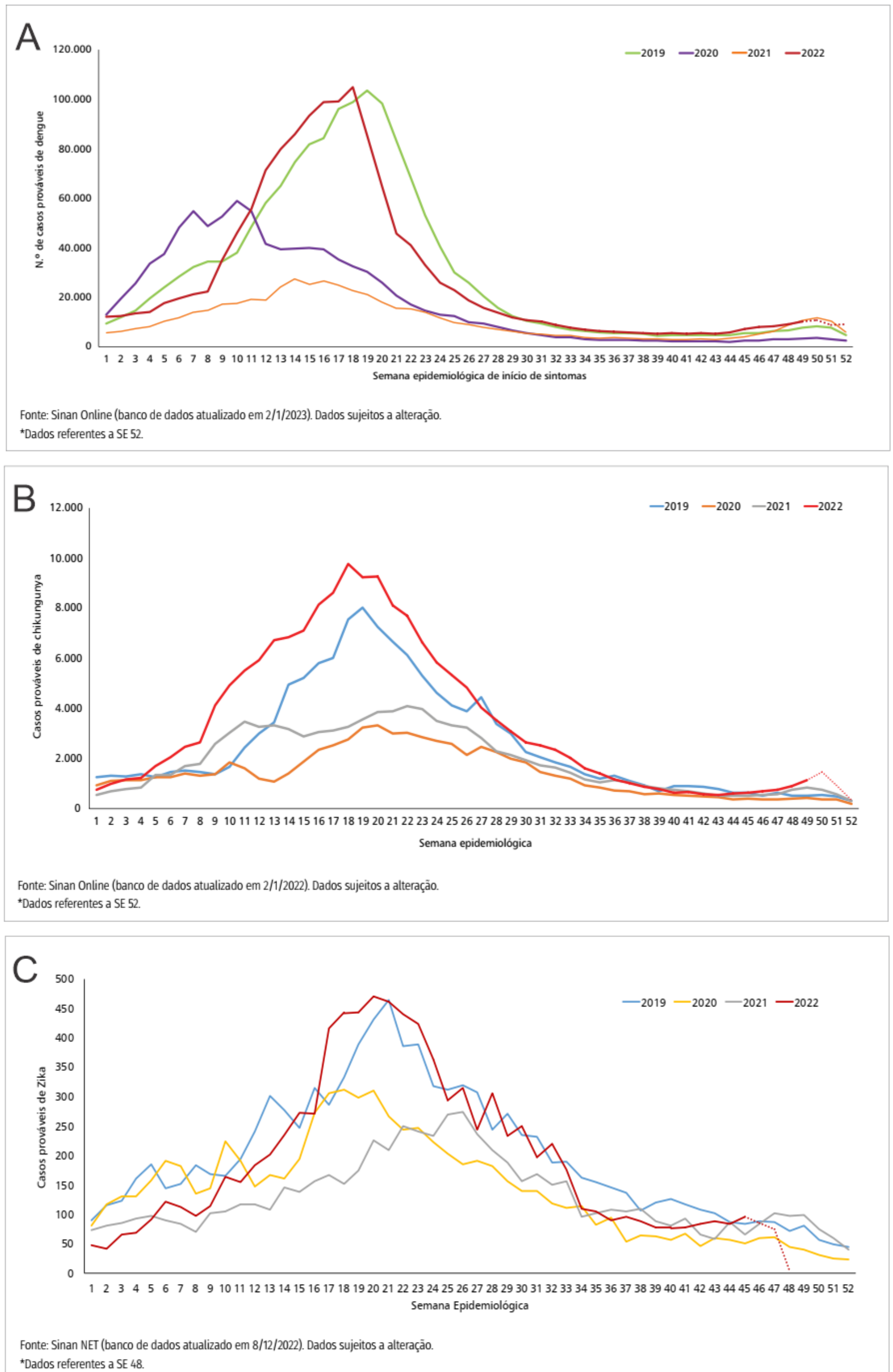


Figura 3: Curva epidêmica dos prováveis casos de dengue (A), chikungunya (B) e zika (C), por semana epidemiológica de início de sintomas, Brasil. 2019 a semana 52 de 2022. Fonte: Boletim epidemiológico volume 54, janeiro de 2023.

1.3 Esquistossomose

A esquistossomose, é uma doença parasitária crônica transmitida por trematodas do gênero *Schistosoma spp*. A doença é propagada pela contaminação de fontes de água por pessoas e/ou animais infectados. As principais espécies responsáveis pela transmissão são *Schistosoma hematobium*, *Schistosoma japonicum* e *Schistosoma mansoni* (Gomes *et al.*, 2022).

A doença é conhecida popularmente por barriga d'água devido a hepatoesplenomegalia, sintoma característico. É uma doença tropical negligenciada predominante em populações de baixa renda com saneamento básico precário, causando o maior impacto socioeconômico dentre as DNs, ficando atrás apenas da malária (Rocha *et al.*, 2021). Estima-se que 779 milhões de pessoas em 78 países vivam em áreas endêmicas de risco. Desta forma a OMS estabeleceu a erradicação da esquistossomose como uma prioridade para saúde pública mundial (WHO, 2022a).

Resumidamente, o ciclo de transmissão da esquistossomose inicia com a eclosão dos ovos em água, liberando os miracídios, forma infectante que penetra no tecido de caramujos hospedeiros, normalmente pertencentes ao gênero *Biomphalaria sp.* Dentro dos caramujos os miracídios se desenvolvem para esporocistos que eventualmente dão origem a cercarias, forma infectante humana com deslocamento aquático livre. Estes por sua vez, penetram ativamente o tegumento do hospedeiro humano perdendo a cauda e passam por transformações morfológicas dando origem a esquistossômulos. Estes deslocam pela epiderme e derme em procura de vasos linfáticos ou sanguíneos. Ao alcançar a circulação sanguínea, aproximadamente 21 dias após a infecção, chegam nas veias do sistema porta-hepático, onde completam o desenvolvimento para forma de verme adulto sexuado e, por fim, macho e fêmea migram para vasos mesentéricos, com exceção de *S. haematobium* que migra para o plexo venoso da bexiga, onde ocorre ovoposição, que vão para as fezes ou urina e, sem saneamento básico adequado, recomeçam o ciclo de transmissão (Figura 4) (Miranda *et al.*, 2022).

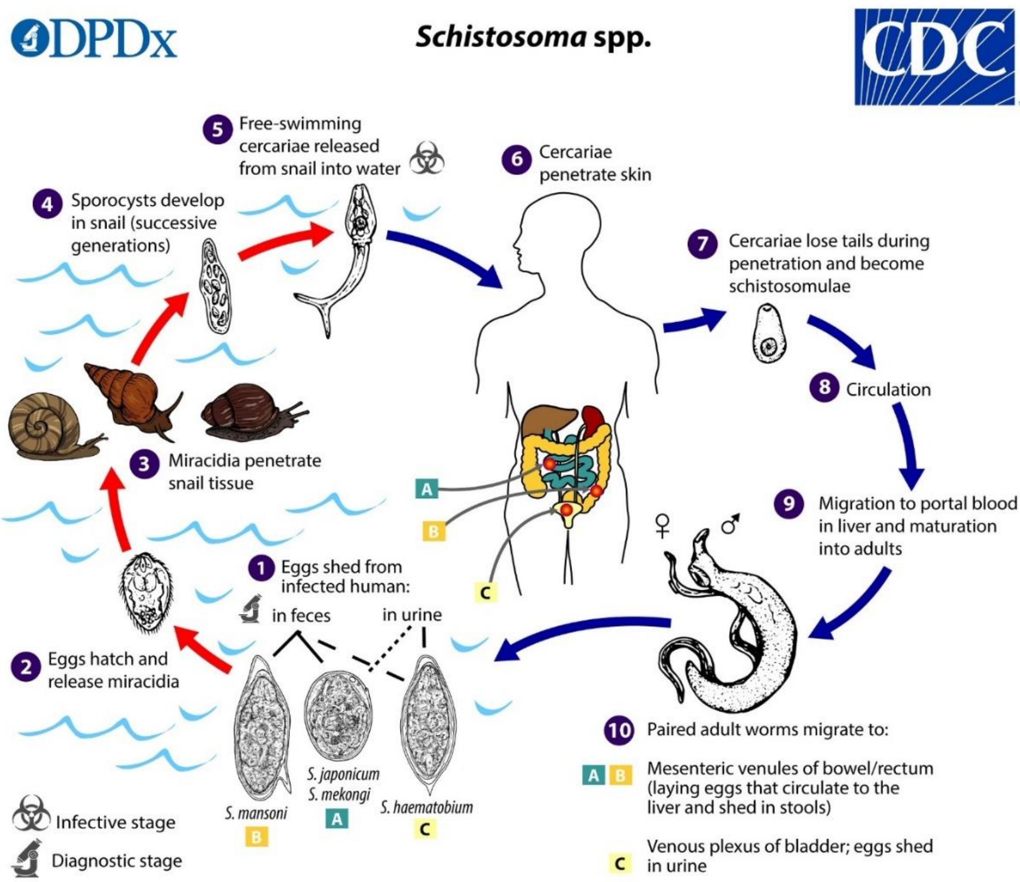


Figura 4: Ciclo de transmissão da esquistossomose por trematodas do gênero *Schistosoma spp*. Fonte: < https://www.cdc.gov/dpdx/schistosomiasis/index.html> acessado em 06 de janeiro de 2023.

Em 2001 a 54° Assembleia Mundial da Saúde estabeleceu a resolução WHA54.19 e endossou que a quimioterapia preventiva, especialmente em crianças em idade escolar, é a estratégia mais eficaz para reduzir mortalidade e morbidades relacionado a esquistossomose em comunidades contaminadas (WHO, 2022a). Em 2015 a esquistossomose custou ao Brasil aproximadamente 41 milhões de dólares em despesas decorrentes da doença (Silva *et al.*, 2020). O praziquantel é o fármaco de primeira escolha para o tratamento de casos de esquistossomose, desempenhando papel fundamental no controle da doença devido sua efetividade, baixo custo e dose-única, facilitando a adesão dos pacientes ao tratamento. Apresenta amplo espectro de atividade em trematodas e cestodas, e possui mecanismo de ação associado a interação na subunidade β dos canais de Ca^{2+} voltagem dependentes em parasitos *Schistosoma sp.* acarretando a disruptura da homeostase de cálcio (NOGUEIRA, LIRA, et al., 2022).

No Brasil os estados considerados endêmicos da doença são Alagoas, Bahia, Maranhão, Pernambuco, Rio Grande do Norte, Paraíba, Sergipe, Espírito Santo e Minas Gerais, sendo a região Nordeste com o maior número de casos do país. No período de 2015 a 2019 foi registrado 2.517 óbitos decorrente da esquistossomose, prevalecendo alta mortalidade para indivíduos da terceira idade com baixa ou nenhuma escolaridade (BRASIL, 2022).

Dentre outras estratégias recomendadas pela OMS para o controle da esquistossomose, encontra-se o controle de caramujos hospedeiros intermediários do gênero *Biomphalaria*, mais especificamente *Biomphalaria glabrata* (Figura 5), por meio de agentes químicos moluscicidas (WHO, 2020). O único agente moluscicida químico recomendado pela OMS é a niclosamida (Baylucid, Bayer). Entretanto, deve ser último recurso a ser utilizado em casos de epidemias devido a toxidez, não seletividade em organismo não alvo e suspensão de uso dos recursos hídricos após aplicação (Friani *et al.*, 2023).

Figura 5: *Biomphalaria glabrata* o caramujo hospedeiro intermediário da esquistossomose. Fonte: < https://portal.fiocruz.br/noticia/genoma-de-caramujo-transmissor-do-schistosoma-mansoni-e-sequenciado > acessado em 07 de janeiro de 2023.

1.4 Pesticidas

Os pesticidas são uma variedade de substâncias de origem química ou biológica utilizados no ambiente para prevenir, controlar e reduzir populações de insetos, roedores, planta invasoras ou outras pestes que acarretam perda econômica agrícola ou que seja danoso para saúde pública (Serrão *et al.*, 2022). Os pesticidas podem atuar de modo destrutivo, repelente ou atenuador de pragas e são convencionalmente classificados como fungicidas, herbicidas, inseticidas, moluscicidas, nematicidas, acaricidas e rodenticidas (Tabela 3). Atualmente, os herbicidas e inseticidas são os mais utilizados representando 47,5% do uso de pesticidas (Syafrudin *et al.*, 2021).

Tabela 3: Pesticidas mais comuns categorizados por espécie alvo.

Pesticidas	**Classe: Substância**
Inseticidas	Organoclorados: Endosulfan
	Organofosforados: Diazinon, malathion, parathion, chlorpyrifos
	Carbamatos: Aldicab, carbofuran, carbaryl
	Piretróides: Deltamethrin, fenpropathrin
	Neonicotinoides: Acetamiprid, thiamethoxam
	Piretróides: permetrina, deltametrina, cipermetrina
Herbicidas	Triazina: Atrazine, cyanazine
	Cloroacetamida: alachor, butachlor, dimethenamid, metolachlor
Fungicidas	Benzamida: Fluopicolide, zoxamide
	Carboxamida: Boscalid captofol
	Hidrocarboneto clorado: Hexachlorbenzeno
	Organofosfato: Edifenphos, iprobenfos
	Clorofenil: Dichloran, quintozene

O uso racional destes agentes é de grande importância para garantia da qualidade de vida na sociedade sendo diretamente relacionados a quantidade e qualidade da produção agrícola base da alimentação humana, se tornando uma ferramenta fundamental para redução da insegurança alimentar e redução da morbidade e mortalidade de epidemias relacionadas a insetos vetores (Serrão *et al.*, 2022).

Entretanto, o uso desenfreado de pesticidas frequentemente acarreta a contaminação ambiental e dos alimentos produzidos. Desta forma, os benefícios da utilização destes agentes químicos são suprimidos pelos efeitos tóxicos para saúde humana e ambiental. A falta de seletividade de pesticidas, como inseticidas, leva a perda drástica da biodiversidade entomológica benéfica em regiões de plantações e loteamentos rurais. Os pesticidas são desenvolvidos para afetar pestes específicas, entretanto, atuam em organismos não alvo como polinizadores, insetos predadores naturais e parasitoides pertencentes do ecossistema local que poderiam contribuir positivamente para sustentabilidade da produção agrícola (Serrão *et al.*, 2022). Além das concentrações letais observadas *in loco*, deve-se atentar sobre as concentrações subletais que podem afetar funções fisiológicas e, desta forma, impedir a permeação das espécies não alvo, por meio de modificações da dinâmica natural do inseto-organismo (Fiaz *et al.*, 2018).

No que diz respeito a polinizadores, as abelhas são o grupo mais representativo. Diversos estudos tem reportado o aumento da mortalidade e colapso das colônias de abelhas. Dentre os possíveis fatores está o uso de pesticidas, as abelhas apresentam alta sensibilidade a agentes pesticidas devido possuir aparato enzimático desintoxicante limitado, quando em relação a outros insetos. Adicionalmente, doses subletais afetam a orientação e capacidade de voo, muitas vezes levando as moléculas de inseticida para dentro da colônia, e ao consequente enfraquecimento da saúde colonial ou impedindo o retorno da abelha (Abati *et al.*, 2021).

Outro fato que deve ser levado em consideração é o acumulo de pesticidas no solo, que acabam sendo diretamente relacionados à contaminação hídrica. Os pesticidas presentes no solo são carreados para água, contaminando rios, lagos e lençóis freáticos, que por sua vez acabam em diferentes níveis tróficos acarretando em biomagnificação (Syafrudin *et al.*, 2021). Por estes e outros motivos é necessário o desenvolvimento de novos produtos, mais seletivos, menos tóxicos e eficientes para substituição e utilização no meio ambiente.

1.4.1 Inseticidas

Os inseticidas podem ser definidos como qualquer substância ou mistura de agentes químicos ou biológicos para o combate de insetos prejudiciais ao homem. Quando se trata de saúde pública, o uso de inseticidas são voltados ao controle de insetos vetores de interesse médico, por exemplo, *Anopheles darlingi* principal transmissor da Malária no Brasil, *Rhodnius prolixus* e *Triatoma infestans*, responsáveis pela transmissão da Doença de Chagas, *Culex quinquefasciatus* vetor transmissor da filariose linfática, flebotomíneos responsáveis pela transmissão de leishmaniose e o *Aedes aegypti*, transmissor de uma série de arboviroses urbanas (Almeida, de *et al.*, 2022; Baronas, 2019; Gómez *et al.*, 2022; Gope e Rawani, 2022; Penrice-Randal *et al.*, 2022; Weslati *et al.*, 2022).

A última medida a ser utilizada nos programas de controle de vetores é o controle químico, devido à possibilidade de causar efeitos prejudiciais ao ser humano e ao meio ambiente. Seu uso é muito difundido devido à necessidade de controle de epidemias, onde atua de maneira rápida e efetiva, diminuindo novas infecções e, por consequente, a mortalidade nas populações expostas à Doenças Transmitidas por Vetores (DTV) (Silva, Guimarães e Ferreira, 2001).

Os organoclorados, também conhecidos como hidrocarbonetos clorados ou inseticidas clorados, são substâncias químicas que contém em sua estrutura química apenas cloro (Cl), hidrogênio (H) e carbono (C), possuindo uma grande estabilidade devido à presença de diversas ligações H-C, C-Cl e C-C em suas moléculas. Entretanto, tiveram seu uso descontinuado devido à acumulação em tecidos lipofílicos e à sua grande persistência no ambiente. São classificados em quatro grupos principais: os difenil-alifáticos, são os inseticidas de primeira geração, sendo os mais antigos. O mais conhecido nesse grupo é o diclorodifeniltricloroetano (DDT), que atua no sistema nervoso central (SNC), na abertura dos canais de sódio, alterando o equilíbrio dos íons sódio e potássio nos axônios, modificando a propagação do impulso nervoso nos insetos; os hexaclorociclohexanos (HCH), possuem mecanismo de ação semelhante ao DDT; ciclodienos, possuem ação antagonista em receptores do ácido gama-aminobutírico (GABA), impedindo a permeabilidade aos íons cloreto nos neurônios; e policloroterpenos que possuem mecanismo de ação equivalente aos ciclodienos (Figura 6) (Rezende-Teixeira *et al.*, 2022).

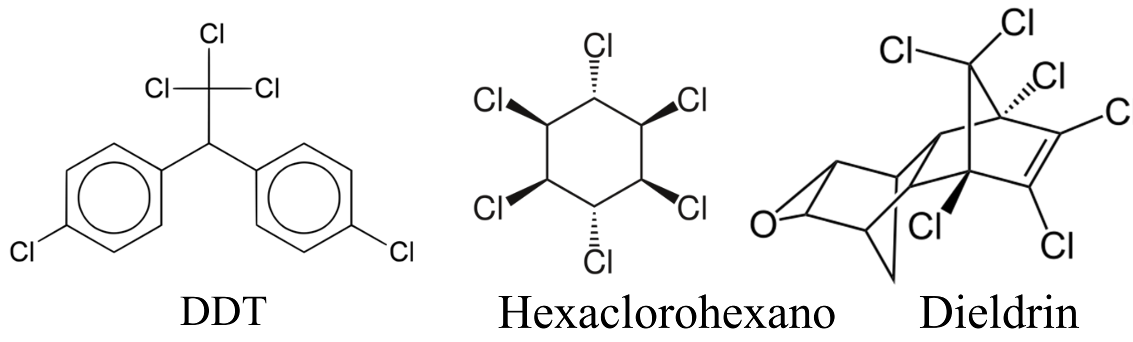


Figura 6: Estruturas químicas do DDT, Hexaclorohexano e Ciclodieno dieldrin. Fonte: Autor.

Organofosforados (OF), são derivados do ácido fosfórico. Apresentam esse nome porque possuem fósforo em sua estrutura química. Podem ser classificados em três subgrupos: alifáticos, derivados do fenil e heterocíclos (Figura 7). Não são acumulados em tecidos e são passíveis de reação de hidrólise dos grupamentos éster, os tornando mais biodegradáveis em relação ao grupo dos organoclorados. Esse fato, porém, torna necessário à sua reaplicação periódica no ambiente. São mais tóxicos a vertebrados devido ao seu mecanismo de ação, uma reação de fosforilação resultando na inibição da acetilcolinesterase (AchE). Os organofosforados se ligam covalentemente no sítio da serina presente na acetilcolinesterase inativando-a irreversivelmente, resultando uma síndrome colinérgica que pode levar à morte (Rezende-Teixeira *et al.*, 2022).

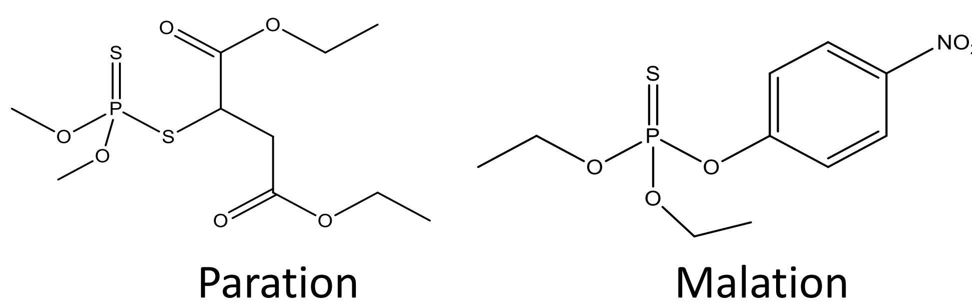
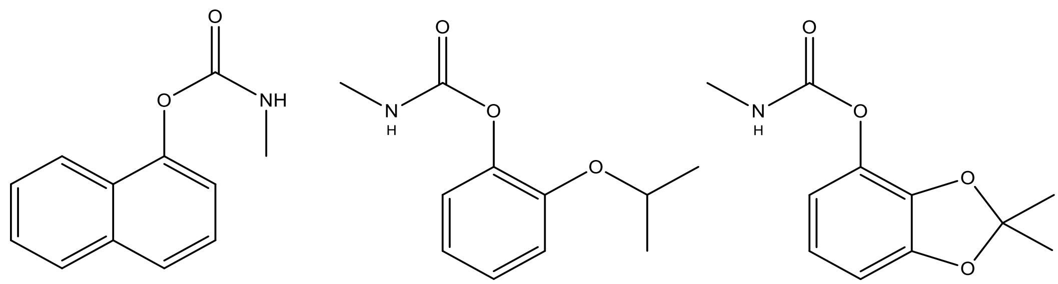


Figura 7: Estruturas químicas dos organofosforados Paration e Malation. Fonte: Autor.

Os carbamatos, são ésteres derivados do ácido carbâmico, cujo grupo funcional é o -$NH(CO)O^-$(Figura 8). São solúveis em solventes orgânicos e sistêmicos para plantas, devido à sua solubilidade relativa em água. São instáveis devido a possibilidade de hidrólise do grupamento éster. Os produtos do metabolismo são tóxicos e possui mecanismo de ação baseado na inibição da acetilcolinesterase, da mesma forma que os organofosforados.

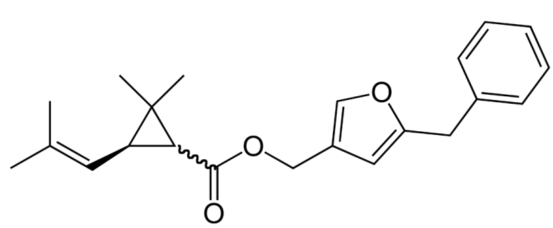
Entretanto, nesse caso a reação envolvida é a carbamilação. A inibição da AchE ocorre de forma reversível (Eddleston, 2019).



Carbaryl Propoxur Bendiocarb

Figura 8: Estruturas químicas dos carbamatos carbaryl, propoxur e bendiocarb. Fonte: Autor

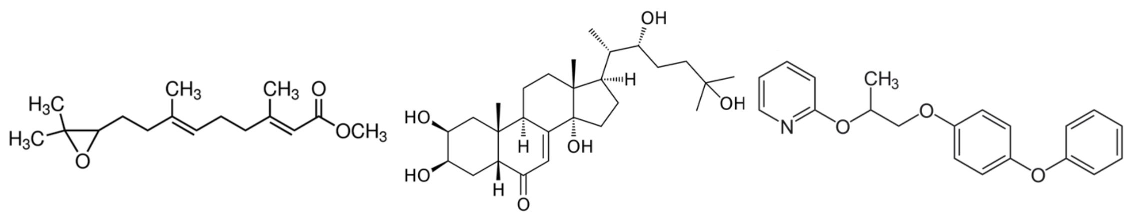
Os piretróides são substâncias sintéticas derivadas das piretrinas, ésteres tóxicos com ação inseticida natural, isoladas de extratos de flores da espécie vegetal *Chrysanthemum cinerariaefolium.* As piretrinas naturais foram submetidas a modificações estruturais devido à sua instabilidade à luz e reações oxidativas. Para isso foram adicionados átomos de nitrogênio, enxofre e halogênio, gerando a resmetrina (Figura 9), o primeiro piretróide com características físico-químicas ideais, solucionando os problemas relacionados à fotoestabilidade, aumentando a atividade inseticida e gerando os piretróides. Gradativamente os inseticidas de primeira e segunda geração foram sendo substituídos pelos inseticidas piretróides, devido à sua baixa toxidez em mamíferos e boa eficácia em diferentes insetos. Apresenta seu modo de ação similar ao do DDT, atuando na abertura dos canais de sódio em membranas neuronais. Esta classe de inseticida exemplifica o potencial biotecnológico que pode ser correlacionado ao metabolismo secundário vegetal (Singh *et al.*, 2022).



Resmetrina

Figura 9: Estrutura química do piretróide Resmetrina. Fonte: autor.

Outros grupos inseticidas alternativos aos produtos químicos que vem sendo utilizados no controle de vetores, são os análogos de hormônio juvenil (AHJ) e reguladores de crescimento (RC). Os RC inibem a síntese de quitina dos insetos, como o diflubenzuron e triflumuron, impedindo a larva de eliminar a cutícula velha, devido a não ocorrência de rigidez da mesma. As larvas sobrevivem por um determinado tempo, mas eventualmente morrem (Parthasarathy e Palli, 2021).



Hormônio Juvenil III Ecdisona Piriproxifeno

Figura 10: Estruturas químicas do hormônio juvenil III, ecdisona e piriproxifeno. Fonte: autor

A metamorfose da fase larvar para a sua passagem à forma adulta ocorre somente na presença de ecdisona e na ausência do HJ. Um inseticida exemplo dessa classe é o piriproxifen (Figura 10), um AHJ utilizado como larvicida contra o *Aedes aegypti,* que atua na inibição da emergência de adultos. Esta classe de inseticida atua no controle de vetores de forma ecologicamente segura, uma vez que o inseto não desenvolve mecanismos de resistência, devido às substâncias mimetizarem uma resposta hormonal endógena (Fiaz *et al.*, 2019).

1.4.2 Moluscicidas

Moluscicidas são pesticidas utilizados para o controle da população de moluscos como caramujos e lesmas, seja para proteção de plantações ou da saúde humana. A niclosamida (Bayluscide®, Bayer, Alemanha) é o agente moluscicida preconizado pela OMS para controlar a esquistossomose causando redução substancial na incidência de casos (Figura 11) (SANTOS, CAVALCANTE, et al., 2017). Em 1950 foi observado uma redução de 10% nos casos de esquistossomose utilizando essa estratégia (Coelho e Caldeira, 2016). Os moluscos comumente associados ao controle da esquistossomose são *Biomphalaria glabrata*, *Biomphalaria* *tenagophila* e *Biomphalaria straminea* (Araújo *et al.*, 2019). Entretanto, diversas desvantagens são associadas ao uso da niclosamida, como o alto valor agregado, desenvolvimento de resistência dos moluscos, reaplicação frequente e toxicidade em organismos não alvo (SANTOS, CAVALCANTE, et al., 2017).

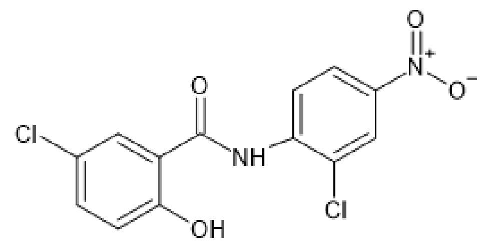


Figura 11: Estrutura química do moluscicida niclosamida (Fonte: autor).

1.5 Nanotecnologia

A nanotecnologia pode ser definida como o *design* intencional para caracterização, produção e aplicação de materiais, estruturas, dispositivos e sistemas com grandeza nanométrica. A nanotecnologia tem sido considerada um dos grandes avanços tecnológicos dos últimos anos, capaz de atender os objetivos do Milênio das Organizações das Nações Unidas (ONU), devido ao seu enorme potencial inovador, industrial e econômico (ABDI, 2010). Em 2005 o consumo mundial de nanomateriais foi de 9 milhões de toneladas métricas e 13,1 bilhões de dólares com expectativa de aumento para 10,3 milhões de toneladas métricas e 20,5 bilhões de dólares em 2010, mostrando uma margem de crescimento anual de 9,3%(ABDI, 2010; Romero Soares Sousa *et al.*, 2018)

Essa ciência tem sido amplamente utilizada na área de tecnologia de alimentos, medicamentos, cosméticos e pesticidas. Entre as formulações mais utilizadas em nanotecnologia podem-se citar os lipossomas, nanoemulsões, nanoesferas, nanocápsulas, nanopartículas metálicas e ciclodextrinas (Mazayen *et al.*, 2022).

Dentre as diversas vantagens que podem ser associadas aos nanoprodutos em relação às formulações comuns, podemos citar o aumento da estabilidade, biodisponibilidade, penetração em membranas biológicas, alterações físico-químicas do ativo na formulação e liberação controlada, permitindo fazer novamente uso de substâncias que caíram em desuso por apresentar alta toxidez (Mahmud *et al.*, 2022).

Dentro das aplicabilidades da nanotecnologia, uma área promissora é o desenvolvimento de biopraguicidas contendo ativos de origem natural, sendo uma opção mais biosustentável, causando menos riscos à saúde humana e menor impacto ambiental, tendo em vista o crescente uso de defensivos agrícolas dos últimos anos, indicando um mercado crescente e a necessidade de desenvolvimento de produtos ambientalmente menos agressivos e/ou seletivos (Mustafa e Hussein, 2020; Pavoni *et al.*, 2019a; b; Sharma *et al.*, 2020)

1.5.1 Nanoemulsões

As nanoemulsões ou miniemulsões são sistemas coloidais amplamente utilizados em indústrias farmacêuticas e de alimentos como mecanismo de encapsular e viabilizar componentes apolar em meio aquoso. São compostas por uma fase aquosa, uma fase oleosa e surfactantes. Podem ser água em óleo (A/O), quando a fase aquosa é dispersa, ou óleo em água (O/A), quando a fase aquosa é a dispersante, sendo esta última mais comum por facilitar a solubilidade de substâncias de caráter lipofílico (Marhamati, Ranjbar e Rezaie, 2021). São sistemas homogêneos de escala nanométrica, possuindo tamanho de gotícula entre 20 a 200 nm. Devido ao tamanho de partícula reduzido as nanoemulsões se apresentam cineticamente estáveis. Possuem uma série de vantagens como, o aumento da biodisponibilidade, estabilidade e melhor absorção (Barradas e Holanda e Silva, de, 2021).

Podem ser preparadas por método de alto aporte energético, que dispõe de equipamentos como homogeneizadores de alta pressão ou geradores de ultrassom, que atuam induzindo uma força de cisalhamento, possibilitando então, o rompimento de gotículas prévias na fase dispersa (Mustafa e Hussein, 2020). E o método de baixo aporte energético de emulsificação que utilizam as propriedades físico-químicas do sistema para obtenção da nanoemulsão. Podem ser preparadas utilizando os métodos de inversão de fases e emulsificação espontânea. A emulsificação por inversão de fase consiste em gotejar lentamente a fase dispersante sobre a fase dispersa com agitação, até a ocorrência da inversão das fases. O processo ocorre com a curvatura de inversão entre as fases se modificando gradualmente. No ponto de inversão a tensão interfacial diminui favorecendo a emulsificação (Mustafa e Hussein, 2020; Ostertag, Weiss e McClements, 2012a).

As nanoemulsões não são termodinamicamente estáveis, apresentando como mecanismo de instabilidade mais relevante o efeito de amadurecimento de Ostwald (*Ostwald ripening* - OR) ou destilação isotérmica (Figura 12), é o fenômeno de transferência de massa entre gotículas de menor diâmetro para de maior diâmetro pela diferença entre os raios de curvatura das gotículas, levando ao aumento do tamanho de gotículas e também na mudança das características óticas macroscópicas do produto (Koroleva e Yurtov, 2021).

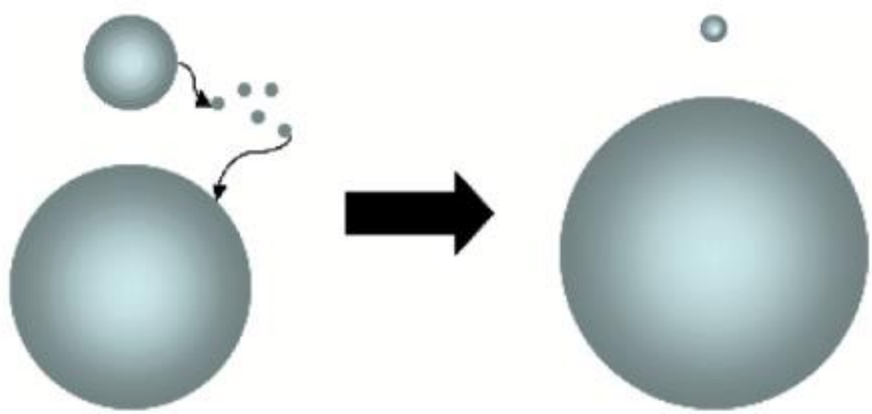


Figura 12: Efeito de amadurecimento de Ostwald. Fonte: (Franzol e Rezende, 2015).

Os sistemas nanoemulsionados tem chamado atenção para formulação de produtos inovadores contendo derivados de origem natural, especialmente os óleos essenciais. A capacidade de viabilizar estes óleos ativo lipofílicos em meio aquoso se tornou um campo de pesquisa favorável para o desenvolvimento de pesticidas para controle de pestes com ciclo ou estágio de vida em meio aquoso, como o *Aedes aegypti*, *Biomphalaria glabrata* ou *Schistosoma mansoni* (Mustafa e Hussein, 2020; Tomiotto-Pellissier *et al.*, 2017).

1.6 O Parque Nacional da Restinga de Jurubatiba

A concepção da criação da unidade de conservação (UC) do Parque Nacional da Restinga de Jurubatiba (PNRJ), surgiu como uma intervenção governamental para preservação e reversão de ecossistemas naturais, em razão à crescente devastação ambiental associada à especulação imobiliária e industrial de regiões de restinga próximas ao litoral de forma a assegurar a preservação do patrimônio biológico (Costa *et al.*, 2022).

A criação do parque teve início na década de 80, com pesquisadores da Universidade Federal do Rio de Janeiro (UFRJ) desenvolvendo pesquisas com restinga devido à sua grande preservação e elevada biodiversidade. O Art. 3°. inciso XVI do Novo Código Florestal (Lei Federal nº 12.651, de 25 de maio de 2012) define a região de restinga como:

> depósito arenoso paralelo à linha da costa, de forma geralmente alongada, produzido por processos de sedimentação, onde se encontram diferentes 1comunidades que recebem influência marinha, com cobertura vegetal em mosaico, encontrada em praias, cordões arenosos, dunas e depressões, apresentando, de acordo com o estágio sucessional, estrato herbáceo, arbustivo e arbóreo, este último mais interiorizado (BRASIL, 2012).

O PNRJ recebeu esse nome devido ao ecossistema costeiro de restinga, localização geográfica pertencente do bioma Mata Atlântica. A palavra Jurubatiba, se deve à “Jiribá”, que segundo nativos indígenas deriva de “Jarybá”, um tipo de palmeira encontrada na região e “tiba”, que significa “em abundância”, desta forma, palmeiras em abundância (SANTOS, L., BOZELLI, 2003).

O parque foi criado em 29 de abril de 1998 e resguarda a maior área de restinga incluída em uma unidade de conservação no Estado do Rio de Janeiro, compreendendo os municípios de Macaé, Carapebus e Quissamã. São 44 quilômetros de costa, contendo 18 lagoas costeiras ao longo de sua extensão e abrangendo um total de 14.860 hectares (Figura 13), onde podem ser encontradas espécies raras e endêmicas de fauna e vegetação (Luz *et al.*, 2022; Santos e Bozelli, 2003)

Figura 13: Mapa representativo do Parque Nacional da Restinga de Jurubatiba. Fonte:DE SOUZA, LEMES MARTINS, et al., (2020).

Um levantamento etnobotânico realizado na restinga de Carapebus das espécies vegetais com utilização popular regional demonstrou o uso de 49 famílias botânicas, 100 gêneros e 119 espécies. Os usos atribuídos as plantas foram: medicinal, comestível, ornamental, têxtil, higiênico, corante, combustível, ritualístico e madeireiro. O uso mais citado foi o medicinal, compreendendo 45 espécies, sendo 29 delas com uso exclusivamente medicinal e relatadas 18 propriedades medicinais diferentes (SANTOS, 2009).

Nesse trabalho se pretende, portanto, utilizar o potencial existente na flora do Parque Nacional da Restinga de Jurubatiba para o controle o de mosquito *Aedes aegypti*, *Biomphalaria glabrata* e *Schistossoma mansoni*.

1.6.1 Myrciaria floribunda

A espécie vegetal *Myrciaria floribunda* (H. West ex Willd.) O. Berg é uma planta nativa, não endêmica do Brasil com ampla distribuição geográfica com ocorrências confirmadas no Norte, Nordeste, Centro-Oeste, Sudeste e Sul do país (Figura 14) (Stadnik, Proença e Caldas, 2023). Entretanto pode ser encontrada mais comumente no bioma da Mata Atlântica (García *et al.*, 2021). No estado do Rio de janeiro pode ser encontrada em regiões de restinga próximo ao litoral em locais com mata nativa remanescente.

Figura 14: Distribuição geográfica da espécie vegetal *Myrciaria floribunda* no Brasil. Fonte: <https://floradobrasil.jbrj.gov.br/FB10792> acessado em 06 de janeiro de 2023.

É pertencente da família Myrtaceae e conhecida popularmente no norte fluminense do estado do Rio de Janeiro como camboim ou camboim amarelo. Entretanto pode ser chamada em outros estados por jabuticabinha, cambuí, murta, goiabaran, araçazeiro, duque e *rumberry* (Azevedo, de *et al.*, 2019; García *et al.*, 2021; Moraes, de *et al.*, 2022). Os frutos de *M. floribunda* são comestíveis, apresentando sabor doce, cítrico e levemente ácido (Figura 16) (García *et al.*, 2021). São popularmente consumidos *in natura* ou para flavorizar bebidas alcoólicas baseadas em cachaça (Magro *et al.*, 2013; Moraes, de *et al.*, 2022). Recentemente o cultivo de *M. floribunda* tem aumentado interesse devido seu potencial econômico e alimentar, para exportação e comercialização no mercado de frutas brasileiras nativas (García *et al.*, 2021).

Figura 15: Folhas e Frutos de *Myrciaria floribunda* no Parque Nacional da Restinga de Jurubatiba (Carapebus, RJ). (Autor: Francisco P. Machado)

Nos últimos anos o perfil químico da *M. floribunda* vem sido amplamente descrito. As substâncias majoritárias presentes no óleo essencial de *M. floribunda* de diversas partes da planta se encontra na tabela 4.

Tabela 4: Rendimento e substâncias majoritárias de óleo essencial de diferentes partes de *M. floribunda*.

Parte da planta	Rendimento (%)	Substâncias majoritárias	Referência
Folhas frescas	0,70	nerolidol (32,4%), β-selineno (9,8%) e 1,8-cineol (5.8%)	Ramos et. al. (2011)
Folhas frescas	0,37	1,8-cineol (38,4%), γ-himachaleno (7%) e α-terpineol (5,5%)	
Caule	0,02	(2E,6E)-acetato de farnesila (19,9%) e (2E,6Z)-farnesol (13,1%)	Tietbohl et. al. (2012)

Flores	0,64	1,8-cineol (22,8%), linalool (12,7%) e (2E,6E) -acetato de farnesila (13,4%)	
Folhas frescas	0,37	1,8-cineol (38,4%), γ-himachaleno (7%) e α-terpineol (5,5%)	Tietbohl et. al. (2019)
Frutos	0,6	δ-Cadinene (26,8%), γ-Cadinene (15,7%), Muurolene (6.2%) e α-Selinene (6,11%)	Barbosa et. al. (2020)
Frutos	-	Cariofileno (21,59-49,3%) e γ-selineno (11,3-16,3%)	Garcia et. al. (2021)
Brotos	1,02	α-phellandrene (22,19%), 1,8-cineol (23,3%) e terpinoleno (22,2%)	Moraes et. al. (2022)

O perfil químico do óleo essencial de folhas foi descrito por Ramos et. al. (2011) apresentando 18.8% de monoterpenos e 75,2% de sesquiterpenos em sua composição. Os componentes majoritários foram nerolidol (32,4%), β-selineno (9,8%) e 1,8-cineol (5,8%) (Ramos *et al.*, 2010). Por outro lado, Tietbohl et al. (2012) descreveram o óleo essencial de folhas com 53,9% de monoterpenos e 39,6% de sesquiterpenos. Os metabólitos majoritários por sua vez foram 1,8-cineol (38,4%), γ-himachaleno (7%) e α-terpineol (5,5%) (Tietbohl *et al.*, 2012). Adicionalmente, outro estudo realizado pela mesma equipe descreveu o óleo essencial de folhas de *M. floribunda* com 19,2% de monoterpenos e 70% de sesquiterpenos com a presença de 1,8-cineol (10,4%), β-selineno (9,8%) e α -selineno (7,4%) e, desta forma, corroborando com o perfil químico descrito por Ramos et al. (2011) (Tietbohl *et al.*, 2020a; b).

Em relação ao potencial biológico da espécie vegetal *M. floribunda*, estudos anteriores descreveram a ação anticolinesterásica, antioxidante, antibacteriano, antifúngico, antiproliferativo, antinociceptivo, inseticida, anti-inflamatório de diferentes extratos, frações e óleos essenciais de partes diversas da planta (Azevedo, de *et al.*, 2019; Moraes, de *et al.*, 2022; Santos *et al.*, 2020; Silva Barbosa, da *et al.*, 2020; Tietbohl *et al.*, 2017).

No que se refere ao potencial inseticida da *M. floribunda*, o óleo essencial de folhas apresentou mortalidade e alterações no desenvolvimento de ninfas de 5° instar de *Rhodnius prolixus*, vetor transmissor da doença de Chagas, apresentando DL_{50} de 19,51 µg/inseto após 30 dias de exposição, deformidades entre mudas e interrupção de metamorfose (Tietbohl *et al.*, 2020b). Similarmente, a ação inseticida do óleo essencial de folhas foi avaliada frente a percevejos pragas do algodão *Dysdercus peruvianus* (percevejo manchador do algodoeiro) e *Oncopeltus fasciatus* apresentando LD_{50} de 94,42 e 72,18 µg/inseto, respectivamente (Tietbohl *et al.*, 2014). Estes relatos sugerem uma ação inseticida em potencial vinculado ao óleo essencial de *Myrciaria floribunda*.

1.6.2 Ocotea indecora

A *Ocotea indecora* (Schott) Mez pertence à família Lauraceae e é popularmente conhecida como Canela-sassafrás (Figura 17) (Nascimento *et al.*, 2020). É uma planta nativa e endêmica do Brasil, pode ser encontrada como arbusto ou árvore e possui como sinonímia *Ocotea elegans* Mez, *Persea indecora* Schott, *Mespilodaphne indecora* (Schott) Meisn e *Oreodaphne indecora* (Schott) Nees (Assis, 2009).

Figura 16: Folhas, frutos e inflorescências de *Ocotea indecora* no Parque Nacional da Restinga de Jurubatiba (RJ). (Autor: Francisco P. Machado)

A Canela-sassafrás é pertencente dos biomas Mata Atlântica e Pampa e possui distribuição geográfica limitada com ocorrências no Nordeste, Sudeste e Sul nos estados da Bahia, Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Rio Grande do Sul e Santa Catarina (Figura 18) (Quinet *et al.*, 2015). No Rio de janeiro pode ser encontrada no Norte Fluminense, nos municípios Macaé, Quissamã e Carapebus em áreas de restinga próximo ao litoral (SANTOS, FEVEREIRO, et al., 2009).

Figura 17: Distribuição geográfica da espécie vegetal *Ocotea indecora* no Brasil. Fonte: <https://floradobrasil.jbrj.gov.br/FB8463> acessado em 06 de janeiro de 2023.

O perfil químico do óleo essencial de folhas de *O. indecora* foi descrito por Figueredo et al. (2018) e Nascimento et. al. (2020), ambas autoras coletaram o material vegetal no Parque Nacional da Restinga de Jurubatiba (RJ) e descreveram o furanoterpenoide sesquirosefurano como o metabólito amplamente majoritário (±92%), seguido de β-Farneseno (±3%) e traços de biciclogermacreno e (2E,6E)-metil farnesoato (Figura 19) (Figueiredo *et al.*, 2018; Nascimento *et al.*, 2020). Por outro lado, Gonçalves et. al. (2018) descreveu o perfil químico do óleo essencial de folhas de *O. indecora* coletada no Parque Estadual Intervales (SP) apresentando biciclogermacreno (29,79%), espatulenol (11,16%), valerianol (15.12%) e β-pineno (11,41%) como metabólitos majoritários (Gonçalves *et al.*, 2018).

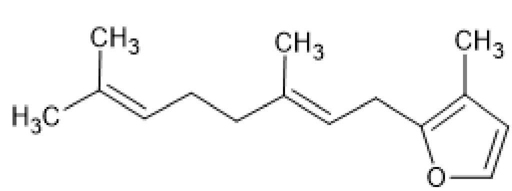


Figura 18: Estrutura química do metabólito sesquirosefurano. (Fonte: autor)

O potencial biológico desta espécie é pouco abordada na literatura. Figueiredo et. al. (2018) avaliou a ação repelente e acaricida do óleo essencial em larvas e adultos de carrapatos bovinos (*Rhipicephalus (Boophilus) microplus*). A repelência observada foi 95,8% em concentrações entre 0,78 e 100 mg/mL. A CL_{50} em carrapatos adultos e larvas após 48h foi de 4,96 mg/mL e 50 mg/mL, respectivamente (Figueiredo *et al.*, 2018). Adicionalmente, o óleo essencial a ação inseticida em percevejos pragas do algodão *Dysdercus peruvianus* resultando em LD_{50} de 162,2 µg/inseto e indicativo de inibição da enzima acetilcolinesterase (CI=1,37 mg/mL) (Nascimento *et al.*, 2020).

2. JUSTIFICATIVA

A estratégia convencional para diminuir os casos de doenças transmitidas por vetores e hospedeiros, como o *Aedes aegypti* para arboviroses e *Biomphalaria glabrata* para esquistossomose, é a prevenção por meio de medicamentos e vacinas. Porém, este método depende da disponibilidade dos produtos e é uma estratégia de redução de casos à longo prazo (Dusfour *et al.*, 2019). Entretanto, em ocorrência de surtos locais ou regionais a estratégia global imediata é o uso de inseticidas ou moluscicidas para o controle das populações de vetores e hospedeiros. Desta forma, as opções inseticidas e moluscicidas disponíveis para utilização na saúde pública são limitados. Para mosquitos os inseticidas preconizados são o temephós ou metoprene e apenas niclosamida para os caramujos *Biomphalaria sp.* (FUNASA, 2001).

O programa de controle de vetores no Brasil é realizado a décadas. Contudo, a utilização destes pesticidas a longo prazo levou a seleção de cepas resistentes, relatos descrevem *Aedes aegypti* temephós resistente desde 1998 (Santos Dias, dos *et al.*, 2017). O mesmo fenômeno de resistência pode ser observado em caramujos *Biomphalaria sp.* transmissores da esquistossomose, uma vez que apenas a niclosamida é recomendada pela OMS para controle de moluscos (Coelho e Caldeira, 2016; Faria *et al.*, 2018).

A toxidez dos pesticidas também é uma problemática que deve ser levada em consideração. Apesar de os inseticidas e moluscicidas serem desenvolvidos para atuação em alvos específicos, não são seletivos, ocasionando grande impacto ambiental em espécies não alvo. Além de apresentarem efeitos tóxicos aos humanos. O consumo humano continuado de doses subletais de pesticidas vem sido associado ao aumento dos casos de câncer na sociedade (Ansari, Moraiet e Ahmad, 2014).

Desta forma, novas alternativas para o controle de *Aedes aegypti* e *Biomphalaria sp* são necessárias. O metabolismo secundário vegetal pode atuar como modificador natural de respostas biológicas. Sendo assim, a caracterização química de novas espécies vegetais pode gerar a descoberta de novas substâncias bioativas, e por conseguinte, novas formulações podem vir a ser desenvolvidas gerando novos bioprodutos. Entre as espécies do Parque Nacional da Restinga de Jurubatiba (PNRJ) estudadas no Laboratório de Tecnologia de Produtos Naturais, cita-se a *Myrciaria floribunda* que apresentou ação inseticida frente à *Dysdercus peruvianus* e

Oncopeltus fasciatus, *Zanthoxylum caribaeum* que possuiu ação inseticida frente à *Rhodnius prolixus e Baccharis reticularia* que apresentou ação larvicida contra *Aedes aegypti*, *Manilkara subsericea* e *Xylopia ochrantha* apresentaram letalidade moluscicida em *Biomphalaria glabrata* sugerindo que estas ou outras espécies da Mata Atlântica podem ser utilizadas biotecnologicamente para obtenção de produtos com efeito inseticida e moluscicida significativo e ecologicamente sustentável (Araújo *et al.*, 2019; Botas, da *et al.*, 2017; Faria *et al.*, 2018; Nogueira *et al.*, 2014; Tietbohl *et al.*, 2014).

3. OBJETIVOS

3.1 Objetivo geral

Preparar nanoemulsões contendo óleo essencial de *Ocotea indecora* e *Myrciaria floribunda* para o controle de *Aedes aegypti* e da esquistossomose.

3.2 Objetivos específicos

- Extrair e caracterizar quimicamente os óleos essenciais;
- Preparar e caracterizar nanoemulsões com óleo essencial de *Myrciaria floribunda* e *Ocotea indecora;*
- Avaliar ação larvicida da nanoemulsão de *Myrciaria floribunda e Ocotea indecora* em larvas de *Aedes aegypti;*
- Avaliar ação moluscicida e cercaricida da nanoemulsão de *Myrciaria floribunda* em *Biomphalaria glabrata* e *Schistosma mansoni;*
- Avaliar ação moluscicida da nanoemulsão de *Ocotea indecora* em *Biomphalaria glabrata;*
- Avaliar toxidez da nanoemulsão de *Myrciaria floribunda* em *Danio rerio;*
- Avaliar toxidez da nanoemulsão de *Ocotea indecora* em *Apis mellifera;*

CAPÍTULO II – MATERIAIS E MÉTODOS

4. MATERIAIS E MÉTODOS

4.1 Coleta do material vegetal

As folhas de *Ocotea indecora* e *Myrciaria floribunda* foram coletadas no Parque Nacional da Restinga de Jurubatiba (PNRJ) no Rio de Janeiro (coordenadas: “22º12.683’S” “41º35.283’O”, “22º12.703’S” e “41º35.336’O”), a coleta e a pesquisa do material vegetal foi autorizada pelo SISBIO/ICMBio (13659–18) e SisGen (A491A56). A identificação da espécie foi realizada pelo botânico Professor Doutor Marcelo Guerra, da Universidade Estadual do Rio de Janeiro. A herborização dos materiais vegetais e as exsicatas foram depositadas no Herbário da Faculdade de Formação de Professores (FFP) sob número de registro RFFP: 16.873 e 13.789 da Universidade Estadual do Rio de Janeiro (UERJ).

4.2 Extração dos óleos essenciais

As folhas frescas de *O. indecora* e *M. floribunda* foram separadas do caule e turbolizadas em água destilada. O material vegetal turbolizado foi adicionado em um balão de fundo redondo de 5 L sob manta térmica e submetido à hidrodestilação em aparato do tipo Clevenger modificado durante 4h para a extração do óleo essencial. Ao final da extração os óleos essenciais foram secos com sulfato de sódio anidro, acondicionados em frasco de vidro âmbar e armazenados em freezer (-20 °C).

4.3 Caracterização química dos óleos essenciais

Os óleos essenciais foram analisados pelo GCMS-QP2010 (SHIMADZU), cromatógrafo a gás equipado com detector de massas por ionização por impacto de elétrons (70 eV) para identificação das substâncias químicas. As condições da cromatografia gasosa serão: temperatura de injeção, 260 °C; Hélio como gás de arraste, taxa de fluxo de 1 mL/min e injeção split com 01:40. Temperatura do forno foi inicialmente 60 °C e depois aumentou até 260°C a uma taxa de 3°C/min. Um microlitro de cada amostra, dissolvida em CH_2Cl_2 (1:100 mg/mL), foi injetado na coluna DB-5MS (id = 0,25 mm, comprimento 30 m, espessura = 0,25 µm). As condições da espectrometria de massa (MS) foram: voltagem de 70 eV e taxa de varredura 1 scan/s.

A composição percentual dos óleos será calculada pelo método de normalização das áreas de pico obtido por cromatografia gasosa. A identificação das substâncias será realizada por comparação do índice aritmético (IA), determinado em relação ao tempo de retenção de uma série de n-alcanos, com dados de referência correspondente pelo banco de dados do ADAMS (2017) e PHEROBASE (2020). O padrão de fragmentação do espectro de massas será comparado com bibliotecas de espectro de massa do *National Institute of Standards and Technology* (NIST).

4.4 Preparo e caracterização das formulações

As formulações contendo óleo essencial de *O. indecora* e *M. floribunda* foram individualmente preparadas pelo método de baixo aporte energético por inversão e fases como descrito por Ostertag et. al. (2012) com algumas modificações (Ostertag, Weiss e McClements, 2012b). A fase oleosa consistiu em 5 % (p/p) de óleo essencial, 5 % (p/p) de mistura de surfactantes (monooleato de sorbitano e polissorbato 20) e a fase aquosa consistiu de 90% (p/p) de água destilada. A composição das formulações está descrita na tabela 5. A fase oleosa foi homogeneizada sob agitação mecânica (600 rpm) por 30 minutos. Em seguida, a fase aquosa lentamente gotejada sob a fase oleosa com agitação mecânica contínua a 600 rpm por 60 min.

O tamanho de gotícula (nm), índice de polidispersão (IP) e potencial zeta (PZ) das formulações foram determinados pela técnica do Espalhamento Dinâmico da Luz (EDL) em

Zetasizer ZS90 (Malvern, UK). As formulações foram diluídas em água destilada (1:50). Após análise dos parâmetros, uma formulação foi selecionada com base no critério tamanho de partícula <200 nm e índice de polidispersão <0.3 para dar continuidade nos estudos de estabilidade e bioatividade. Todas análises foram realizadas em triplicata.

Tabela 5: Composição das formulações descrita no item 2.6.

Formulações	FO % (p/p)	FA % (p/p)	OE % (p/p)	Polisorbato 20 % (p/p)	Monooleato de sorbitano % (p/p)
F1	10,0	90,0	5,0	5,0	0,0
F2	10,0	90,0	5,0	4,5	0,5
F3	10,0	90,0	5,0	4,0	1,0
F4	10,0	90,0	5,0	3,5	1,5
F5	10,0	90,0	5,0	3,0	2,0
F6	10,0	90,0	5,0	2,5	2,5
F7	10,0	90,0	5,0	2,0	3,0
F8	10,0	90,0	5,0	1,5	3,5
F9	10,0	90,0	5,0	1,0	4,0
F10	10,0	90,0	5,0	0,5	4,5
F11	10,0	90,0	5,0	0,0	5,0

*FO, fase oleosa; FA, fase aquosa; OE, óleo essencial.

4.5 Determinação do requerido Equilíbrio Hidrófilo-Lipófilo (EHL) das fases oleosas

As formulações foram preparadas com faixa de EHL entre 4,3 (monooleato de sorbitano) e 16,7 (polisorbato 20). O valor de EHLr referente a mistura de surfactantes de cada formulação foi calculada pela equação (a):

$$EHLr = \frac{(HLBa\ x\ A\%+HLBb\ x\ B\%)}{100} \qquad (a)$$

Onde:

EHLr é o valor de EHL resultante da mistura de dois surfactantes;

EHLa é o valor de EHL do surfactante mais hidrofóbico;

EHLb é o valor de EHL do surfactante mais hidrofílico;

A% é o percentual do surfactante mais hidrofóbico;

B% é o percentual do surfactante mais hidrofílico;

A% + B% = 100%;

4.6 Estabilidade das nanoemulsões

Os estudos de estabilidade foram realizados a partir das nanoemulsões selecionadas. Desta forma, foram preparadas três nanoemulsões, para cada óleo essencial, e acondicionadas em frascos de vidro âmbar (5 mL) com batoque e tampa e acondicionadas em temperatura ambiente (25 °C), sob refrigeração (8 °C) e estufa (42 °C) no período entre 0 e 365 dias após a preparo. Foram avaliados os aspectos macroscópicos das nanoemulsões (cor, separação de fase, cremagem, floculação e sedimentação). As análises relacionadas ao tamanho de partícula e índice de polidispersão foram realizados como descrito no item 2.6.

4.7 Microscopia eletrônica de transmissão (MET)

As nanoemulsões promissoras do óleo essencial de *O. indecora* e *M. floribunda* foram submetidas a microscopia eletrônica de transmissão (MET) em modelo Morgagni 268/FEI. As nanoemulsões foram individualmente diluídas em água destila em proporção 1:1. Então, 5 µL foram adicionados em uma grade de cobre revestido como *formar*, secos em dessecador por 60 min, e por fim submetido para análise.

4.8 Planejamento fatorial da nanoemulsão de *O. indecora*

Um planejamento fatorial 2^3 foi realizado no programa *Statistica 12* com a nanoemulsão mais promissora do óleo essencial de *O. indecora*. A nanoemulsão inicial foi utilizada como ponto central e foi avaliado a influência e interação da quantidade de óleo essencial, quantidade de surfactantes e rotações por minuto (RPM) nas variáveis dependentes (variáveis resposta) tamanho de gotículas (nm) e índice de polidispersão de forma a otimizar a nanoemulsão. O planejamento experimental foi realizado como descrito na tabela 6. Todas formulações foram preparadas pelo método de baixo aporte de energia por inversão de fases descrito no item 2.6.

Tabela 6: Planejamento fatorial para otimização da nanoemulsão de *Ocotea indecora*.

Fatores	Níveis		
	Low (-1)	*Medium* (0)	*High* (+1)
Variáveis independentes			
Óleo essencial (%)	2,5	5,0	7,5
Surfactantes (%)	2,5	5,0	7,5
RPM	500	700	900
Variáveis dependents			
Tamanho de gotícula			
IP			

*IP: índice de polidispersão; RPM: rotações por minuto.

4.9 Cepas de *Aedes aegypti*

Os ovos de *Aedes aegypti* cepas Rockefeller susceptíveis a deltametrina e temephos foram cedidos pelo Laboratório de Fisiologia e Controle de Artrópodes Vetores (LAFICAVE) do Instituto Oswaldo Cruz, Rio de Janeiro, Brasil, para execução do item 2.12.

4.10 Avaliação Larvicida em *Aedes aegypti*

As propriedades larvicidas da nanoemulsão otimizada de *O. indecora* e *M. floribunda* foi realizado de acordo com a OMS (WHO, 2005), com modificações. O ensaio foi realizado no Laboratório de Biologia de Insetos da Universidade Federal Fluminense. Larvas de *Ae. aegypti* no terceiro instar (L3) (n=210) foram separadas e depositadas em recipientes plásticos de polipropileno (30 mL) com 10 mL da nanoemulsão em concentrações de 200 a 12.5 µg/mL (valores expressos em óleo essencial). Os grupos experimentais consistiram em: controle negativo (água destilada), branco da nanoemulsão a 200 µg/mL (sem óleo essencial) e o controle positivo imidacloprid (1 µg/mL). Todos grupos experimentais foram avaliados até o controle negativo chegar na idade adulto (6 dias). Todo ensaio foi realizado em triplicata. A estimativa da concentração letal média (CL_{50}) foi realizado por análise de probit com programa SAS (*SAS institute* Inc., 2008). O tempo letal médio (TL_{50}) foi obtido através dos estimadores Kaplan-Meier (método Log-rank) por meio do programa Sigmaplot 12.0 (*Systat software*, San Jose, Califórnia, EUA). A curva de sobrevivência foi obtida utilizando o método de Holm-Sidak.

4.11 Avaliação morfológica das larvas de *Aedes aegypti*

A morfologia das larvas de *Ae. aegypti* foi obtida como descrito por Pessoa et. al. (2018) (Pessoa *et al.*, 2018). As larvas foram incubadas por 24 h com a nanoemulsão otimizada de *O. indecora* (250 µg/mL), com exceção do controle negativo. Após isso, as larvas foram fixadas com etanol 70%, secas a temperatura ambiente, e avaliado em microscópio eletrônico de varredura à baixo vácuo modelo Tabletop TM3030Plus (Hitachi, Ibaraki, Japão).

4.12 Docagem molecular de sesquirosefuran em enzima acetilcolinesterase de *Ae. aegypti*

As sequências de aminoácidos para enzima acetilcolinesterase foram encontradas no banco de dados *National Center for Biotechnology Information* (NCBI). Desta forma, foi utilizado a sequência UniProtKB da *Swiss-Prot*: Q6A2E2. Depois disso, identificamos o modelo do Banco de dados *Protein Data Bank* (PDB) das sequências de aminoácidos usando a ferramenta BLASTp, baixada do Banco de Dados de Proteínas (https://www.rcsb.org/), considerando o método experimental, a resolução e o valor de R como parâmetros de qualidade. O modelo PDB baixado foi 6ARY referente à *Anopheles gambiae* com 92,41% de identidade. A enzima acetilcolinesterase foi construída usando uma abordagem de modelagem homológica usando o *Swiss Model Workspace* (https://swissmodel.expasy.org/), com quebras na estrutura de proteínas, e posições de aminoácidos nos sítios de ligação (Waterhouse *et al.*, 2018), as parcelas Ramachandran (Haas *et al.*, 2018; Ramachandran, Illinois e Sasisekharan, 1968), e o fator QMEAN (Benkert, Biasini e Schwede, 2011) também foram considerados. Em seguida, foram preparadas as substâncias majoritárias presentes no óleo essencial de *O. indecora* β-farneseno e sesquirosefurano usando PubChem (Kim *et al.*, 2019) no NCBI e os armazenamos em SDF (*Structure Data Format*) para previsões de docagem molecular. Estas moléculas e receptores foram preparados com o programa *Autodock Tools* 1.5.7.44 (Sanner, 1999). O melhor complexo ligante-receptor, e seus valores de energia de afinidade (kcal/mol) por meio do *AutoDock Vina* (Trott e Olson, 2009), foi utilizado para gerar mapas de interação 2D com o programa *Discovery Studio* (Dassault Systemes, 2017).

4.13 Toxidez oral aguda da nanoemulsão de *O. indecora* em inseto não-alvo *Apis mellifera*

O bioensaio de segurança será realizado pela exposição oral de abelha *Apis mellifera* à concentração de 250 µg/mL. Os bioensaios foram realizados na Universidade Federal de Viçosa (UFV, Viçosa, MG, Brazil [20°45′ S, 42°52′ O]). A nanoemulsão otimizada foi diluída em solução de xarope a base de açúcar (50%, v/v) e oferecidos às abelhas em tubos Eppendorf de 2 ml inseridos em recipientes plásticos de baixa densidade (500 mL). Cada recipiente plástico foi usado como uma unidade experimental (n=10), onde as abelhas foram alimentadas com 1 mL de solução açucarada contendo a nanoemulsão à 5% de óleo essencial de folhas de *O.*

indecora na concentração de 250 µg/mL (exceto para abelhas não tratadas, ou seja, controle). As abelhas permaneceram em jejum durante 1 h antes de permitirem o acesso à dieta contaminada com a nanoemulsão. Após 5 horas de exposição oral à nanoemulsão, as abelhas receberam uma dieta livre de contaminação, e a mortalidade foi registrada 24 horas após a reposição da dieta. As abelhas foram consideradas mortas se não conseguissem se mover quando tocadas com uma pinça. Todas análises foram realizados em quadruplicata. Cada réplica consistiu de um recipiente plástico contendo abelhas da mesma colônia, e três a seis colônias diferentes foram usadas nos bioensaios para explicar a variação intercolônica na resposta (Tomé *et al.*, 2017).

4.14 Avaliação ovicida de nanoemulsão de *M. floribunda* em *Biomphalaria glabrata*

A capacidade ovicida da nanoemulsão de *M. floribunda* foi testada em embriões de *B. glabrata* com 72 h de vida. Para isto, em triplicata, seguindo a metodologia de (Araújo et. al. 2019) utilizando placas de 24 poços descartáveis, os embriões (n=225) foram colocados em poços e expostos a 2 mL da nanoemulsão de *M. floribunda* (Araújo *et al.*, 2019). Os embriões foram expostos durante 48 h à concentrações de 100 a 10 µg/mL (expresso em óleo essencial). A mortalidade foi avaliada após 24 h e 48 h. O controle negativo foi água destilada, o controle positivo foi niclosamida (2 µg/mL) e o branco da nanoemulsão (sem óleo essencial) à 100 µg/mL também foi avaliado.

4.15 Avaliação moluscicida da nanoemulsão de *O. indecora* e *M. floribunda* em *Biomphalaria glabrata*

Os moluscos *Biomphalaria glabrata* foram coletados em Sumidoro (22º02'59"S, 42º40'29"O), Rio de Janeiro, Brasil, e foram mantidos em tanques de reprodução no Pavilhão

Lauro Travassos do Instituto Oswaldo Cruz, no estado do Rio de Janeiro. Os moluscos foram mantidos em água sem cloro e alimentados com alface fresca (*Lactuca sativa* L.).

Os ensaios moluscicida (n=162) foram realizados utilizando placas de 24 poços descartáveis contendo nanoemulsão de *M. floribunda* em concentrações de 100 à 10 µg/mL (expresso em óleo essencial). Depois disso, moluscos juvenis (6 - 8 mm) e adultos (10 – 12 mm) foram colocados individualmente nos poços. O mesmo volume foi utilizado para o controle negativo (água destilada), branco da nanoemulsão à 100 µg/mL (sem óleo essencial) e controle positivo (niclosamida à 2 µg/mL). A mortalidade foi avaliada após 24 h e 48 h após exposição. Os critérios de mortalidade foram liberação de hemolinfa, ausência de retração ou retração exagerada na concha.

4.16 Avaliação cercaricida da nanoemulsão de *M. floribunda* em *Schistossoma mansoni*

A nanoemulsão de *M. floribunda* também foi avaliado contra cercarias do parasita S. mansoni (n=2160). Para este fim, em triplicata, a mortalidade das cercarárias em suspensão na nanoemulsão (2 mL) de *M. floribunda* em placas de 24 poços descartáveis. Cada grupo experimental (n=80) foi exposto a nanoemulsão em concentrações de 100 a 10 µg/mL (expresso em óleo essencial) por 4 h. A mortalidade foi avaliada a cada hora após exposição. O controle negativo consistiu de água destilada, o controle positivo foi niclosamida à 2 µg/mL e o branco da nanoemulsão (sem óleo essencial) à 100 µg/mL.

4.17 Toxidez ambiental *in silico* do óleo essencial de *M. floribunda*

A avaliação da toxidez ambiental foi realizada no programa *ADMET Predictor*^{TM} (versão 9.5 *Simulations Plus*, Lancaster, Califórnia) para avaliar a estrutura molecular e dados experimentais para criar modelos *QSAR* para prever as propriedades biológicas das substâncias majoritárias do óleo essencial de *M. floribunda*. Os parâmetros avaliados foram bioconcentração, biodegradação, toxicidade aquática em diferentes níveis tróficos (*Tetrahymena pyriformis, Daphnia magna e Pimephales promelas*), toxicidade endócrina e risco toxicológico.

4.18 Toxidez aguda oral da nanoemulsão de *M. floribunda* em *Danio rerio*

O experimento seguiu a regulamentação de bem-estar animal, The ARRIVE guidelines (Kilkenny *et al.*, 2010) e, aprovado pelo Comitê de Ética do Instituto Vital Brazil protocolo número 003/2019. *Danio rerio* (*Zebrafish*) machos pesando 300 - 400 mg, fornecidos pelo Laboratório de Métodos Alternativos ao Uso de Animais do Instituto Vital Brazil e foram mantidos em *rack*, com os parâmetros de controle da água pH = 7,0 ± 1, temperatura 26 ± 2 o C, amônia = 0 ppm, fotoperíodo (claro/escuro) = 12h/12h.

Dez animais foram distribuídos em igual número e de forma aleatória em cinco aquários de experimentação (15 x 8 x 12 cm) contendo 1L de água. A dose correspondendo à 50 µg do óleo essencial de folhas de *Myrciaria floribunda* contido na NE /animal foi administrada por via oral (gavagem). Observou-se os sinais clínicos relacionados a equilíbrio, comportamento natatório, função ventilatória, pigmentação da pele e anormalidade visíveis nos intervalos 0, 3, 24, 30 e 48 h após administração oral. Os animais foram pesados antes e após o experimento (OECD, 2019). A avaliação e o uso de sinais clínicos para avaliação de pontos finais humanizados foram observados (OECD, 2000). Ao final, os animais foram eutanasiados utilizando solução de eugenol (CONCEA, 2015).

4.19 Análise estatística

A estimativa dos valores de CL_{50} foi realizado por análise de Probit com o programa *SAS* (*SAS institute Inc*., 2008), e a análise de variância unidirecional (ANOVA) seguido pelo pós-teste de Tukey foi realizado usando o *GraphPad Prism* (versão 8) com nível de significância de $p < 0,05$.

5. CAPÍTULO III - ARTIGO 1

5.1 Nanoemulsion of *Ocotea indecora* (Shott) Mez essential oil: larvicidal effects against *Aedes aegypti*

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Nanoemulsion of *Ocotea indecora* (Shott) Mez essential oil: larvicidal effects against *Aedes aegypti*

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Abstract

The widespread use of insecticide can lead to the resistance of the *Aedes aegypti* mosquito and adverse effects on non-target organisms, such as humans, other animals, and insects. In this sense, nanotechnology associated with natural products is a promising alternative to obtaining safer and more sustainable insecticide formulations against this vector. Therefore, in this research, we developed and optimized a nanoemulsion with essential oil from *Ocotea indecora* (Shott) Mez leaves and evaluated its larvicidal properties against *Ae. aegypti* larvae. In addition, oral toxicity assays were performed to test the nanoemulsion safety of the non-target organism *Apis mellifera*. The major constituent found was sesquirosefuran (81.4 %). The nanoemulsions were prepared by the low-energy method by phase inversion and characterized by the dynamic light scattering technique. The most suitable surfactant mixture was in hydrophilic-lipophilic balance 14.22, presenting droplets size of 122.8 nm and polydispersity index of 0.262. Then a 2^3 factorial design was realized to optimize the formulation suggesting the variables conditions of 1:1 of essential oil (5% w/w) and surfactants at 500 rotations per minute. This led to spherical nanoemulsions with mean size and PdI of 105.3 nm and 0.263, respectively. The optimized nanoemulsion presented stability when stored at room temperature and refrigerated for up to one year. The LC_{50} values against *Ae. aegypti larvae* were 61.4, and 26.8 µg/mL after, 48, and 144 h, respectively. Scanning electron micrography showed morphological body alterations on the larvae *Ae. aegypti* treated with the nanoemulsion. Regarding the ecotoxicological evaluation, the nanoemulsion showed no toxicity against *Apis mellifera*. Therefore, this work demonstrated a simple method to obtain *O. indecora* nanoemulsion as an environmental-friendlier alternative to the *Aedes aegypti* control.

Keywords*:* Vector control; larvicide; Lauraceae; Nanotechnology; Nanodispersion; Insecticide

1. Introduction

Aedes aegypti (Diptera: Culicidae), popularly known as “Dengue Mosquito”, is considered of medical importance in public health due to its ability to transmit several arboviruses, such as Dengue, Zika, Chikungunya, urban yellow fever, and Mayaro (Silvério et al., 2020). The *Ae. aegypti* life cycle is holometabolic and consists of 4 stages: egg, larvae, pupa, and adult. Even though the adult mosquito flies freely in terrestrial environments, *Ae. aegypti* spends its entire immature development in aquatic settings. Initially, the eggs hatch with water contact and start the aquatic period, with four larval stages (L1-L4) followed by the pupal stage (Silvério et al., 2020). The complete aquatic cycle lasts 10 to 15 days and varies according to environmental conditions. For instance, a higher temperature can speed the life cycle of *Ae. aegypti*. The aquatic phases of *Ae. aegypti* present an opportunity to manage this pest more efficiently than in the winged adult form (Moura et al., 2021).

The control of insects that can cause epidemic diseases in humans is often carried with insecticides. However, these products can implicate harmful effects on human health and the environment (van den Berg et al., 2021). Insecticides act quickly and effectively, reducing new infections and mortality in populations exposed to vector-transmitted diseases (Duarte et al., 2020). Unfortunately, in addition to their high toxicity, most insecticides are non-selective to other insects such as pollinators, reducing biodiversity (Senthil-Nathan, 2020; van den Berg et al., 2021).

Therefore, the search for new sustainable alternatives for the *Ae. aegypti* control is crucial. Nanotechnology of natural products, such as essential oils, is a biotechnological approach to developing new pesticides (Pavoni et al., 2019). These biorational strategies present a more sustainable option with less risk to human health and the environment (Duarte et al., 2020). Nanoemulsions are dispersed systems constituted by two immiscible liquids stabilized by one or more surfactants(Mustafa and Hussein, 2020). Theyre kinetically stable and thermodynamically unstable, with droplet size between 20-200 nm and polydispersity index (PdI) below 0.3 (Marhamati et al., 2021). They present improved cell penetration and increased stability of the bioactive compounds. In addition, due to the low water miscibility of essential oils, nanoemulsions allow better dispersion of its compounds in aqueous media, thus optimizing larvicidal activity. (Folly et al., 2021; Martins et al., 2021; Sharma et al., 2020).

Ocotea indecora (Shott) Mez belongs to the Lauraceae family and is popularly known as “Canela-sassafrás”. This plant is native and endemic to Brazil and is found in the Restinga de Jurubatiba National Park in Rio de Janeiro, Brazil. Phytochemical studies conducted on the essential oil of *O. indecora* demonstrated the presence of the sesquiterpene sesquirosefuran as the major component. (Figueiredo et al., 2018; Nascimento et al., 2020). Insecticidal activities of this essential oil were previously reported against the tick *Rhipicephalus microplus* and *Dysdercus peruvianus* (Figueiredo et al., 2018; Nascimento et al., 2020). The present work aimed to prepare and characterize nanoemulsions with *Ocotea indecora* essential oil using an organic solvent-free method and test the optimized formulation for its larvicidal activity against *Ae. Aegypti*. Finally, the safety of the nanoemulsion was tested via ecotoxicological assays against the non-target pollinator *Apis mellifera* (Hymenoptera: Apidae).

.2. Methodology

2.1 Plant material

The leaves of *O. indecora* were collected in the Restinga de Jurubatiba National Park in Rio de Janeiro on August 25, 2019 ("22º12.683’S", "41º35.283’O", "22º12.703’S" and "41º35.336’O"). SisBio/ICMBio (13659-14) and SisGen (A0D648D) authorized the collection and research of the plant material. In addition, a voucher for the specimen was deposited in the Herbarium of the Faculty of Teacher Training (FFP) (RFFP: 16.873) of the State University of Rio de Janeiro (UERJ), Brazil.

2.2 Essential oil extraction

The fresh *O. indecora* leaves were separated from the stem and crushed in distilled water. The plant material was placed in a 5 L bottom-round flask and subjected to hydrodistillation in a modified Clevenger-type apparatus for 4 hours. The essential oil obtained was stored at 4 ºC and protected from light.

2.3 Essential oil characterization

The essential oil was analyzed using GC-MS QP2010 (Shimadzu) gas chromatograph equipped with a mass spectrometer and a GC-2014 (Shimadzu) gas chromatograph equipped with a flame ionization detector (FID). Gas chromatographic (GC) conditions were as follows: injector temperature, 260 °C; Helium as carrier gas; flow rate, 1 ml/min and split injection with split ratio 1:40. The oven temperature was initially 60 °C and then increased to 290 °C at a 3 °C/min rate. One microliter of the sample, dissolved in dichloromethane (1:100 mg/µl) was injected into an RTX-5 column (0.25 mm ID, 30 m in length, 0.25 µm film thickness). Mass spectrometry (MS) electron ionization was 70 eV, and the scan rate was 1 scan/s. GC-FID conditions were similar to the MS, except for the injection in an RTX-5 column (0.25 mm ID, 30 m in length, 0.25 µm film thickness) and FID temperature at 290 °C. The arithmetic Index (AI) was calculated by interpolating the retention times of a mixture of aliphatic hydrocarbons (C9-C30) analyzed under the same condition. Substances were identified by comparing their retention indices and mass spectra with those reported in the literature (Adams, 2017; El-Sayed, 2021). MS fragmentation pattern of compounds was also compared with NIST mass spectrum libraries. The relative abundance of the chemical constituents was performed by flame ionization gas chromatography (GC-FID) at a GC-2014 (Shimadzu) under the same conditions as GC-MS. The FID peak area normalization method obtained the analysis and percentages of these compounds.

2.4 Nanoemulsion preparation and required Hydrophile Lipophile Balance (HLB) determination

The nanoemulsions were prepared by the low-energy method by phase inversion (Ostertag et al., 2012). Eleven formulations were prepared to contain different proportions of the surfactants sorbitan monooleate 20 and polysorbate 80, with the Hydrophile Lipophile Balance (HLB) range between 4.3 and 16.7 (Table 1S). The formulations contained 5% (w/w) essential oil, 5% (w/w) of surfactant blend, and 90% aqueous phase. The essential oil and surfactants were homogenized by mechanical agitation for 30 minutes. Then, the aqueous phase was slowly dropped onto the oil phase with the same mechanical agitation for 60 min.

2.5 Nanoemulsion characterization

The nanoemulsions were characterized by Dynamic Light Scattering (DLS) in a Zetasizer 5000 (Malvern, UK). The nanoemulsions were diluted in distilled water (1:50) and evaluated the parameters droplet size (nm) and polydispersity index (PdI). All measurements were made in triplicate. Values reported refers to the means ± standard deviation of at least three different batches of each formulation.

1.0 2.6 Factorial design 2^3

An experimental design 2^3 was performed by the software *Statistica 12* with the most promisor formulation of item 2.5 as a center point to evaluate the influence and the interaction of the amount of the independent variable of essential oil, surfactants, and the rotation per minute (RPM) on the dependent variables droplet size (nm) and polydispersion index. The amount of essential oil and surfactants used were 2.5% (low level), 5% (center point) and 7.5% (high level). The RPM were 500 (low level), 700 (center point) and 900 (high level) (Table 1). The criteria to determine the optimum formulation of *O. indecora* essential oil was based on the smallest droplets size and polydispersity index. All nanoemulsions were prepared by the low-energy method described in item 2.5.

Table 1: Factorial design for preparation of *Ocotea indecora* nanoemulsion.

Factor	Level		
	Low (-1)	Medium (0)	High (+1)
Independent variables			
Essential oil (%)	2.5	5.0	7.5
Surfactants (%)	2.5	5.0	7.5
RPM	500	700	900
Dependent variables			
Droplets size (nm)			
Polydispersity index			

2.7 Long-term stability

Three optimized nanoemulsions were prepared and stored in amber glass vials at room temperature (25 °C), under refrigeration (8 °C), and climatic chamber (42 °C). The size and PdI analysis were realized in different time intervals after preparation by DLS and Zeta potential (ZP). In addition, the parameters color, appearance, phase separation, presence of cremation, and sedimentation were evaluated macroscopically.

2.8 Transmission Electron Microscopy (TEM)

The nanoemulsion optimized (NEOI-OPT) was submitted to morphology characterization in a transmission electron microscope (TEM) model Morgagni 268/FEI. First, the nanoemulsion was diluted in distilled water at a 1:1 ratio. Then 5 µL were added to a copper grid with formvar, dried in a desiccator for 1h, and then submitted to analysis.

2.9 Larvicidal activity against *Aedes aegypti*

The larvicidal properties of the optimal nanoemulsion (NEOI-OPT) of the essential oil from *O. indecora* leaves were evaluated according to WHO (2005) with some modifications. Third-instar larvae (L3) of *Ae. aegypti* (n = 210) were separated and deposited in polypropylene plastic containers (30 mL) with 10 mL of the nanoemulsion at concentrations of 200, 100, 50, 25, and 12.5 µg/mL (expressed in essential oil). The negative control consisted of 10 mL of distilled water and the positive control was imidacloprid at 1 µg/mL. The blank nanoemulsion (without essential oil) at 200 µg/mL was also evaluated. All experimental groups were recorded until the control group reached adulthood which occurred in 6 days. All trials were performed in triplicate. The LC_{50} estimations were carried out by Probit analysis using the SAS software (SAS Institute Inc., 2018). The median lethal-time (LT_{50}) estimations were achieved by applying the Kaplan–Meier estimators (Log-rank method. Available at SigmaPlot 12.0 software - Systat Software, San Jose, California, USA) to the analyze the survival bioassays results. The survival curve comparisons were achieved using Holm-Sidak’s method.

2.10 Larvae morphological study

The morphology of *Aedes aegypti* larvae was obtained according to Pessoa et al. (2018). Briefly, the larvae were incubated with essential oil optimal nanoemulsion of *O. indecora* at 250 µg/mL, except for the control group. Then, after 24 h they were fixed on ethanol 70%, dried in the air, and evaluated by scanning electron microscopy under a low vacuum by a Tabletop Microscope TM3030Plus (Hitachi, Ibaraki, Japan).

2.11 Molecular docking between essential oil biomolecules of *Ocotea indecora* and acetylcholinesterase enzyme of *Aedes aegypti*

Amino acid sequences of the acetylcholinesterase for predictions were found in the National Center for Biotechnology Information (NCBI) database with complete annotation. Hence, we used the sequence UniProtKB of Swiss-Prot: Q6A2E2. After that, we identified the Protein Data Bank (PDB) template of the amino acid sequences using the BLASTp tool, downloaded from the Protein Databank (https://www.rcsb.org/), considering the experimental method, resolution, and R-value as quality parameters. The PDB template downloaded was 6ARY of *Anopheles gambiae* with 92.41% identity. The acetylcholinesterase enzyme was built using a homology modeling approach using the Swiss Model Workspace (https://swissmodel.expasy.org/), with protein structure crashes, and amino acid positions at the binding pockets (Waterhouse et al., 2018), the Ramachandran plots (Haas et al., 2018; Ramachandran et al., 1968), and QMEAN factor (Benkert et al., 2011) were inspected. Next, we prepared the β-farnesene and sesquirosefuran molecules of *O. indecora* oil using PubChem (Kim et al., 2019) at NCBI and stored them in SDF (Structure Data Format) for molecular docking predictions. These molecules and receptors were prepared with Autodock Tools 1.5.7.44(Sanner, 1999). The best ligand-receptor complex, which returned affinity energy values (kcal/mol) using the AutoDock Vina (Trott and Olson, 2009), was used to generate 2D interaction maps with Discovery Studio (Dassault Systemes, 2017).

2.12 Acute toxicity of the nanoemulsion to non-target insect *Apis mellifera*

The safety bioassay was performed by the oral exposure of the *O. indecora* nanoemulsion to *Apis mellifera* at 250 µg/mL. Bioassays were held at the Federal University of Viçosa (UFV, Viçosa, MG, Brazil (20° 45', 42° 52' W). The NEOI-OPT was diluted in sugar-based syrup (50 %, v/v) and offered to the bees in 2 mL Eppendorf tubes inserted into low-density plastic containers (500 mL). Each plastic container was used as an experimental unit (n = 10), where bees were fed with 1 mL of a sugary solution containing nanoemulsion at 250 µg/mL. Control treatment received sugary solution only. Bees remained fastened for 1 h before accessing the diets. After 5 hours, bees that were fed the nanoemulsion-contaminated diet received an uncontaminated diet (sugary solution only), and mortality was recorded 24 hours after the beginning of the exposure. The bees were considered dead if they could not move when touched with forceps. Four replicates were performed for each treatment. Each replicate consisted of a plastic container containing bees from the same colony. Four to six different colonies were used in each treatment to explain the intercolonial variation in the response. All experimental groups were replicated three times (Tomé et al., 2017).

3. Results

3.1 Chemical characterization of the *Ocotea indecora* essential oil

The essential oil presented a yield of 0.69 % (w/w). It was possible to characterize 88.2 % of the *O. indecora* essential oil components. The chemical profile was composed of sesquirosefuran (81.4 %), β-farnesene (4.7 %), dehydro-aromadendrene (0.62 %), bicyclogermacrene (0.95 %), and (2E,6E) -methyl farnesoate (0.51 %) (Table 2S).

3.2 Nanoemulsion preparation and required Hydrophile Lipophile Balance HLB determination

From the 11 formulations prepared, 7 formulations had high mean droplet size and polydispersity index (Table 3S). The best results were obtained with the formulation F3 with HLB 14.22, which had the smallest droplet size (122.8 ± 1.07 nm) and PdI (0.262 ± 0.029). Furthermore, the F3 showed a slightly bluish-white coloration. Formulations F10 and F11 showed phase separation and were discarded from the DLS analysis.

3.3 Factorial design 2^3

Table 2 shows the matrix of the three-factor, two-level factorial design and the responses in the dependent variable’s droplet size and PdI. Figure 1 shows Pareto's charts, the interaction between the independent variable amount of essential oil and surfactants significantly influenced (*p*-value of 0.0153) the size of the nanoemulsion droplets. However, it did not affect the polydispersity index. On the other hand, figure 2 shows three-dimensional graphs of independent variables' effects on the droplets' size. The nanoemulsion with the lower essential oil amount, with a higher amount of surfactants, leads to smaller values of droplets and PdI (Figures 2A and D). The RPM did not influence the diameter of the droplets or PdI but indicated better values with lower RPM (figure 2B, C, E, and F). These data revealed the optimal formulation conditions of 500 RPM, 7.5 % of surfactants, and 2.5 % of essential oil. However, the formulation chosen as the optimal nanoemulsion for the *O. indecora* oil (NEOI-OPT) presented 500 RPM, 5.0 % of essential oils, and surfactants. Figure 3A shows the size distribution of the NEOI-OPT with an average size of 105.3 ± 1.36 nm and PdI of 0.263 ± 0.004. The models fitted well with R^2 of 0.88, and 0.85 to mean size, and PdI, respectively.

Table 2: Observed responses from the factorial design of *O. indecora* nanoemulsion.

	Independent variables			Dependent variables	
	Essential oil (%)	Surfactants (%)	RPM	Droplet size (nm)	PdI
1	2.5	2.5	500	125.3±0.5	0.273±0.011
2	7.5	2.5	500	373.4±20.4	0.269±0.258
3	2.5	7.5	500	176.8±3.8	0.299±0.028
4	7.5	7.5	500	123.2±1.7	0.268±0.014
5	2.5	2.5	900	122.2±2.7	0.252±0.016
6	7.5	2.5	900	431.6±5.7	0.510±0.093
7	2.5	7.5	900	202.5±3.7	0.259±0.010
8	7.5	7.5	900	122.2±1.9	0.282±0.010
9 (C)	5.0	5.0	700	131.9±1.6	0.272±0.020
10 (C)	5.0	5.0	700	134.5±2.4	0.262±0.011
11 (C)	5.0	5.0	700	126.7±2.6	0.263±0.001

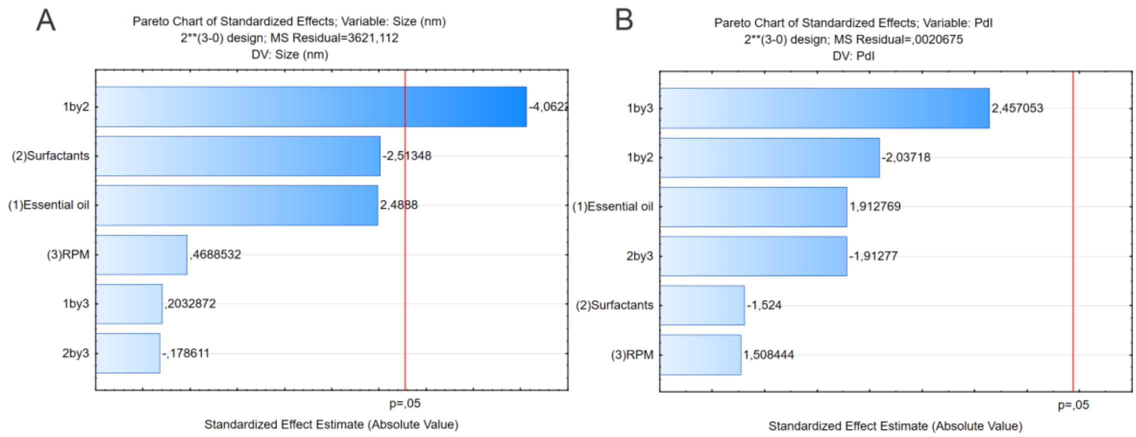

Figure 1: Pareto charts for the variable (A) droplet size and (B) polydispersity index.

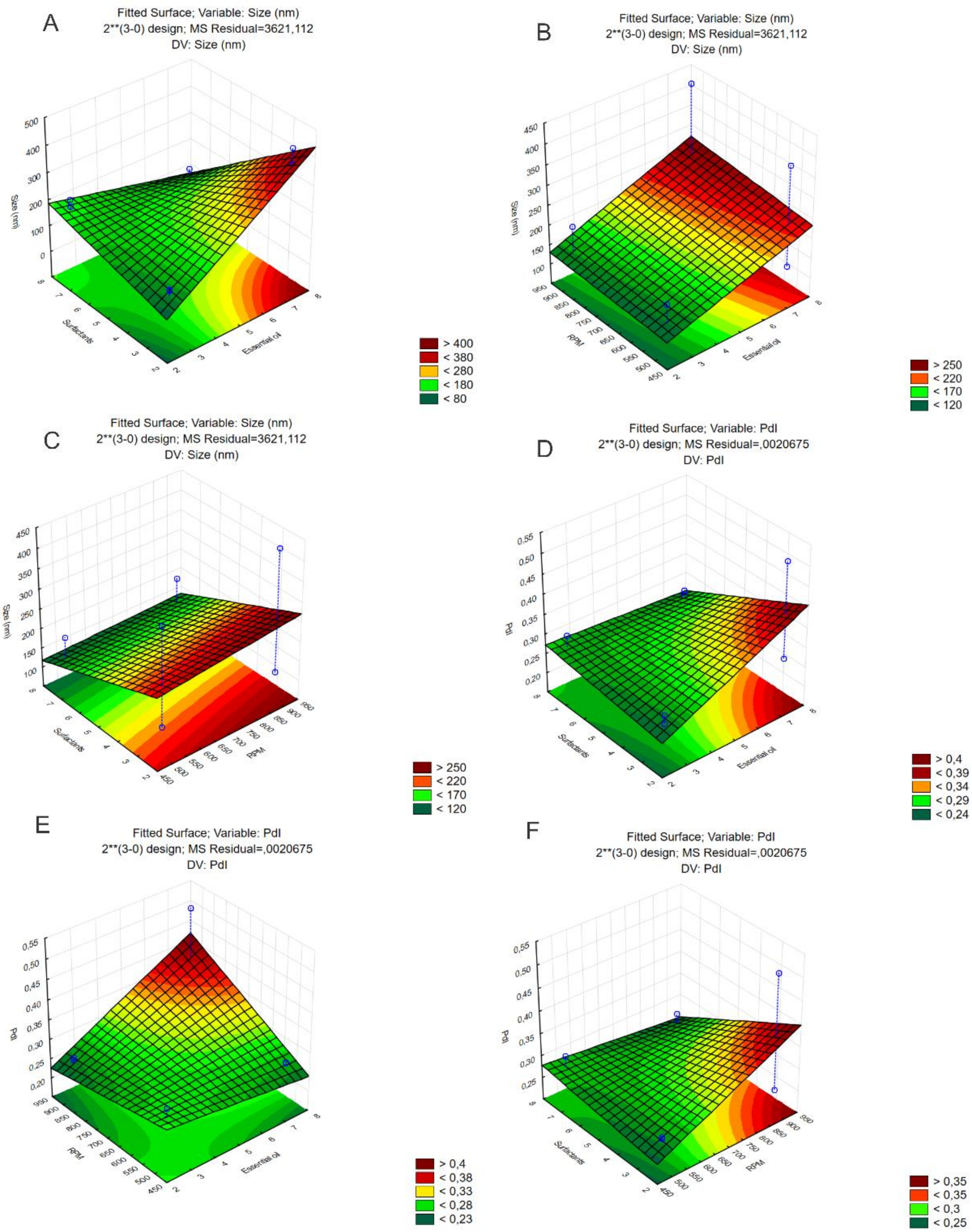

Figure 2: Surface graph for (A, B, and C) mean size (nm) and (D, E, and F) polydispersity index from the 2^3 experimental designs.

3.4 Long-term stability

The stability of the NEOI-OPT is shown in table 3. All three batches presented bluish-white coloration after preparation. The nanoemulsions stored at 25 °C and 4 °C maintained the initial aspect after 365 days of preparation. However, the nanoemulsion stored at 40 °C presented color alteration and instability signals, such as an increase in the size and reduced PdI values over time.

Table 3: NEOI-OPT average droplet size (nm) and PdI after 365 days of storage in three different temperatures

	25 °C ± 2		08 °C ± 2		42 °C ± 2	
Days	Size (nm)	PdI	Size (nm)	PdI	Size (nm)	PdI
T00	112.7±0.96	0.247±0.004	115.1±1.44	0.256±0.012	110.3±1.00	0.241±0.013
T07	111.3±1.05	0.242±0.026	115.9±1.12	0.267±0.003	105.0±0.56	0.243±0.014
T15	108.8±0.83	0.255±0.006	111.2±0.62	0.272±0.006	102.5±0.72	0.253±0.019
T30	106.8±1.36	0.260±0.004	110.8±3.50	0.296±0.035	96.8±3.21	0.267±0.009
T60	105.6±0.55	0.254±0.008	107.8±0.80	0.274±0.022	98.2±0.49	0.244±0.008
T90	101.3±0.70	0.232±0.005	106.5±1.00	0.279±0.005	96.2±0.75	0.209±0.023
T240	101.3±0.40	0.256±0.013	109.6±0.28	0.286±0.002	112.3±0.65	0.160±0.010
T365	95.19±0.84	0.229±0.010	101.7±0.35	0.291±0.021	160.7±0.77	0.075±0.018

*Zeta potencial = -23.8 ± 2.01 mV (T00) to -27.7±0.95 (T365).

3.5 Transmission Electron Microscopy (TEM)

Figure 3B shows spherical droplets of the NEOI-OPT in 89-K magnification. It can be seen in two droplets with a size of approximately 100 nm and several smaller droplets as subproducts of the laser beam degradation of the TEM.

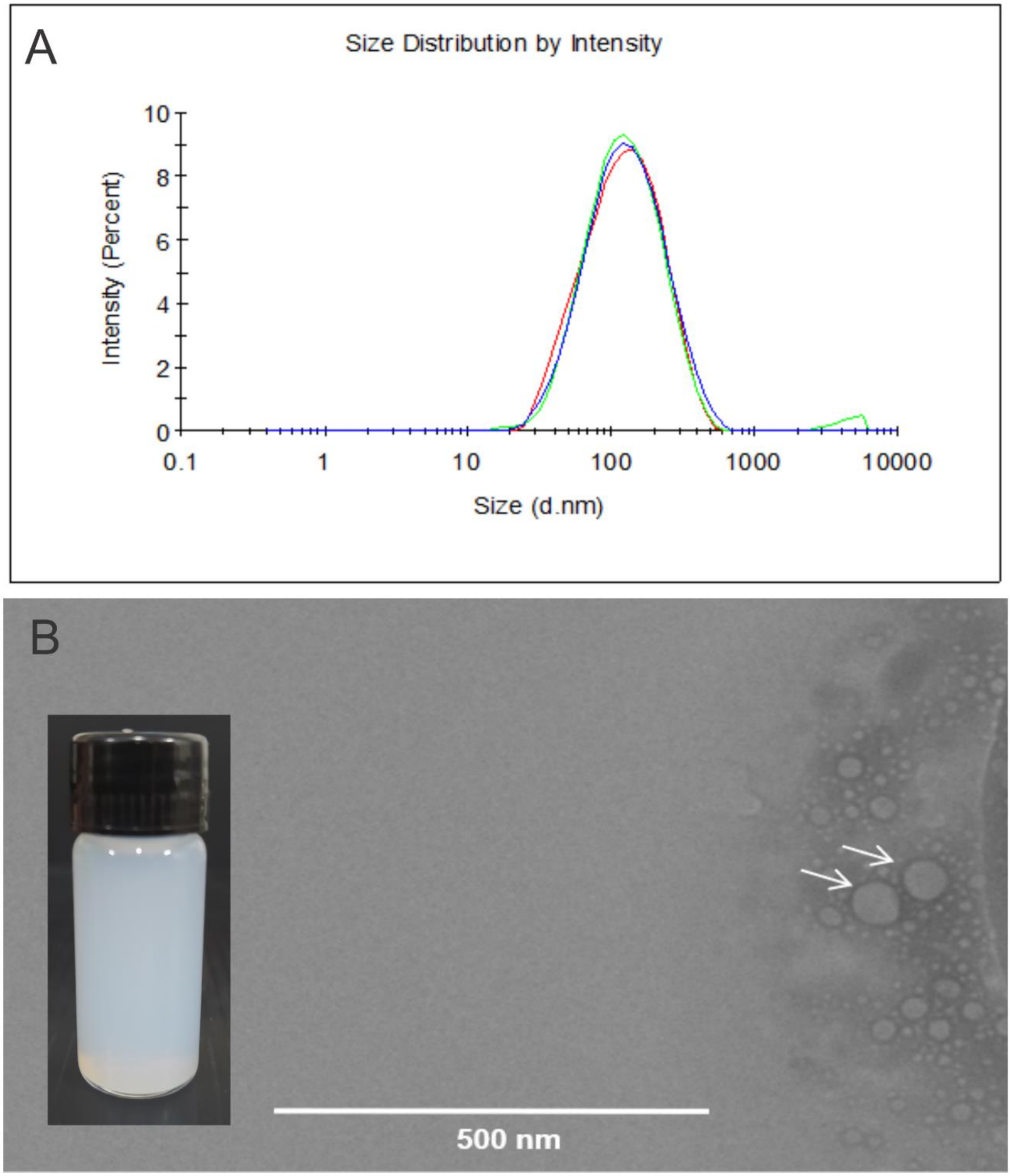

Figure 3: (A) NEOI-OPT size distribution by intensity; (B) TEM image of the NEOI-OPT, arrows show spheric droplets with approximately 100 nm.

3.6 Larvicidal activity against *Aedes aegypti*

Figure 4A shows the estimated lethal time (LTs) for NEOI-OPT against *Ae. aegypti* larvae. The negative control, the blank nanoemulsion, and the nanoemulsion at 12.5 µg/mL did not allow the estimation of a median lethal time (LT_{50}) as both of them were unable to cause 50

% of mortality. The positive control (imidacloprid) at 1 µg/mL presented 100% of mortality in 24 h. The nanoemulsion concentrations of 25 µg/mL (LT_{50} = 133 [128 -137] h) and 50 mg/mL (LT_{50} = 144 [119 -169] h) presented statistical differences compared to the control groups (P < 0.0001), but were less potent that the concentrations of 100 mg/mL (LT_{50} = 96 [92 -100] h) and 200 mg/mL (LT_{50} < 24 h). As demonstrated in (Figure 4B), further estimations of NEOI-OPT toxicity were achieved when the exposure periods were 48h and 144h, resulting in median lethal-concentration (LC_{50}) of 26.8 (21.5 - 32.6) mg/mL for the exposure period of 144 h and of 61.4 (52.4 - 72.0) mg/mL when the larvae were exposed to 48h (table 4).

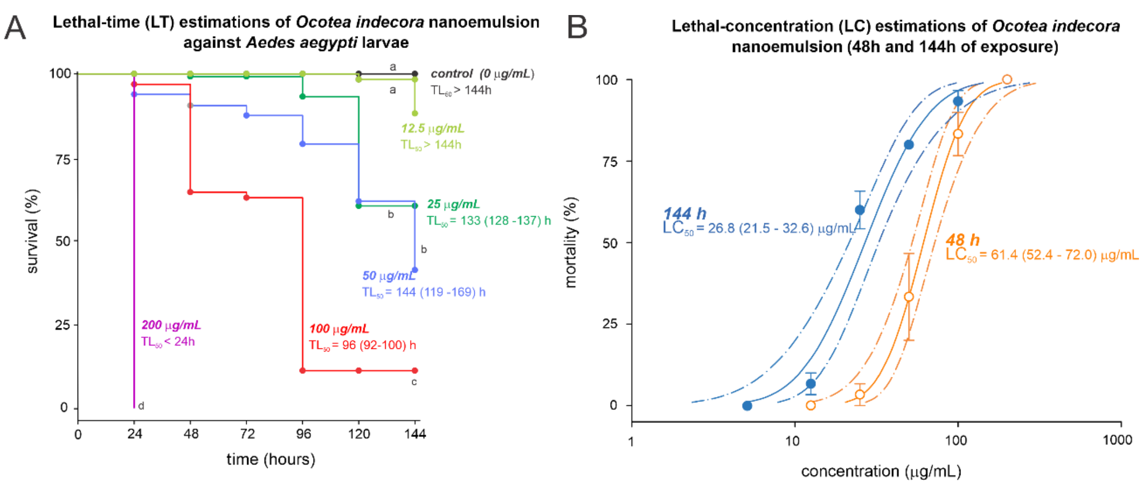

Figure 4: Lethal-time (A) and lethal concentrations (expressed in essential oil) (B) of optimized *Ocotea indecora* nanoemulsion in *Aedes aegypti* larvae.

Table 4: Lethal concentration of the optimal nanoemulsion of *Ocotea indecora* in *Aedes aegypti* larvae (L3) after 48, and 144 h.

Hours	LC_{50} (µg/mL)	χ^2	df	Slope ± S.E	*p-value*
48	61.4 (52.4-72.0)	0.28	3	4.86 ± 0.73	0.96
144	26.8 (21.5-32.6)	4.89	3	3.21 ±0.47	0.18

3.7 Larvae morphological study

Photomicrographs of *Ae. aegypti*, after 24 h of exposure to the NEOI-OPT at 250 µg/mL, presented alterations in the cuticle as shown in figures 5D, 5C, and 5F. Control *Ae. aegypti* larvae showed no alteration in morphology after the incubation period (figure 5A, B, and C).

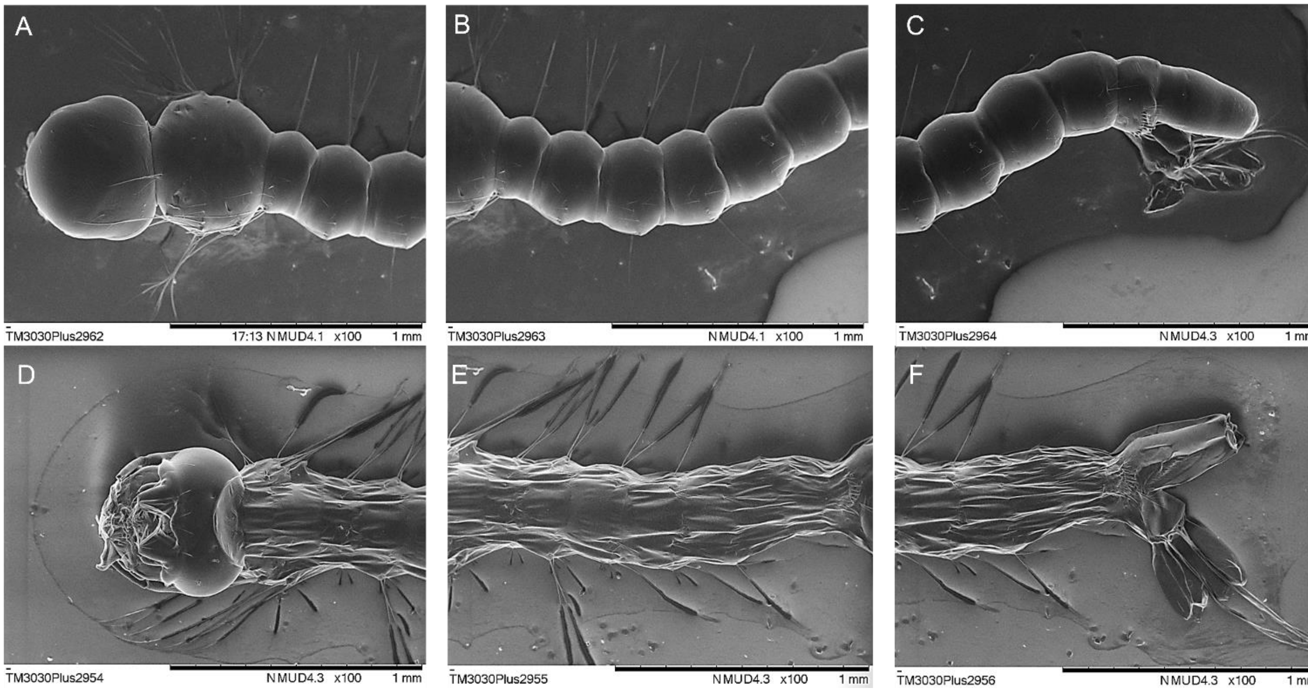

Figure 5: Scanning electron micrograph of *Ae. aegypti* larvae (L3) without treatment showing no alteration on the head and thorax (A), abdomen segments (B), siphon, and anal papillae (C). Larvae exposed to the NEOI-OPT at 250 µg/mL showed cuticle alterations on the head and thorax (D), abdomen segments (E), siphon, and anal papillae (F).

3.8 Molecular docking between essential oil biomolecules of *Ocotea indecora* and acetylcholinesterase enzyme of *Aedes aegypti*

Based on our results of the larvicidal effect against *Aedes aegypti* of *O. indecora*, we suggest that their constituent biomolecules may interact with the acetylcholinesterase enzyme of larvae causing physiological disruption. The constructed acetylcholinesterase protein model of *Ae. aegypti* highlighted the values of Ramachandran favored with 95.33 % and a QMEAN factor of −0.05. The acetylcholinesterase exhibited higher energy affinities (AutoDockVina affinity energy kcal mol^{-1}) when complexed with β-farnesene (-8.8 kcal mol^{-1}) and sesquirosefuran (-6.5 kcal mol^{-1}). The two biomolecules showed an affinity for different binding pockets (figure 6A). The interactions between Alkyl and van der Waals dominated the complex AChE-biomolecules. β-farnesene showed van der Waals interactions with LYS655, GLN536, ARG629, LEU647, ASN532, GLU533, and Alkyl interactions with LYS654. Sesquirosefuran showed van der Waals interactions with CYS650, ASN532, GLN536, GLU533, and ARG629, Pi-Pi shaped interactions with TRP505, Alkyl interactions with LEU647, LYS655, LYS654, ALA651, and PRO508 (figure 6B).

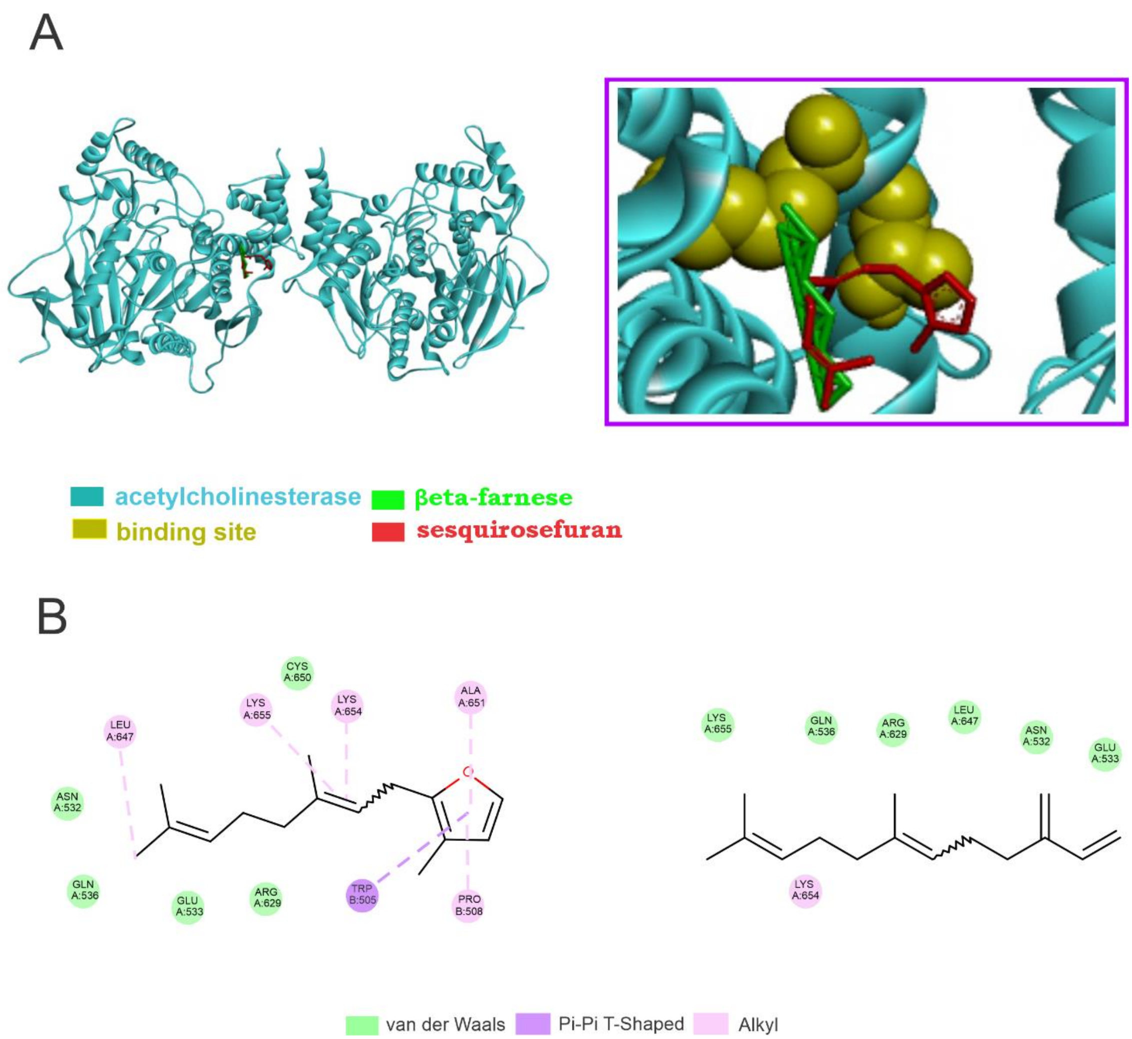

Figure 6: *Ocotea indecora* major constituents bind to acetylcholinesterase enzyme related to *Aedes aegypti* (A) Docking views of the sesquirosefuran and β-farnesene with acetylcholinesterase binding sites. (B) 2D interaction maps of acetylcholinesterase enzyme interaction sites with sesquirosefuran, and β-farnesene.

3.9 Acute toxicity of the nanoemulsion to the non-target organism *Apis mellifera*

The bioassay of acute oral toxicity in *Apis mellifera* NEOI-OPT was based on approximately four times the LC_{50} after 24 h. There was 100% of survival after 24 hours in the groups treated with the NEOI-OPT, control, and nanoemulsion blank.

4.0 Discussion

The uncontrolled use of conventional insecticides has led to the selection of mosquitoes resistant to field doses. Further, these chemical agents are considered by Word Health Organization (WHO) a major public health problem (Pavela, 2015). For this reason, the search and development of new alternatives for controlling insects of medical interest have been considered of great importance. These new strategies should be effective, biodegradable, less toxic, and environmentally less aggressive (Senthil-Nathan, 2020). Among the alternatives that have been considered for the development of new pesticides are insecticides based on natural products. Plant extracts and essential oils are highlighted because they result from a natural coevolutionary interaction between plants and herbivores insects, reflected in the chemical constitution of the plant's secondary metabolism (Pavela, 2015). Formulating nanoemulsions based on vegetal oils allows hydrophobic dispersion of the essential oil in an aqueous medium which is desirable for *Ae. aegypti* since most of its development occurs in water. In urban environments, *Ae. aegypti* larvae usually develop in a restricted container, making it easier to control them in immature stages than in their winged form (Sharma et al., 2020).

Essential oils are mainly composed of monoterpenes, sesquiterpenes, and phenylpropanoids. Several authors have described the potential of terpenes from essential oils as sustainable biopesticides (Pavela, 2015; Duarte et al., 2020; Senthil-Nathan, 2020). Gonçalves et al. (2018) described the essential oil of *O. indecora* containing byciclogermacrene (29.8 %), valerianol (15.1 %), and β-pinene (11.4 %) as major compounds. However, Nascimento et al. (2020) and Figueredo et al. (2018) described the sesquiterpene sesquirosefuran as the major component comprising values above 90 % in the essential oil of leaves. The present study with *O. indecora* corroborates the results of Nascimento et al. (2020) and Figueredo et al. (2018), showing the sesquirosefuran as the major substance in the essential oil of leaves (81.4%), suggesting a key role of this metabolite in its larvicidal activity. However, some variations in the composition of essential oils from the same plant species usually occur due to several extrinsic factors modulating the secondary metabolism pathways to produce metabolites in response to environmental pressures (Gobbo-Neto and Lopes, 2007).

In this work, the low-energy method by phase inversion was chosen to prepare the nanoemulsion since it does not use organic solvents or high temperatures, preventing volatilization and degradation of the low-weight terpenoids of the essential oil. Further, this method is more suitable for large-scale production (Gledovic et al., 2021; Sharma et al., 2020). Our results highlight the formulation F3, with the proportion of 4:1 for the surfactants polysorbate 20 and sorbitan monooleate 80, together with the smaller particle size and PdI values for the nanoemulsion of essential oil from *O. indecora*. The HLB of 14.22 indicates the formulation with a better capacity to reduce the interfacial tension between the essential oil of

O. indecora and water (Marhamati et al., 2021). The proportion of the surfactants polysorbate 20 (4%) and sorbitan monooleate 80 (1%) suggest the most stable dispersion between the eleven formulations prepared because the particle size is a parameter directly related to the stability of the nanoemulsion, so the smaller the size, the more stable the dispersion will be (Sharma et al., 2020).

To formulate and optimize the *O. indecora* nanoemulsion, a factorial design was realized to evaluate the effects of the independent variable's RPM, amount of essential oil, and surfactants on the dependent variable's droplet size and PdI. This experimental design allowed us to evaluate these factors and their interactions simultaneously. Despite several pharmaceutical articles on nanoemulsion development, few use the Design of Experiments (DoE) tool to improve process conditions, better understanding, and product optimization (Cunha et al., 2020). The Pareto charts (figure 1) showed that the interaction of the amount of essential oil with the surfactants was significant (p-value <0.05) and influenced the droplet's diameter. However, it did not present an influence on the PdI. The interactions of the oil phase with the non-ionic surfactants are a critical step in the nanoemulsion formation since it reduces the surface tension to stabilize the interfacial surface of the droplets (Barradas and de Holanda e Silva, 2021). In this context, it is expected that the interactions of those independent variables may present significance in the formulation process.

The factorial design established an inversely proportional correlation between the independent variable's amount of essential oil and surfactants. The lower the concentration of essential oil with a higher concentration of surfactants, the more desirable the average size and PdI values will be. Therefore, the optimized nanoemulsion of *O. indecora* (NEOI-OPT) was determined using the lowest concentration of surfactants to reduce the toxicity of the formulation, capable of achieving the highest concentration of essential oil to present reduced PdI and mean size values.

The predicted interactions with a droplet size of approximately 100 nm suggested a 1:1 (w/w) proportion to the essential oil and the mixture of surfactants. However, the interaction between the factors showed lower PdI values at the lowest RPM. For these reasons, the NEOI-OPT with a proportion of 1:1 to essential oil (5 % w/w) and surfactants (5 % w/w) at 500 RPM was selected to continue the stability and TEM studies. As a result, the size distribution (figure 3A) of the NEOI-OPT presented a mean size of 105.3 ± 1.36 and PdI of 0.263 ± 0.004.

The stability study demonstrated that the NEOI-OPT presented stability at room temperature (25 °C) and under refrigeration (8 °C) after 365 days of preparation. However, the nanoemulsion stored in the climatic chamber (42 °C) was only stable for 60 days. Then, a decrease in the PdI values was observed from 90 until day 365. Afterward, an increase in particle size occurred 240 days after preparation. After that, the nanoemulsion showed a gradual increase in droplet diameter. This fact can be explained by the Ostwald ripening occurrence, which transfers the mass of essential oil from smaller droplets to larger droplets, thus causing a decrease in PdI values and increasing particle size, eventually leading to phase separation (Faustino et al., 2020). Therefore, instability of the nanoemulsion under 42 °C is expected due to the acceleration in the collision of the droplets dispersed in the aqueous phase, leading to the destabilization of the nanoemulsion (Cossetin et al., 2021).

The zeta potential after long-term stability of the nanoemulsion was -27.7 ± 0.95 mV. This result, when compared to the day 0 of the optimal nanoemulsion (-23.8 ± 2.01 mV) shows a low variation of zeta potential, as expected for kinetic stable colloids prepared with non-ionic surfactants. ZP is an important parameter for the interpretation of surface of droplets. Natural product-based nanoemulsions are supposed to have adsorbed ions related to the compounds of the nanostructured herbal oil (Dias et al., 2014). In the present study, this negative charge may be related to at least partially, to the resonance hybrid of the sesquirosefuran, the main compound of the essential oil used for the preparation of the optimal nanoemulsion.

The TEM image (Figure 3B) showed circular-shaped droplets aggregated. This occurs due to the drying process in the sample preparation. Therefore, it is possible to observe larger droplets with particle sizes of approximately 100 nm, which corroborates with the NEOI-OPT DLS values. Thus, smaller droplets produced by the electron beam can also be observed (Ho et al., 2021).

The NEOI-OPT reduced 100% of the larvae population (n=30) at 200 µg/mL after 24 h and 83.33 ± 15.28 % at 100 µg/mL after 48 h, presenting effectiveness at higher concentrations in the first two days. But, then, the mortality increased over 144 h showing 80 % of lethality at 50 µg/mL and 60 ± 10 % at 25 µg/mL, suggesting that the NEOI-OPT increased the effect time of *O. indecora* essential oil. Several authors reported the potential of essential oils nanoemulsions as an alternative to *Ae. aegypti* control. For instance, Da Botas et al. (2017) described the activity of the nanoemulsion of essential oil of *Baccharis reticularia* leaves on 3rd

instar larvae with LC_{50} of 144.7 µg mL^{-1} after 48 h. Martins et al. (2021) described the nanoemulsion of essential oil from *Aeollanthus suaveolens* against 3rd instar with LC_{50} of 54.2 µg/mL after 24 h. In addition, Folly et al. (2021) described the larvicidal action of *Annona acutiflora* essential oil nanoemulsion in the 3rd instar with LC_{50} of 66.1 µg/mL after 48 h. Our results showed the LC_{50} of the *O. indecora* nanoemulsion decreased as a function of time (61.4 µg/mL after 48h to 26.8 µg/mL after 144h). Similarly, Jesus et al. (2017) described the residual effects of the nanoemulsion of *Carapa guianensis* oil in the 3rd instar larvae of *Ae. aegypti* that presented a reduction in the mortality rate of 53.3 % after 144 h. This can be explained because the nanoemulsion has controlled release, gradually releasing the droplets into the aqueous media, decreasing the LC_{50} over time, and prolonging the action time (da Silva and Ricci-Júnior, 2020).

No specific value is defined by WHO to classify a good larvicidal activity obtained by plant-based products. However, several authors agree with the LC_{50} value of <100 µg/mL to categorize a larvicidal agent as significant (Dias and Moraes, 2014; Folly et al., 2021; Pavela, 2015). In this sense, the NEOI-OPT can be characterized as a promisor larvicide, presenting LC_{50} values of 61.4 µg/mL after 48 h. In addition, the mechanism of action of nanostructured plant-based larvicides could be associated with larvae morphological alterations, the formation of reactive oxygen species that cause genotoxicity, and inhibiting acetylcholinesterase (Duarte et al., 2020). Our in-silico analysis suggests that the larvicide property of *O. indecora* essential oil is related to acetylcholinesterase enzyme inhibition by sesquirosefuran, as it is widely predominant in the oil and can be considered fundamental in the larvicide activity observed. The morphological alterations observed in the *Ae. aegypti* larvae by SEM reinforce that the NEOI-OPT may act in the cuticle, influencing motility, development, and assisting lethality. Other authors have found similar results, showing alterations in the cuticle of the head, thorax, abdomen, and siphon in larvae of *Ae. aegypti* and *Culex quinquefasciatus* with other essential oils nanoemulsions (Da Botas et al., 2017; Pessoa et al., 2018).

Accessing the environmental safety of pesticide formulations, including their toxicity to non-target organisms, is critical when envisioning their future adoption (Carneiro et al., 2020). Larvicidal agents applied in urban aquatic environments to control *Ae. aegypti* present risks to non-target insects that can utilize those resources, such as pollinator bees. Hence, it is vital to search for selective molecules and formulations to prevent potential environmental harm since the extensive use of insecticides has decreased the population of pollinators, such as the honey bee, *Apis mellifera*, over the years (Tomé et al., 2017). The nanoemulsion (NEOI-OPT) developed in this study, besides showing strong biological activity against *Ae. aegypti* caused no mortality to honeybees (*A. mellifera*) via oral administration for 24 hours. It is worth noting that the nanoemulsion was tested in a concentration that is at least 4-fold the LC_{50} found for *Ae. aegypti* within the same exposure period, showing the high selective potential of our formulation. Despite not having studies concerning *O. indecora* essential oil and *Ae. aegypti*, Nascimento et al. (2020) demonstrated the insecticidal effect of another nanoemulsion of *Ocotea indecora* on *Dysdercus peruvianus,* and Figueiredo et al. (2018) described the effects of the *O. indecora* essential oil against *Rhipicephalus (Boophilus) microplus*. Our study presents an innovative approach for using *O. indecora* nanoemulsions as larvicides against *Ae. aegypti* in aquatic settings coupled with its safety to pollinator bees

5. Conclusion

The essential oil of *O. indecora* contained sesquirosefuran as the major constituent. The study enabled the development and optimization of a stable nanoemulsion (NEOI-OPT) with small size distribution values that showed promising larvicidal effects against *Aedes aegypti* larvae, inducing external morphological alterations. Conversely, NEOI-OPT exhibited no acute oral toxicity to *Apis mellifera*. In this context, this work permitted the development of an effective, low cost and eco-friendly larvicide to be used as an alternative to control *Aedes aegypti*.

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6. CAPÍTULO IV – ARTIGO 2

6.1 Molluscicidal and cercaricidal effects of *Myrciaria floribunda* (H. West ex Willd.) O. Berg essential oil nanoemulsion

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Molluscicidal and cercaricidal effects of *Myrciaria floribunda* essential oil nanoemulsion

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Abstract: Schistosomiasis is a tropical disease transmitted in an aqueous environment by cercariae from the *Schistosoma* genus. This disease affects 200 million people living in risk areas in the world. The control of the schistosomiasis is realized by chemotherapy, wastewater sanitation, health education, and mollusk control using molluscicidal agents. This work evaluates the effects of a nanoemulsion containing essential oil from *Myrciaria floribunda* leaves as a molluscicidal and cercaricidal agent against *Biomphalaria glabrata* mollusks and *Schistosoma mansoni* cercariae. The *Myrciaria floribunda* essential oil from leaves showed nerolidol, β-selinene, 1,8 cineol, and zonarene as major constituents. The formulation study

suggested the F3 formulation as the most promising nanoemulsion with polysorbate 20 and sorbitan monooleate 80 (4:1) with 5% (w/w) essential oil as it showed the smaller droplet size of approximately 100 nm with a PDI lower than 0.3, and prominent bluish reflection. Furthermore, this nanoemulsion showed stability after 200 days under refrigeration. The *Myrciaria floribunda* nanoemulsion showed LC_{50} values of 48.11 µg/mL, 29.66 µg/mL, and 47.02 µg/mL in *Biomphalaria glabrata* embryos, juveniles, and adult mollusks, respectively, after 48 h and 83.88 µg/mL for *Schistossoma mansoni* cercariae after 2 h. In addition, an in-silico toxicity assay showed lower overall human toxicity potential to the major compounds in the essential oil compared to the reference molluscicide niclosamide. These results suggest that the nanoemulsion of *Myrciaria floribunda* leaves may be a promising alternative for schistosomiasis control.
Keywords: Myrtaceae; pesticides; essential oil; schistosomiasis control; nanodispersion; natural products

1. Introduction

Schistosomiasis is a disease transmitted by parasites from *Schistosoma* genus. The main species responsible for the disease are *Schistosoma hematobium*, *Schistosoma mansoni*, and *Schistosoma japonicum* [1]. The life cycle of the *S. mansoni* parasite is divided between two hosts: the first cycle occurs in aqueous media in mollusks of the genus *Biomphalaria*, especially *Biomphalaria glabrata*, and the second and definitive cycle occurs in humans [2]. In the environment, the, e.g., of the parasite hatch and liberate the first larval stage miracidia, which invade the mollusks of the *Biomphalaria* genus. After a few weeks, the trematode matures into the second larval stage, which is responsible for human infection, and leaves the mollusk as cercariae [2,3]. In humans, cercariae travel through the blood vessels until they reach the liver and become mature worms. Then, *Schistosoma* sp. realizes the sexual cycle and lays its, e.g., into the mesenteric vessels, being excreted in the feces [4]. Without sanitation, excreta can contaminate the aquatic environment again and restart the cycle [1,2,4].

The principal areas affected by schistosomiasis are tropical and subtropical regions, mainly poor communities with low or no access to drinking water and basic sanitation [2]. This disease affects over 290 million people in the world living in endemic areas [3]. Strategies for the prevention and control of bilharzia are based on treating the population living in risk areas, improving basic sanitation, access to safe drinking water, hygiene education, chemotherapy, and snail control [3]. Niclosamide is the molluscicide agent recommended to control *B. glabrata* mollusks[2]. However, it is toxic to aquatic species and undergoes photodegradation [2,5]. Furthermore, niclosamide frequently needs reapplication [5]. Therefore, developing new products as an alternative to niclosamide is necessary. For this reason, research into novel products of natural origin represents an interesting alternative to obtaining a biorational molluscicide with a less negative impact on public and environmental health [1,5–8].

Nanostructured systems are in the spotlight for developing novel pesticide alternatives [9,10]. Nanoemulsions (NEs) are thermodynamically unstable colloidal dispersions of two immiscible liquids containing nanometer-sized droplets between 20-200 nm [11,12]. Nanoemulsification is a strategy that has been adopted for the application of essential oils or lipophilic extracts in aqueous media due to their hydrophobicity [13–15]. Volatile oils are added to surfactants to form droplets and prevent the separation of the oil and aqueous phases, thus favoring the kinetic stability of the dispersion [16,17]. There are several advantages of nanoemulsions with essential oils, such as prevention of degradation and volatilization of low molecular weight substances, enabling use in aqueous media, increased bioavailability, and improved bioactivity [18].

Myrciaria floribunda (H. West ex Willd.) O. Berg (Myrtaceae) is a native plant from Brazil and is popularly known as “Cambui”, “Camboim amarelo” or “camboim” and is distributed in sandbank areas next to the coast in the Restinga de Jurubatiba National Park in Rio de Janeiro, Brazil [19]. The fruits are edible and popularly used in northern Rio de Janeiro to flavor alcoholic beverages based on the distilled spirit from sugarcane known as “cachaça” [20]. Previous studies described the pharmacological properties of this essential oil with insecticidal and anticholinesterase effects [21–23]. Other authors described the antiproliferative, antioxidant, antifungal, antibacterial, anti-inflammatory, and antinociceptive effects of different extract fractions from this plant [19,24–26]. Based on this scenario, this work aims to obtain a novel nanoemulsion with molluscicidal and cercaricidal activity from the essential oil of *Myrciaria floribunda* leaves as an alternative against *Biomphalaria glabrata* and *Schistossoma mansoni* to control schistosomiasis.

2. Results and Discussion

The essential oil from *Myrciaria floribunda* yielded 0.9% (w/w) and showed a clear transparent aspect. The chemical characterization showed 11.33% monoterpene and 80.49% sesquiterpene fractions (Table 1). In addition, 27 substances were identified, and nerolidol (15.4%), β-selinene (13.9%), 1,8-cineole (10.7%), and zonarene (7.67%) were the major components (Figure 1).

Table 1: Chemical characterization of the essential oil from leaves of *Myrciaria floribunda* GC–MS and GC-FID.

	Retention index	Arithmetic index	Arithmetic index calculated	Substances	%
1	8.321	1023	1022	O-Cymene	0.19
2	8.459	1027	1026	1,8-Cineole	10.70
3	14.963	1194	1186	α-Terpineol	0.44
4	22.355	1369	1373	α-Ylangene	3.43
5	24.126	1412	1417	β-Caryophylenne	2.28
6	24.920	1431	1439	Aromadendrene	0.27
7	25.581	1447	1452	α-Humulene	1.15
8	26.333	1466	1476	β-Chamigrene	1.87
9	26.937	1481	1489	β-Selinene	13.28
10	27.235	1488	1498	α-Selinene	6.13
11	27.387	1492	1500	α-Muurolene	0.49
12	27.493	1495	1509	α-Bulnesene	0.45
13	28.136	1511	1511	δ-Amorphene	4.80
14	28.292	1515	1521	trans-Calamenene	0.43
15	28.819	1529	1528	Zonarene	7.69
16	29.010	1534	1545	Selina-3,7(11)-diene	6.54
17	29.654	1550	1559	Germacrene B	0.50
18	29.886	1556	1561	Nerolidol	15.43
19	30.560	1574	1582	Caryophyllene oxide	0.76
20	31.176	1590	1595	Cubeban-11-ol	0.29

21	32.506	1625	1622	10-epi-γ-Eudesmol	0.66
22	33.311	1647	1649	β-Eudesmol	1.24
23	33.439	1651	1658	neo-Intermedeol	4.41
24	33.836	1661	1665	Intermedeol	1.56
25	34.892	1690	1700	Eudesm-7(11)-en-4-ol	1.48
26	35.702	1713	1714	Farnesol	2.05
27	39.756	1829	1821	(2Z,6E)-Farnesyl acetate	3.33
	Total identified				91.82
	Monoterpene hydrocarbons				0.19
	Oxygenated monoterpenes				11.14
	Monoterpenes: total				11.33
	Sesquiterpenes hydrocarbons				49.31
	Oxygenated sesquiterpenes				31.18
	Sesquiterpenes: total				80.49

The chemical composition of the essential oil of *Myrciaria floribunda* leaves was described by Ramos et al. (2011) [27]. They reported 18.8% monoterpenes and 75.2% sesquiterpenes in the oil. Additionally, the major constituents were nerolidol (32.4%), β-selinene (9.8%), 1,8 cineol (5.8%), and β-terpinene (4.6%) [27]. Another study (Tietbohl et al., 2012) reported 53.9% monoterpenes and 39.6% sesquiterpenes [23]. Additionally, the major components were 1,8 cineole (38.4%), ɣ-himachalene (7%), α-terpineol (5.5%), and zonarene (4.6%) [23]. More recently, Tietbohl et al. (2019) described essential oil with 19.2% monoterpenes and 70% sesquiterpenes and 1,8 cineol (10.4%), β-selinene (8.4%), and α-selinene (7.4%) as major components [22].

The fraction of terpenes in the present study was 11.33% for monoterpenes and 80.5% for sesquiterpenes, which follows Ramos et al. (2010) and Tietbohl et al. (2019) but differs from the terpene fractions described by Tietbohl et al. (2012) [22,23,27]. The major constituents found in this study corroborated the major compounds in the *M. floribunda* essential oil described by Ramos et al. (2010) and Tietbohl et al. (2012; 2019) [22,23,27]. The variations in the chemical compositions of the essential oils were quantitative and qualitative, probably because individual plants suffer different environmental pressures, leading to different adaptations of secondary plant metabolism [28]. Factors such as temperature, collection time, season, extraction, processing, and storage of the raw plant material can lead to variations in the chemical profile of *M. floribunda* essential oil[28].

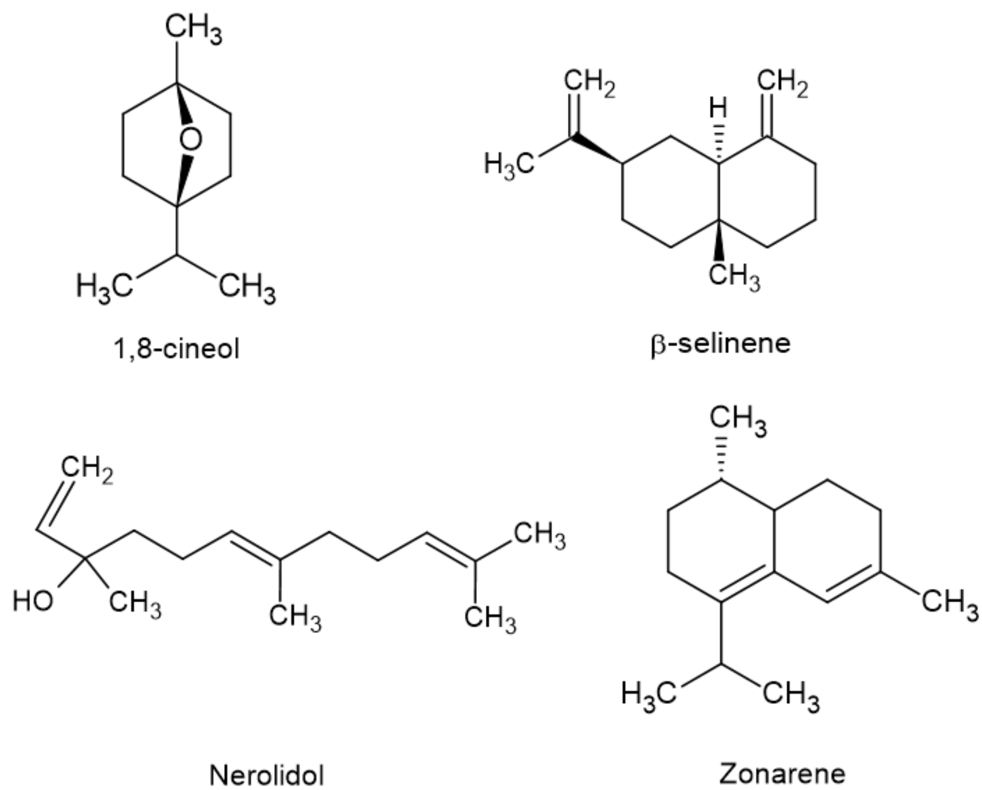

Figure 1: Major components of *Myrciaria floribunda* essential oil from leaves.

Essential oils are mainly composed of terpenes and phenylpropanoids, substances of low molecular weight from plant metabolism [28]. They are widely described in the literature as having promising biological properties, generating biotechnological interest in several areas, among them, as molluscicide agents for presenting low cost, high efficiency, biodegradability, and less chemical resistance from parasites or hosts [29]. However, the lipophilic properties of these volatile oils prevent their use for the control of aquatic mollusks such as *Biomphalaria glabrata,* requiring the use of drug delivery systems such as nanoemulsions for their viability to control schistosomiasis [30].

The low-energy by phase inversion nanoemulsification method was chosen because it does not use organic solvent and avoids degradation or evaporation of the thermosensitive substances in essential oils [31]. In this work, 11 formulations were prepared with different proportions of surfactants. The criteria to characterize a formulation as a nanoemulsion were an average droplet size < 200 nm and PdI < 0.3 to be considered a monodisperse system [12,32,33]. The formulations with smaller nanodroplets (F1-F3) comprehended 4-5% polysorbate 20 and 0-1% sorbitan monooleate (Table 2). Additionally, it showed a bluish-white coloration and a 16.70-14.22 HLB range. To rationalize the experiments, only the F3 formulation (4:1 polysorbate 20 and sorbitan monooleate) was selected to continue the work due to its pronounced macroscopic characteristics and smaller droplet size. Also, showed a typical bluish-white reflection characteristic of light scattering due to the Tyndall effect in nanodispersions (Figure 2) [34]. In addition, showed a smaller average droplet size of 99.71 ± 0.5220 nm, and the PDI was 0.262 ± 0.013 (Figure 3A). The HLB of 14.22 suggests relatively hydrophilic characteristics of the *M. floribunda* essential oil [17]. The other F5-F9 formulations showed a milky white color, droplet size >200 nm, and PDI>0.3. The F10 and F11 formulations (HLB 5.54-4.3) presented visual phase separation and were discarded from the analysis.

Table 2: Average droplet size, polydispersity index (PDI), and required hydrophilic-lipophilic balance (HLB) values of formulations F1-F9 of *Myrciaria floribunda* essential oil.

Formulation	Droplet size (nm)	Polydispersity index	Hydrophilc-Liphilic Balance
F1	171.8 ± 1.3	0.271 ± 0.011	16.7
F2	120.5 ± 0.92	0.249 ± 0.009	15.46
F3	99.71 ± 0.52	0.262 ± 0.013	14.22
F4	196.4 ± 1.4	0.322 ± 0.105	12.98
F5	311.5 ± 12.80	0.204 ± 0.134	11.74
F6	592.8 ± 84.40	0.672 ± 0.340	10.5
F7	1532± 646.25	0.520 ±0.481	9.26
F8	1750 ± 817.41	0.439 ± 0.430	8.02
F9	1461 ± 263.65	1.0 ± 0	6.78

Figure 2: *Myrciaria floribunda* essential oil nanoemulsion (formulation F3) with bluish reflection (dilution 1:40).

Figure 3A shows the size distribution by the intensity of formulation F3, showing the superposition of three measures with an average size of 100 nm, and PDI lesser than 0.3, suggesting a monomodal behavior of the nanoemulsion. The TEM image of the F3 formulation after 1h of preparation at an 89-K magnification (Figure 3B) shows an agglomeration of the droplets due to the sample drying in the copper grid during its preparation and spherical-shaped droplets of approximately 100 nm, which corroborates the mean droplet size values observed in the DLS analysis. Smaller droplets are products of the degradation of larger particles due to the incidence of the TEM electron beam [32,35].

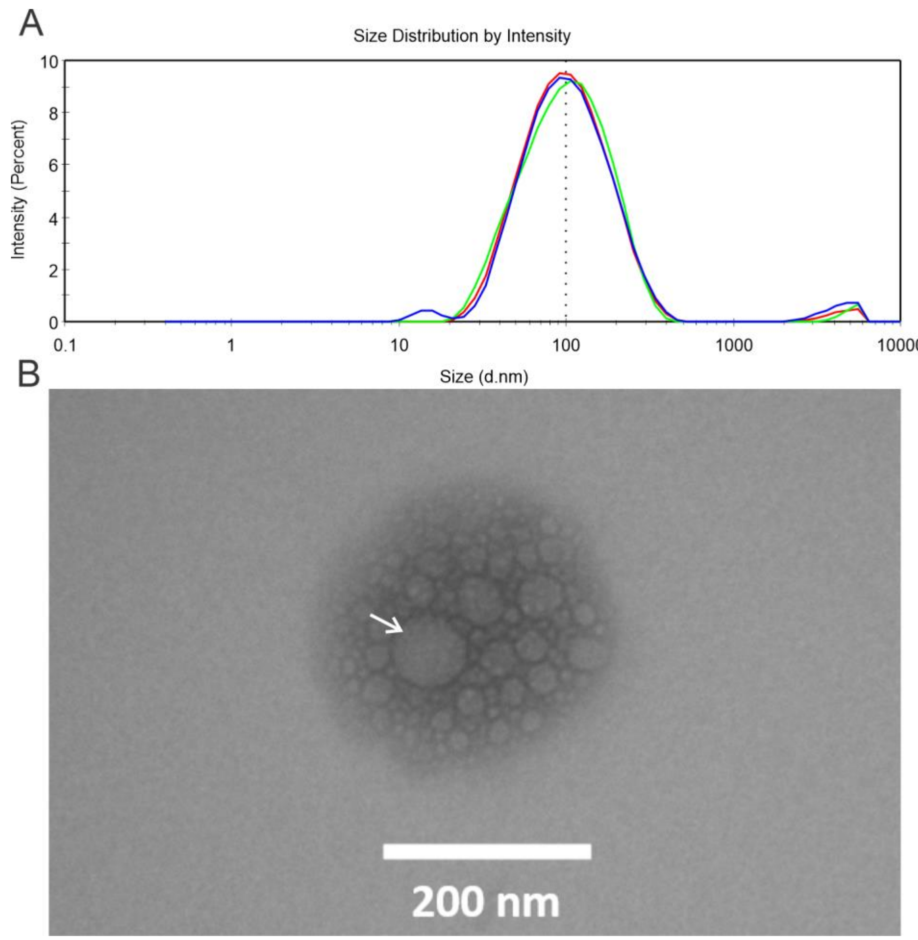

Figure 3: Formulation F3 size distribution by intensity (A) and transmission electron microscopy (TEM) image showing spherical droplets after 1h of preparation (B).

New batches of the F3 formulation were prepared and submitted to the stability study over 200 days (Table 3). The NEs must maintain the mean droplet size and PDI values to be considered stable. The NE stored at room temperature (25 °C) was stable until day 30, presenting a homogeneous particle size and PDI. Then at 30 to 60 days the NE decreased the PDI and maintained the size, suggesting the migration of the more hydrophilic compounds of the oil to the aqueous phase. From that point, a gradual increase in the size of the droplets and a decrease in the PDI values were observed, maintaining the characteristic values of the nanoemulsion until day 150, it can be seen in Figure 4A. After that, it had a particle size >200 nm, milky white color, and initial phase separation. This can be explained by the fact that more water-soluble oils, such as essential oils, gradually increase the droplet diameter by the Ostwald ripening effect. This mechanism is responsible for the mass transfer from smaller to larger radius droplets [36]. In this way, it reduces the number of dispersed droplets, presents monomodal behavior, and eventually leads to phase separation, as observed after 200 days in this experiment [36,37].

In the climatic chamber (42 °C), the nanoemulsion showed polymodal behavior and was considered unstable throughout the analysis period (Figure 4B). This formulation presented a milky white color 60 days after preparation. This effect is expected due to the increased energy in the system, leading to the acceleration of the collision of the dispersed droplets, influencing the thermodynamic equilibrium, increasing the solubility of the more hydrophilic constituents and thus accelerating the Ostwald ripening effect, increasing the coalescence speed, and destabilizing the dispersion [37,38]. However, the formulation stored under refrigeration (8 °C) was stable over time and maintained a bluish-white color, homogeneous particle size values, and PDI the same monomodal behavior overlayed after 200 days of preparation (Figure 4C). This phenomenon occurs due to the possibility of an increase in viscosity and a decrease in droplet collision in the nanoemulsified system, minimizing coalescence and increasing the viability of the formulation over time [37]. These results suggest that the *M. floribunda* NE in order to maintain its physicochemical properties in the long term should preferably be stored and transported under refrigerated conditions.

Table 3: Stability study of *Myrciaria floribunda* nanoemulsion (formulation F3) at different temperatures.

	25 °C		08 °C		42 °C	
	Average size (nm)	Polydispersity index	Average size (nm)	Polydispersity index	Average size (nm)	Polydispersity index
T00	87.2 ± 1.363	0.267 ± 0.010	96.6 ± 0.9917	0.263 ± 0.009	69.6 ± 1.767	0.283 ± 0.013
T07	84.4 ± 1.05	0.268 ± 0.008	98.1 ± 0.615	0.261 ± 0.003	72.0 ± 0.87	0.186 ± 0.010
T15	84.5 ± 1.517	0.261 ± 0.010	98.2 ± 1.103	0.248 ± 0.0	91.9 ± 0.2553	0.112 ± 0.021
T30	80.4 ± 0.9721	0.241 ± 0.008	94.3 ± 0.6274	0.268 ± 0.007	161.2 ± 2.237	0.148 ± 0.017
T60	91.4 ± 1.231	0.178 ± 0.008	97.8 ± 1.250	0.273 ± 0.009	533.8 ± 32.51	0.151 ± 0.080

T90	130 ± 0.8505	0.098 ± 0.008	100.4 ± 1.195	0.271 ± 0.004	843.5 ± 67.30	0.417 ± 0.519
T120	188.5 ± 1.801	0.141 ± 0.052	101.8 ± 1.041	0.285 ± 0.019	2603 ± 82.56	0.243 ± 0.031
T150	276.3 ± 5.408	0.126 ± 0.029	97.0 ± 0.6504	0.265 ± 0.013	2988 ± 423	0.676 ± 0.208
T200	580.0 ± 20.46	0.165 ± 0.105	102.4 ± 1.386	0.219 ± 0.008	2998 ± 114	0.370 ± 0.143

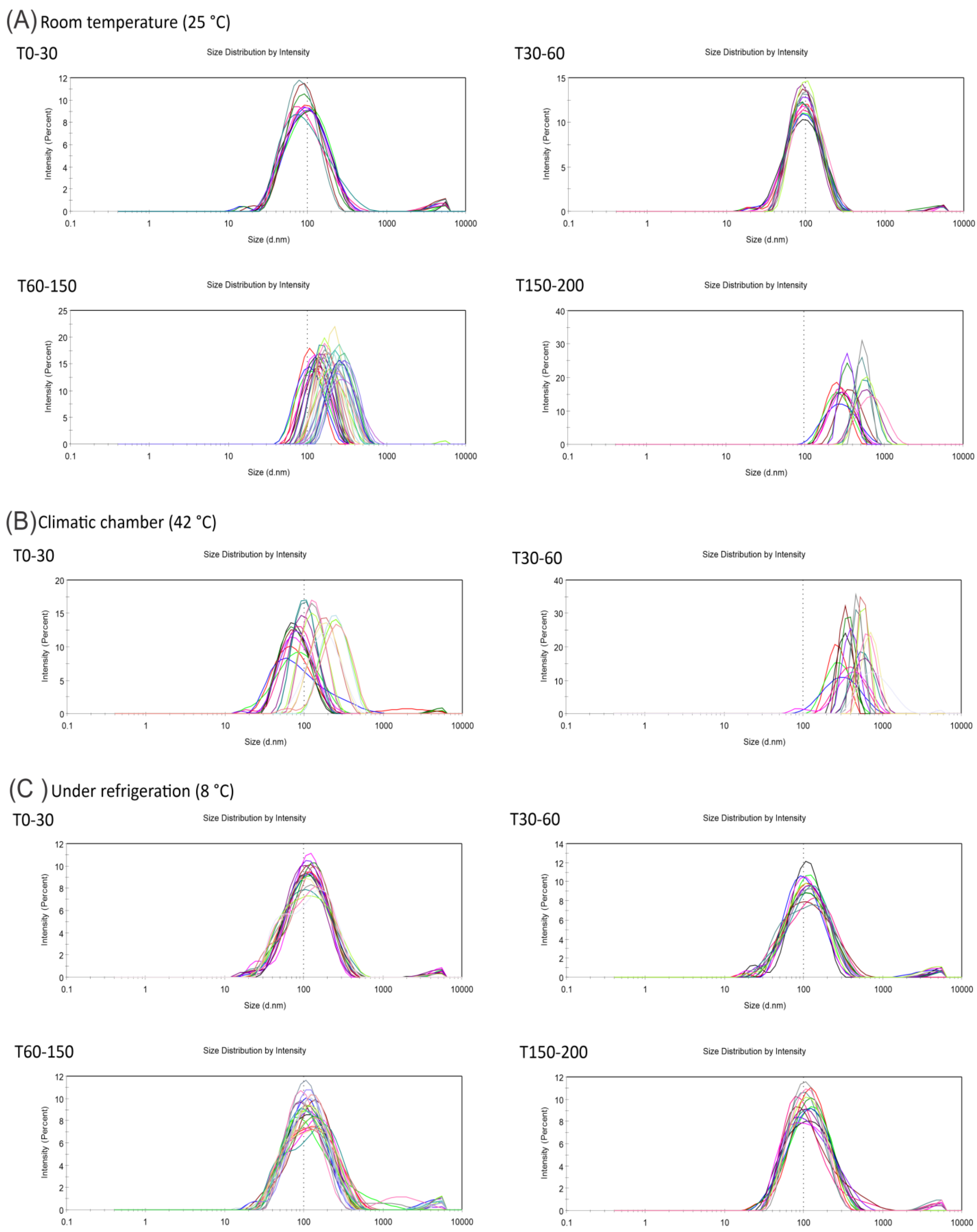
(A) Room temperature (25 °C)
T0-30
T30-60
T60-150
T150-200
(B) Climatic chamber (42 °C)
T0-30
T30-60
(C) Under refrigeration (8 °C)
T0-30
T30-60
T60-150
T150-200
Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
0.1
1
10
100
1000
10000

Figure 4: *M. floribunda* nanoemulsion (formulation F3) stability stored at room temperature (A), climatic chamber (B), and under refrigeration (C) over 200 days.

The NE from *M. floribunda* (formulation F3) showed 100% mortality after 24 h at 80 µg/mL, 40 µg/mL, and 80 µg/mL (expressed in essential oil) to *B. glabrata* embryos, juveniles, and adult mollusks, respectively, and 60 µg/mL to *S. mansoni* cercariae after 3 h. The mortality data can be found in the supplementary material (Figure 1S). The *M. floribunda* NE *B. glabrata* adults (10-12 mm) LC_{50} after 24 h was 48.26 (43.05 – 54.64) µg/mL . The LC_{50} after 48 h was 29.66 (26.81 – 32.62) µg/mL to juvenile mollusks (6-8 mm), 47.02 (41.94 – 52.84) µg/mL to adults (10-12 mm), 48.11 (45.15- 50.87) µg/mL to embryos and 83.88 (75.04 – 95.52) µg/mL to cercariae after 2 h (Figure 5). Regarding the lethal effect of the positive control, niclosamide showed 100% cercaria and mollusk mortality (embryos, juvenile, and adults) after 24 h in all groups ($p < 0.001$). At the same time, no mortality was observed in the negative control (distilled water) and NE blank (without essential oil) ($p < 0.001$). The nanoemulsion *of M. floribunda* was effective in all phases of the life cycle of the mollusk *B. glabrata*, showing a more significant effect on juvenile mollusks. This fact probably occurs because the juvenile organism is not fully developed and is more susceptible to the effects of the nanoemulsion and essential oil components [15].

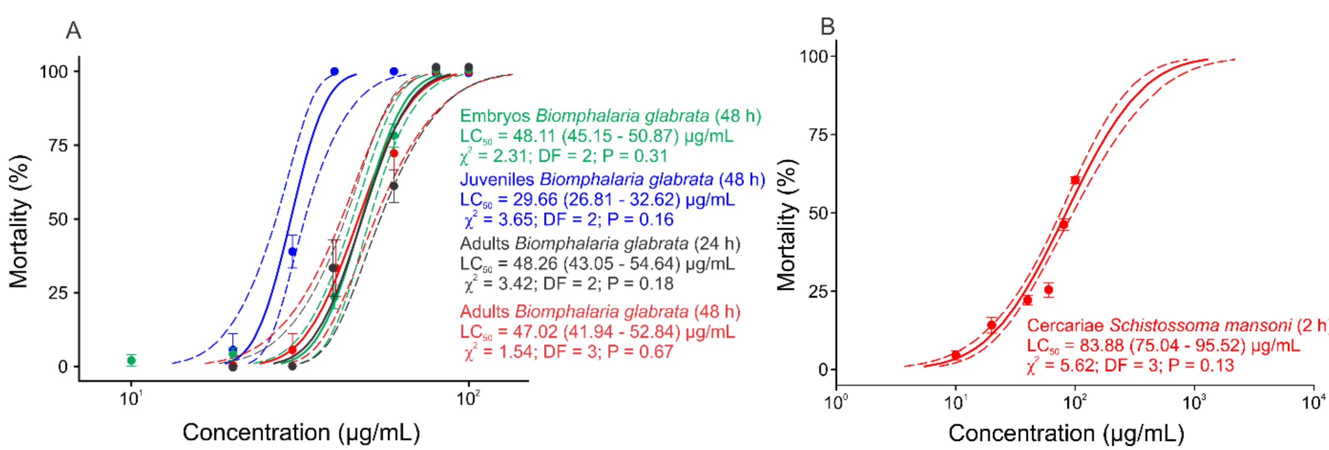

Figure 5: Lethal concentrations of the *Myrciaria floribunda* nanoemulsion (expressed in essential oil) in *Biomphalaria glabrata* embryos, juveniles, and adults (A) and *Schistosoma mansoni* cercariae (B).

Several studies for the control of mollusks, especially *B. glabrata* and the parasite *S. mansoni*, have been conducted with products derived from plant origin, generating important information and showing the potential of plants as a source of bioactive metabolites to support the combat of schistosomiasis [5,7,29,39–43]. However, few studies have approached the formulation of these novel natural products for the functional control of aquatic mollusks since the common limitation in these studies is the physicochemical properties of substances, which are often volatile or insoluble in aqueous media and generally require the use of organic solvents such as dimethyl sulfoxide (DMSO) to enable biological testing [29]. In this context, nanoemulsions present themselves as a potential research field to enable these actives in an aqueous medium, especially essential oils. A few authors described similar nanotechnological approaches with nanoemulsions from *Xylopia ochrantha* (Annonaceae) essential oil (24 h/LC_{50} =50.9 µg/mL), *Ocotea pulchella* (Lauraceae) essential oil (24 h/LC_{50}= 45.8 µg/mL), and *Sideroxylon obtusifolium* (Sapotaceae) extract (24 h/LC_{50}= 75.2 µg/mL) against *B. glabrata* mollusks, thus corroborating the LC_{50} values found in our study [13,15,44].

According to the World Health Organization, for an essential oil to be considered a promising molluscicide agent, it must have a mortality of at least 90% of the adult mollusk population at concentrations equal to or less than 100 µg/mL [29]. In this context, the nanoemulsion of essential oil from *M. floribunda* leaves can be considered a promising molluscicide formulation with 100% mortality at 80 µg/mL after 24 h and an LC_{50} of 47 µg/mL after 48 h in *B. glabrata* adults.

Evaluation of the environmental and human toxicity of molluscicides is crucial to ensure the safety and future adhesion of the product since the human population, as well as non-target organisms, may consume hydric resources from polluted water bodies close to urban or peri-urban environments in endemic regions of tropical diseases, including schistosomiases [1,29]. For this reason, it is important to develop new selective products to avoid harmful effects on the environment or human health [29]. In this study, *Danio rerio* (Zebrafish) was used as an aquatic non-target organism. The Organization for Economic Co-operation and Development (OECD) indicates it as an animal model for toxicological and ecotoxicological studies [45,46]. The *M. floribunda* NE besides presenting promissory molluscicidal activity in the broad spectrum of the *B. glabrata* lifecycle has not shown significant toxicity to Zebrafish. The applied oral dose corresponded to 50 µg/mL (expressed in essential oil), approximately the LC_{50} against the target organism *Biomphalaria glabrata*. In the first 6h, 10% of lethality was observed, then another 10% of lethality occurred at 48 h. There was an 80% in the survival of the adult *Danio rerio*. No changes related to equilibrium, swimming behavior, ventilatory function, skin pigmentation, abnormalities visible, and weight were observed after 48 h of exposure, suggesting low toxicity to the NE with *M. floribunda* essential oil [45].

The in silico ecotoxicity results are in Table 4. The major compounds of the oil were analyzed individually in comparison with the reference molluscicide niclosamide. Regarding the bioconcentration factor (BCF), all the analyzed compounds presented a higher value than niclosamide. From the compounds analyzed, 1,8-cineole is predicted to have a moderate accumulation in aquatic organisms. On the other hand, β-selinene and zonarene are predicted to have a very high BCF. Therefore, these substances are expected to accumulate in lipophilic tissues. Nerolidol was the only terpenoid evaluated as supposedly biodegradable.

Regarding the aquatic toxicity for *Tetrahymena pyriformis* species, all substances are predicted to be more toxic than niclosamide. Regarding *Daphnia magna*, all the analyzed compounds tended to be less toxic than niclosamide, especially 1,8-cineole. Finally, for the *Pimephales promelas* species, all compounds analyzed are predicted to be more toxic than niclosamide, except for 1,8-cineole, which is predicted to have considerably low toxicity for this species. The endocrine toxicological results indicated that niclosamide, β-selinene, and nerolidol could interact with the androgen receptor. This result suggests a low endocrine toxicological event of these compounds.

Most substances from the essential oil of *M. floribunda* leaves showed a lower overall human toxicity potential than niclosamide. Concerning environmental toxicity, only 1,8-cineole exhibited better parameters than niclosamide. However, it must be said that the *M. floribunda* essential oil is a phytocomplex; in other words, it presents different proportions of terpenoids in its composition and, in this context, may reduce the potentially toxic effects of the essential oil em relation to niclosamide.

Table 4: *Myrciaria floribunda* essential oil major compound ecotoxicity results.

Compounds	Bioconcentration factor	Biodegradation	Aquatic toxicity			Endocrine receptor binding		TOX-Risk
			Tetrahymena $pIGC_{50}$	*Daphnia* LC_{50}	Minnow LC_{50}	Androgen receptor	Estrogen receptor	
Niclosamide	6.65	No	1.968	1.752	3.612	Toxic	Nontoxic	2
Zonarene	1497.046	No	1.003	11.855	0.421	Nontoxic	Nontoxic	1
β-selinene	1951.705	No	1.287	2.110	0.369	Toxic	Nontoxic	-
1,8-Cineole	61.434	No	0.041	227.511	149.762	Nontoxic	Nontoxic	0
Nerolidol	566.945	Yes	0.774	2.863	1.531	Toxic	Nontoxic	1

** Th_pyr_pIGC50: median inhibition of Tetrahymena pyriformis after 40 h of exposure; Daphnia_LC50: median lethal concentration (mg/L) Daphina. magna population; Minnow_LC50: median lethal concentration (mg/L) of minnows. Andro_Filter and Estro_Filter: evaluates a substance afinity in binding to the androgen/estrogen receptor. ADMET_Risk: potential liabilities of a substance.*

4.0 Materials and Methods

4.1. Plant material

The collection of the *Myrciaria floribunda* leaves were realized at the Restinga de Jurubatiba National Park, Rio de Janeiro, Brazil (22°12'98.6” S - 41°35'00.7” O, 22°12'99.8” S - 41°35'01.8” O). The collet and and research of the vegetal material were authorized by the Chico Mendes Institute of Biodiversity Conservation (ICMbio/Brazil) under n° 13659-14 and SisGen code A314288. A *M. floribunda* voucher specimen was deposited at the Herbarium of the Faculdade de Formação de Professores (FFP) under registration RFFP: 13.789 of the Universidade do Estado do Rio de Janeiro (UERJ).

4.2. Essential oil extraction

Fresh leaves (3120 g) of *Myrciaria floribunda* were separated from the stem and crushed in distilled water. Then, they were conditioned in a round-bottom flask and hydrodistilled in a modified Clevenger for 4 h to extract the essential oil. Finally, the essential oil was filtered in anhydrous sodium sulfate (Na_2SO_4), collected in an amber glass vial, and stored in a freezer (-20 °C).

4.3. Essential oil characterization

The *M. floribunda* essential oil (1 µL) was solubilized in dichloromethane (GC grade) at proportion 1:100 mg/µL then characterized by a gas chromatograph model QP2010 (Shimadzu) coupled with a mass spectrometer (MS) and a gas chromatograph model GC-2014 (Shimadzu) equipped with a flame ionization detector (FID).

The gas chromatographic conditions were: the column was an RTX-5 column (0.25 mm ID, 30 m in length, 0.25 µm, and film thickness). The carrier gas was helium with a flow rate of 1 mL/min. The temperature of the injector was 260 °C with split injection (ratio 1:40). The temperature in the oven was started at 60 °C and then increased to 290 °C at a rate of 3 °C/min. The mass spectrometry conditions were: electron ionization was 70 eV, and the scan rate was 1 scan/s.

The arithmetic index was calculated with the retention time intervals of a standard mixture of aliphatic hydrocarbons (C7-C40) analyzed under the same gas chromatographic conditions described above. The essential oil components were identified by comparing their retention indices, arithmetic index, and mass spectra with spectral databases (Adams, 2017). The compound's MS fragmentation pattern was analyzed in relation to the suggestion from the NIST GC mass spectrum libraries. The quantification of the terpenoids was realized in a flame ionization gas chromatography (GC-FID) under the same GC–MS conditions with the exception of the FID temperature (290 °C). The percentages of the substances in the essential oil were obtained by the FID peak area normalization method.

4.4. Nanoemulsion preparation and characterization

The formulations were prepared by the low-energy by phase inversion method with modifications [45]. To prepare the formulations and determine the HLB value, eleven mixtures with different ratios of nonionic surfactants polysorbate 20 (HLB 16.7) and sorbitan monooleate 80 (HLB 4.3) were prepared (Table 5). The nonionic surfactants were added to the essential oil (5% w/w) and homogenized by magnetic stirring at 500 rpm for 30 min at room temperature

(25 °C). Then, the aqueous phase was slowly dripped onto the oil phase under the same conditions for 60 min. The parameters for the characterization were average droplet size (nm) and polydispersity index (PDI), analyzed by the dynamic light scattering (DLS) in a Zetasizer Nano, model S90 device (Malvern Panalytical, UK). The measurements were performed with the samples at room temperature (25 °C) in a 1:50 dilution in distilled water. The required HLB for the *M. floribunda* essential oil was calculated by equation (a)[46].

Table 5: *Myrciaria floribunda* formulation (F1-F11) compositions.

	Oil phase %	Aqueous phase % (w/w)	Essential oil (%) (w/w)	Polysorbate 20 (%) (w/w)	Sorbitan monooleate (%) (w/w)
F1	10.0	90.0	5.0	5.0	0.0
F2	10.0	90.0	5.0	4.5	0.5
F3	10.0	90.0	5.0	4.0	1.0
F4	10.0	90.0	5.0	3.5	1.5
F5	10.0	90.0	5.0	3.0	2.0
F6	10.0	90.0	5.0	2.5	2.5
F7	10.0	90.0	5.0	2.0	3.0
F8	10.0	90.0	5.0	1.5	3.5
F9	10.0	90.0	5.0	1.0	4.0
F10	10.0	90.0	5.0	0.5	4.5
F11	10.0	90.0	5.0	0.0	5.0

$$HLBr = \frac{(HLBa\ x\ A\% + HLBb\ x\ B\%)}{100} \qquad \text{(a)}$$

The required *HLBr* is the resulting value of two surfactants mixture. *HLBa* is the more hydrophobic surfactant. *A%* is the percentage of the hydrophobic surfactant. *HLBb* is the hydrophilic surfactant. *B%* is the percentage of the hydrophobic surfactant. A% + B%=100.

4.5. Stability study

The promising NE formulation was selected to carry out the stability study for 200 days. The NEs were prepared and stored under different thermal conditions: room temperature (25 ± 2 °C), refrigeration (8 ± 2 °C), and in a climatic chamber (42 ± 2 °C) [47]. The mean droplet size (nm) and polydispersity index (PdI) were evaluated with NE at room temperature (25 °C) several times (0, 7, 15, 30, 60, 90, 120, 150, and 200 days) after preparation under the three storage conditions.

4.6. Transmission electron microscopy (TEM)

The morphology of the NE with promising DLS values after 1 h of preparation was characterized by transmission electron microscopy (TEM) with a Morgagni 268/FEI (FEI Company, Holland). First, the NE was diluted in a proportion of 1:1 in distilled water. Then, 5 µL was conditioned in a copper grid coated with formvar, dried in a desiccator for 60 min, and analyzed.

4.7. Ovicidal assay

The ovicidal capacity of the nanoemulsion was tested on embryos of *B. glabrata* at 72 h of life. For this, in triplicate, following Araújo et al. (2019) methodology, using 24-well plates, embryos (n=150) were placed in a well and exposed to 2 ml of the nanoemulsion formulation. Embryos were exposed for 48 h to the following NE concentrations: 100, 80, 60, 40, 20, and 10 µg/mL (expressed in essential oil). Mortality was evaluated continuously after 24 h and 48 h. The negative control was distilled water, and niclosamide (2 µg/mL). was used as the positive control. Additionally, the blank NE (without active oil) was evaluated.

4.8. Molluscicidal assays

The mollusks were collected at Sumidouro (RJ, Brazil) and kept in breeding tanks at the Lauro Travassos Pavilion of the Oswaldo Cruz Institute (Rio de Janeiro). They were maintained in chlorine-free water and fed with *Lactuca sativa* L. (1758). For the molluscicidal assays (n=108), 24-well plates containing 2 mL of NE were used in each well at the concentrations: 10, 20, 40, 60, 80, and 100 µg/mL (expressed in essential oil). After that, *B. glabrata* juvenile (6-8 mm) and adult (10-12 mm) mollusks were individually placed in the wells [48]. The same volume was used for the negative control (distilled water), blank NE (without essential oil), and positive control (niclosamide at 2 µg/mL). Mortality was assessed at 24 h and 48 h. The mortality criteria were the release of hemolymph, absence of retraction, and exaggerated retraction into the shell.

4.9. Cercaricidal assay

The *M. floribunda* NE was also evaluated against the cercariae of the parasite *S. mansoni* (n= 480). For this purpose, in triplicate, the mortality of cercariae in suspension exposed to NE was evaluated in a 24-well plate, totaling 2 mL per test well. Each group (n=80) was exposed to NE at 100, 80, 60, 40, 20, and 10 µg/mL (expressed in essential oil) for 4 h. The mortality was evaluated after every hour for 4 h after nanoemulsion exposure. The negative control was distilled water, and the blank NE (without active oil) at 100 µg/mL, and the positive control was niclosamide (2 µg/mL).

4.10 In silico environmental toxicity analysis

ADMET Predictor™ (version 9.5, Simulations Plus, Lancaster, CA) was used to evaluate the reference molluscicide niclosamide and the essential oil terpenoids molecular structure and experimental data to create the QSAR models to predict the biological properties of the essential oil major compounds. The endpoints were bioconcentration, biodegradation, aquatic toxicity at different trophic levels (*Tetrahymena pyriformis*, *Daphnia* (water flea), and *Pimephales promelas*), endocrine toxicity (estrogenic and androgenic hormones) and toxicological risk.

4.11 Acute oral toxicity in non-target Danio rerio

The experiment followed the ARRIVE guidelines of animal welfare regulation and, was approved by the Ethics Committee of Instituto Vital Brazil protocol number 003/2019 [49]. Male *Danio rerio* (Zebrafish) weighing 400 - 450 mg, provided by the Alternative Methods to Animal Use Laboratory of the Vital Brazil Institute were kept in a rack, with the water control parameters pH = 7.0 ± 1, temperature 26 ± 2 oC, ammonia = 0 ppm, photoperiod (light/dark) = 12h/12h.

Ten *Danio rerio* were randomly distributed in equal numbers in five experimental tanks (15 x 8 x 12 cm) containing 1 L of water. The dose corresponding to 50 µg/mL (expressed in essential oil) of the *M. floribunda* nanoemulsion was administered orally (gavage). The clinical signs related to balance, swimming behavior, ventilatory function, skin pigmentation, and visible abnormality were observed at 0, 3, 6, 24, and 48 h after oral administration. The mortality criteria were no visible reaction after touching the caudal peduncle. The animals were weighed before and after the experiment [50]. The evaluation and use of clinical signs for humanized endpoint assessment were observed [51]. In the end, the animals were euthanized using a eugenol solution [52].

4.12 Statistical analysis

The LC_{50} estimations were realized by probit analysis with SAS software [53], and survival and one-way analysis of variance (ANOVA) followed by Tukey's post-test was performed using GraphPad Prism (ver. 8) with a significance level of $p < 0.001$.

5. Conclusions

This study revealed a stable biotechnological nanoemulsion containing *Myrciaria floribunda* (Myrtaceae) essential oil as a potential alternative to control *Biomphalaria glabrata* mollusks, the intermediate host of schistosomiasis, presenting promising mortality effects in a broad spectrum of the mollusk life cycle and cercariaes, the human infective form of *Schistosoma mansoni*.

6. Patents

This work resulted in an invention patent deposited at the Brazilian National Institute of Industrial Property (INPI) process number BR 10 2020 026179 7.

Supplementary Materials: Not applicable.

Author Contributions: Conceptualization, F.PM. ; methodology, F.P.M., L.S. R; software, N.L. R, C.R.R. ; validation, L.S. R, and R.X. F; formal analysis, F.P. M, L.S. R, R.E., D.F., A.P.S. M, M.G.S., J.A.A. S, N.L. R, and C.R. ; investigation, F.P.M., L.S. R, A.P.S. M; resources, L.R. and. R.X. F; data curation, F.P.M. ; writing—original draft preparation, F.P.M. ; writing—review and editing, F.P. M; A.P.S.M., L.R. and. R.X.F. visualization, F.P. M, L.R. and. R.X.F. ; supervision, E.R. J, L.R. and. R.X.F. ; project administration, E.R. J, L.R. and. R.X.F. ; funding acquisition, E.R. J, L.R. and. R.X.F. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: The samples of essential oil were available from the authors.

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7. CAPÍTULO V – ARTIGO 3

7.1 Potential of *Ocotea indecora* (Schott) Mez essential oil nanoemulsion in schistosomiasis control: molluscicidal effects

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Potential of *Ocotea indecora* (Schott) Mez essential oil nanoemulsion in schistosomiasis control: molluscicidal effects

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Abstract

Schistosomiasis is a neglected disease transmitted through contaminated water in populations with low basic sanitation. The World Health Organization recommends controlling the intermediate host snails of the *Biomphalaria* genus with the molluscicide niclosamide. This work aims to evaluate the biocidal potential of the nanoemulsion prepared with the essential oil of *Ocotea indecora* leaves for the control of the mollusk *Biomphalaria glabrata*, intermediate host of the *Schistosoma mansoni*, the etiologic agent of schistosomiasis.

Keywords: Lauraceae; nanotechnology; moluscicide; natural products

1. Introduction

Schistosomiasis is a neglected parasitic disease transmitted by trematodes of the *Schistosoma* genus. The disease is associated to fecal contamination of water sources by infected people and/or animals. The main etiological agents of schistosomiasis are *Schistosoma hematobium*, *Schistosoma japonicum*, and *Schistosoma mansoni* (Gomes *et al.*, 2022). It is a predominant disease in low-income populations with poor sanitation, causing a great socioeconomic impact on developing countries (Rocha *et al.*, 2021). It is estimated that 779 million people in 78 countries live in endemic areas. In this way, the World Health Organization (WHO) established the eradication of schistosomiasis as a priority for world public health (WHO, 2022a).

The WHO recommends the control of intermediate host snails of the *Biomphalaria* genus with molluscicide agents, such as niclosamide, in epidemic areas (WHO, 2022a). However, chemical molluscicides are the last resource to control schistosomiasis due to their high cost, toxicity to non-target organisms, and the emergence of resistance.

An alternative approach is related to searching and developing new plant-based products. Essential oils are in the spotlight of novelty for new products with different applicability, among them as pesticides, to produce cheaper, more effective, biodegradable, safer and environmentally less aggressive new alternatives (Pavoni *et al.*, 2019a). However, these volatile lipophilic oils' have an intrinsic limited use in aqueous media. For this reason, nanoemulsions are an interesting approach enabling utilization of essential oils in aqueous matrices (Tomiotto-Pellissier *et al.*, 2017). They are kinetically stable dispersed systems composed of two immiscible phases with a main difference to kinetic-unstable conventional emulsions due to a reduced droplet size (~20 - 200 nm). Therefore, it has been widely used in several industry segments over the years (Marhamati, Ranjbar e Rezaie, 2021).

The *Ocotea indecora* (Schott) Mez (Lauraceae) is a native and endemic plant from Brazil occurring in the Atlantic Forest. To the best of our knowledge, few studies were performed with the essential oil of *O. indecora* leaves, showing diverse secondary metabolites such sesquirosefuran, bicyclogermacrene, valerianol, and β-pinene (Gonçalves et al., Machado et al., 2023; Pinto et al., 2023). The biological potential was described in term of biocidal action against pests such as the *Aedes aegypti* (dengue mosquito vector), *Rhipicephalus (Boophilus) microplus* (bovine ticks), *Dysdercus peruvianus* (cotton stainer bugs), in addition to antifungal activity against *Aspergillus* species (Figueiredo *et al.*, 2018; Machado *et al.*, 2023; Nascimento *et al.*, 2020; Pinto *et al.*, 2023).

Thus, as part of our ongoing studies with bioactive prospection of *O. indecora* essential oil against pests, this work aimed to evaluate the action of both essential oil and its nanoemulsion against *Biomphalaria glabrata* mollusks, intermediate hosts of human schistosomiasis.

2. Material and methods

2.1 Plant material

The plant material was collected in the Restinga de Jurubatiba National Park ("22º12.683'S", "41º35.283'O") with SisBio 13659-18 and SisGen A491A56 authorization codes. The herbal material was identified, and a voucher of the specimen was deposited in the herbarium of the State University of Rio de Janeiro (UERJ) under code RFFP: 16.873.

2.2 Essential oil extraction and characterization

The essential oil from leaves (300 g) was extracted by hydrodistillation in a modified Clevenger for 4h, dried over anhydrous sodium sulfate, and stored in an amber glass flask under refrigeration (-20 °C). The chemical characterization of the essential oil was realized by gas chromatography coupled to mass spectrometry (GC-MS QP2010, Shimadzu) and quantified in a gas chromatograph (GC-2014, Shimadzu) coupled to a flame ionization detector (FID). The chromatographic conditions for MS and FID were described previously by Machado et al. (2023).

2.3 Nanoemulsion preparation and characterization

The *O. indecora* nanoemulsion was prepared by a low-energy method with following composition: 2% (w/w) of essential oil, 2% of surfactants - sorbitan monooleate and polysorbate 20 at 1:4 proportion (w/w) – and 96% of deionized water (w/w) (Machado *et al.*, 2023). Prior to the nanoemulsification the oil phase (essential oil and surfactants) was homogenized for 2 min in a vortex homogenizator (Phoenix, model AP56). Then, the aqueous phase was slowly dripped to the oil phase at continuous homogenization. The average droplet size (nm) and polydispersity index (PdI) were obtained were from dynamic light scattering (DLS) analysis, while zeta potential (ZP) was obtained from electrophoretic light scattering (ELS). Both DLS and ELS measurements were performed in a Zetasizer Advance Lab Blue (Malvern, UK) at room temperature (25 °C). Prior to the measurements, which were performed in triplicate, the nanoemulsion was diluted at 1:20 in deionized water to avoid multiple scattering.

2.4 Molluscicidal assays

The molluscicidal assay was performed as described by Santos et al., (2017). *B. glabrata* adults with 10-12 mm were separately deposited in 24-well plates with 2 mL of samples (nanoemulsion or essential oil) at concentrations of 100 to 10 µg/mL (expressed in essential oil) in order to evaluate its biocide effect. The nanoemulsion control (dispersion of surfactants), essential oil control (DMSO solution), negative control (distilled water) and positive control (niclosamide 1 µg/mL) were also evaluated in *B. glabrata* after 24 h of exposure. The mortality criteria were the release of hemolymph, absence of retraction, or prolongated retraction in the shell.

2.6 Statistical analysis

The median lethal concentrations were estimated by probit analysis with SAS software, and one-way analysis of variance (ANOVA) followed by the Tuckey post-test was realized with GraphPad prism (ver. 8) with a significance level of $p < 0.05$.

3. Results

3.1 Essential oil extraction and characterization

The chemical characterization of the essential oil from *Ocotea indecora* leaves is described in Table 1. The most predominant terpenoid in the essential oil was sesquirosefuran (83.39 %). (Z)-β-farnesene (3.55 %) and allo-aromadendrene (1.64 %) were also identified in minor proportions.

Table 1: Chemical characterization of the *Ocotea indecora* leaves essential oil by gas chromatography.

	RT	AI_{exp}	AI	Substances	%
1	26.970	1448	1440	(Z)-β-farnesene	3.6
2	27.323	1457	1458	Allo-aromadendrene	1.6
3	30.802	1545	1549	Sesquirosefuran	83.4

Total identified	88.6
Sesquiterpene hydrocarbons	5.2
Sesquiterpene furan	83.4

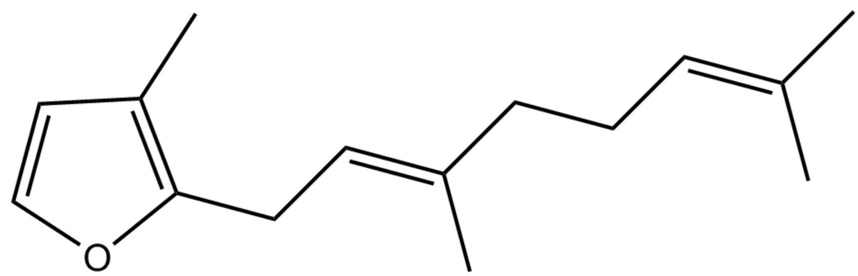


Figure 1: Chemical structure of the sesquirosefuran, the main metabolite of *O. indecora* essential oil.

3.2 Nanoemulsion preparation and characterization

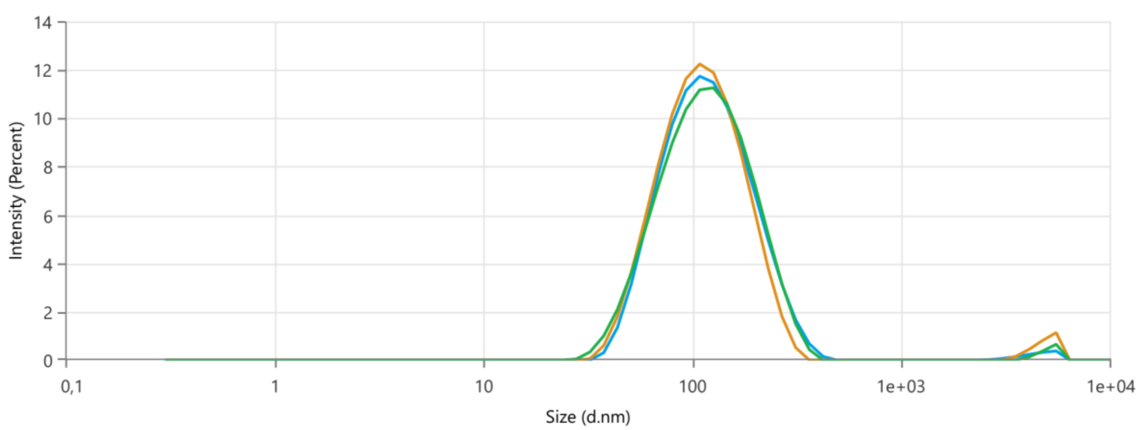


Figure 2: Droplet size distribution by intensity of the *Ocotea indecora* nanoemulsion.

Nanoemulsion macroscopic characteristics were homogeneous blue transparent aspect. These characteristics are related to the reflection of the light and known as Tyndall effect, a typical appearance of colloids. Droplet size distribution graph of the *O. indecora* nanoemulsion after preparation is shown in Figure 2, indicating a monomodal curve. After preparation, it presented droplets size (nm) of 102.8 ± 0.754 with 0.243 ± 0.014 PdI. Zeta potential presented high value (in module) and average value was - 34.66 ± 0.007 mV. The nanoemulsion maintained the physicochemical characteristics for 15 days after preparation (Table 2).

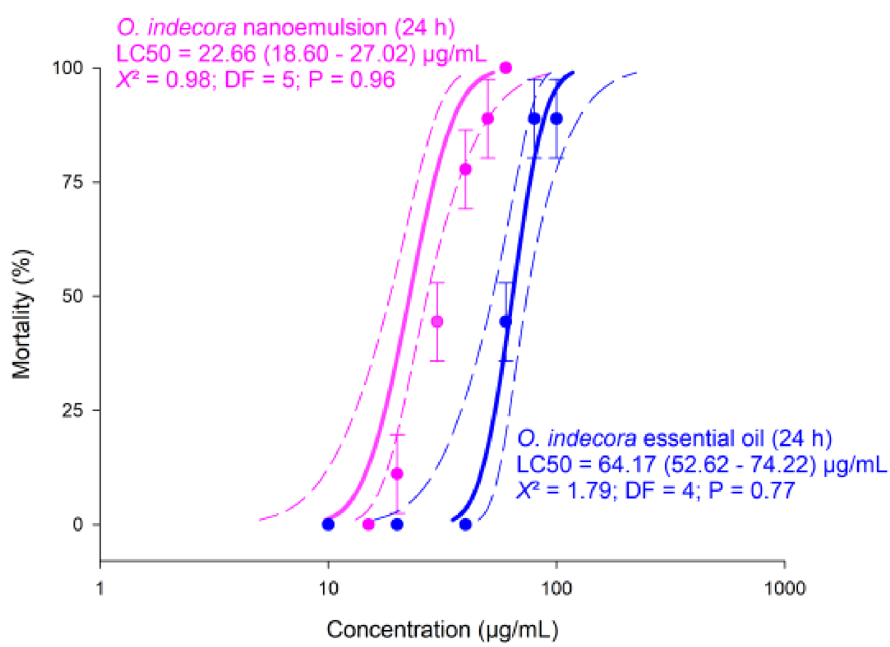
Table 2: *Ocotea indecora* nanoemulsion stability at room temperature for 15 days after preparations.

Days	Average size (nm)	PdI	ZP
0	102.8 ± 0.754	0.243 ± 0.014	-34.66 ± 0.007
7	98.39 ± 1.045	0.275 ± 0.002	-31.25 ± 1.152
15	97.27 ± 0.310	0.310 ± 0.038	-32.28 ± 0.574

3.3 Molluscicidal assays

The nanoemulsion showed 100% mortality at 50 µg/mL, while the essential oil showed 88.8% at 100 µg/mL. Only the lowest concentrations of nanoemulsion and essential oil did not present significant difference ($p>0.05$) to sample controls and negative control. The LC_{50} was 22.66 (18.50 – 27.02) µg/mL and 64.17 (52.62 - 74.22) µg/mL for the nanoemulsion and the essential oil, respectively (figure 3).

Figure 3: Nanoemulsion and essential oil of *O. indecora* median lethal concentration in *B.*



glabrata after 24 h.

4. Discussion

The main metabolite of *Ocotea indecora* essential oil from leaves was the sesquirosefuran comprehending 83.4% of the oil. Therefore, corroborating other studies that also described sesquirosefuran as the most abundant component with 88.2 - 92.2% in the oil (Figueiredo *et al.*, 2018; Machado *et al.*, 2023; Nascimento *et al.*, 2020).

The chosen low-energy nanoemulsification method is solvent-free, without heating and therefore can be considered “green method” and expected to avoid alterations in the chemical profile of *O. indecora* essential (Viana *et al.*, 2023). The dynamic light scattering showed a droplet size of approximately 100 nm, being in accordance with the classification of nanoemulsions (Marhamati, Ranjbar e Rezaie, 2021). The PdI of 0.24 suggests a monodisperse distribution with monomodal behavior over the analyzed days (Santos Matos, dos *et al.*, 2020), suggesting together with the droplet size a suitable stability.

The surface charge of nanoemulsions is also an important parameter associated with the stability of the system. The intermolecular repulsive force between nanodroplets favors the kinetic stability by Brownian motion. The zeta potential results in the present study were negative with values of -34 mV, indicating good coulombic repulsion between nanodroplets over the 15 days observed. Absence of variations (~ ±5 mV) in a short time suggest droplet stabilization (Rai *et al.*, 2018).

Several authors have been describing the potential of natural products and nanotechnology to assist in the development of novel alternatives. This approach converges with the United Nations Millennium Development Goals (Aguiar *et al.*, 2022; Araújo *et al.*, 2019; Faria *et al.*, 2018; Friani *et al.*, 2023; Matos *et al.*, 2020; WHO, 2022b), especially those involving the eradication of neglected diseases, such as schistosomiasis.

The World Health Organization (WHO) determined that an essential oil can be considered a promising molluscicide agent by presenting LC_{50} values lower than 40 µg/mL (Pereira *et al.*, 2020). In this way, the essential oil of *O. indecora* can be considered a promising candidate for controlling intermediate host snails since it presented an LC_{50} of 64.17 µg/mL after 24 h. Sesquirosefuran is a metabolite found in essential oils and poorly explored in relation to its biological potential. Some authors have described an insecticidal effect associated with its presence in essential oils, which may be associated with an allomonic ecological correlation between plant-insect (Machado *et al.*, 2023; Mollo *et al.*, 2017; Nascimento *et al.*, 2020). Also, furanoterpenoids analogue of sesquirosefuran have been found in sea sponges and correlated with defensive mechanisms (Mollo *et al.*, 2017). Therefore, considering it high percentage in the essential oil, it may develop a main role in the molluscicide action.

Notably, the nanoemulsion enhanced about 3x the molluscicidal effects of the *O. indecora* essential oil, showing an LC_{50} of 22.66 µg/mL after 24 h. This phenomenon can be associated with the nanometric scale of the essential oil droplets, which often can improve the biocide activity by increasing the permeability through biological membranes and, consequently, the bioavailability with a lower concentration of a bioactive compound (Tomiotto-Pellissier *et al.*, 2017).

5. Conclusion

This work showed the potential of the essential oil of *Ocotea indecora* for schistosomiasis control. In addition, its nanoemulsion enabled the dispersion in aqueous media and improvement of the molluscicidal effect against *Biomphalaria glabrata* mollusks. One would expect that not merely a physical characterization would define a nanoemulsion, but alteration of a desired property is an important parameter. Therefore, we provide a description of potential molluscicide nanoemulsion that was prepared by a green concept and can be of great interest for prospection of novel molluscicidal systems.

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8. CAPÍTULO VI – ARTIGO 4

8.1 Chemical Profile of *Ocotea indecora* (Shott) Mez Leaves Essential oil: A Brazilian Endemic Specie from the Atlantic Forest

Short comunication submetido no periódico "Chemistry of Natural Compounds"

Em 17 de janeiro de 2023

CHEMICAL PROFILE OF *Ocotea indecora* (Shott) Mez LEAVES ESSENTIAL OIL: A BRAZILIAN ENDEMIC SPECIE FROM THE ATLANTIC FOREST

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Brazil is a country of continental proportions centralizing 15% of the planet's biodiversity. The Atlantic Forest is considered a biodiversity hotspot due to continuous deforestation over the years and currently, only 7% of the forest remains [1]. Therefore, it is necessary to investigate the chemical potential of Atlantic Forest plants, especially endemic ones.

Ocotea indecora (Lauraceae) is a native and endemic plant from Brazil that can be found as a tree or shrub and is popularly known as “Canela-sassafrás” in the Northeast of Rio de Janeiro State. To the best of our knowledge, this plant is almost unexploited in terms of scientific investigations. Thus, the objective of this work was to describe the chemical composition of *Ocotea indecora* essential oil from leaves collected in coastal sandbank areas in different years.

The leaves of *Ocotea indecora* were collected from 2019 to 2022 at the same locality (22°12.983’S, 41°35.283’O) in the Restinga de Jurubatiba National Park, Carapebus, RJ, Brazil. The collection and research of the plant material were authorized by SISGen (code A0D648D) and SISBio (n° 13659-14). The herbal material was identified and a voucher specimen was deposited and registered under the register number RFFP 16.873 at the herbarium from the Rio de Janeiro State University (UERJ, São Gonçalo, RJ, Brazil [-

22.832584542925385, -43.07258158657145]). Fresh leaves (350 g) were subjected to hydrodistillation for 4h in a modified Clevenger. The obtained essential oils were dried with anhydrous sodium sulfate and stored in amber glass vials at -20 °C for analysis. The essential oils obtained from *O. indecora* at different years were slightly yellow and transparent. The yields were 0.63, 0.61, 0.70, and 0.47 % (w/w), respectively for the years 2019, 2020, 2021, and 2022.

The chemical characterization of the essential oils was realized by gas chromatography (GC) coupled to a mass spectrometer (MS) for identification and GC coupled to a flame ionization detector (FID) for quantification as described previously [2]. The GC analysis allowed the identification of 9 constituents of the leaf's essential oils (Table 1).

TABLE 1. Chemical characterization of *Ocotea indecora* essential oil from different years by GC-MS and GC-FID, %.

Compound[a]	AI[b]	2019	2020	2021	2022
α-copaene	1374	-	0.30	-	-
(Z)-β-farnesene	1440	4.48	4.81	5.76	3.33
Allo-aromadendrene	1458	0.73	0.95	1.22	1.55
Bicyclogermacrene	1489	0.67	-	-	-
Sesquirosefuran	1549	87.99	79.84	70.45	86.13
Dendrolasin	1570	0.78	0.98	-	-
Spathulenol	1577	0.19	1.24	-	-
Eremoligenol	1629	0.25	-	-	-
(2E,6E)-methyl farnesoate	1776	0.37	0.39	-	-
Total identified		95.46	88.51	77.43	91.01

[a]Elution on DB-5 column; [b]Arithmetic index.

The identification of the compounds was realized by interpolating the retention times of a standard mixture of aliphatic hydrocarbons (C9-C30) analyzed under the same chromatographic conditions and by comparing the AI and the MS fragmentation pattern with built-in NIST libraries and other mass spectra libraries [3, 4].

In all years, the major compound in *O. indecora* essential oil was the sesquirosefuran (70.43 to 87.99%), a farnesene-type furansesquiterpenoid (Figure 1). It is reported in the Lauraceae family, generally in low quantities in essential oils, with exception of the

Actinodaphne lancifolia which showed 79.2% of this compound in the root oil [5]. Also, small amounts of dendrolasin (0.78 to 0.98%), another furanyl sesquiterpenoid structurally similar to sesquirosefuran, were found in the essential oils from 2019 and 2020.

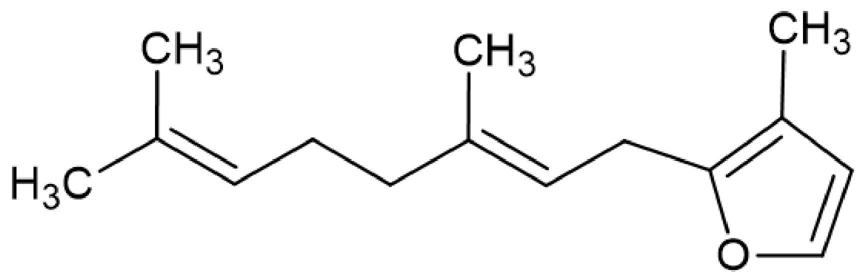

FIGURE 1: Sesquirosefuran chemical structure.

A specimen collected in São Paulo presented bicyclogermacrene (29.79%), valerianol (15.12%), β-pinene (11.41%), and spathulenol (11.16%) as the major compounds [6]. There are few records about the sesquirosefuran bioactivity [7, 8]. However, it is hypothesized that furan-type sesquiterpenoids may be related to a defensive allomone mechanism from plants against herbivorous insects [9].

The high percentage of this compound in the essential oil collected from different years may be attributed to environmental response, since the present work evaluates specimens from a location associated with dramatic conditions, such singular natural pressures, such as temperatures, salinity, hydric resources, soil nutrient among other factors [10]. Therefore, this work contributes to the chemical report of an important compound present in high amounts in an endemic Brazilian species.

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9. CAPÍTULO VII – ARTIGO 5

9.1 Larvicidal effects of *Myrciaria floribunda* (H. West ex Willd.) O. Berg nanoemulsion against *Aedes aegypti*

Artigo a submeter no periódico "Journal of Natural Pesticides Research"

Em 2023

Larvicidal effects of *Myrciaria floribunda* (H. West ex Willd.) O. Berg nanoemulsion against *Aedes aegypti*

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Abstract

Dengue is the major health problem between the arboviruses (e.g Dengue, Zika, Chikungunya, and urban yellow fever) transmitted by the *Aedes aegypti* (Diptera: Culicidae). In 2023 until November a total of 4.5 million of Dengue cases was reported in 80 countries. The World Health Organization (WHO) preconizes the use of insecticidal agents to control the *Aedes aegypti*, as a way to interrupt the transmission cycle of those arboviruses. However, insecticides often are harmful to the environments and humans. The widespread use for an extensive time created selective pressures to the *Aedes aegypti* develop resistance mechanisms to the traditional insecticides, and consequentially, decreasing their effects. Thus, this work aimed to evaluate the insecticidal properties of the *Myrciaria floribunda* essential oil nanoemulsion in 3rd instar larvae. The essential oil showed 1,8 cineole, (E)-nerolidol and β-selinene as major constituents. The nanoemulsion showed bluish white macroscopical characteristics with droplet size of 88.34 ± 0.946, 0.276 ± 0.014 of polydispersity index, and -34.70 ± 1.311 of zeta potential. Also, showed stability after 15 days of preparation and when briefly exposed to rising temperatures. Regarding the larvicidal effect, the nanoemulsion presented LC_{50} = 299.29 (259.1 – 356.8) µg/mL and 233.65 (199.9 - 272.5) µg/mL after 24h and 48h, respectively. This work describes the chemical profile and larvicidal effects of

Myrciaria floribunda essential oil from leaves and contributes to the efforts in develop novel and sustainable alternatives for the control of arboviruses.

Keywords: essential oil; Myrtaceae; vector; dengue; insecticide

1. Introduction

The *Aedes aegypti* (Diptera: Culicidae), popularly known as the mosquito, is considered of medical importance as it is the main vector responsible for transmitting several arboviruses including Dengue, Zika, Chikungunya, Mayaro, and urban yellow fever (Silvério et al., 2020).

Dengue fever is established globally, and is the most prevalent arboviral public health problem, epidemiological estimative suggests that until November of 2023 occurred 4.5 million dengue infections with 4.000 deaths (ECDC, 2023). However, it is suggested that dengue (among other arboviruses) is underreported by countries' surveillance agencies, especially those under development (Junior et al., 2022). Therefore, the use of insecticides for mosquito population control is an alternative recommended by the World Health Organization (WHO) for managing regions with viral endemic (WHO, 2009). The most effective way of chemical control is in the larval stages because the *Aedes aegypti* lifecycle is water-dependent. Also, they are restricted to a container, unlike the adult form with free-winged movement that prevents its exposure to pulverized insecticides (WHO, 2005). However, traditional insecticides, such as the organophosphorus temephos, have long half-lives and are non-selective and environmentally toxic (Chaudhary et al., 2017). In addition, leads to the selection of mosquitos predisposed to resistance, leading to ineffectiveness in *Aedes aegypti* control, a consequence of the continued use over the years (Gupta et al., 2023; WHO, 2009).

An alternative approach is the use of plant-derived substances in the development of pesticides more organic, biodegradable, and sustainable when compared to traditional synthetic ones (Esmaili et al., 2021). The search for essential oils for the development of novel insecticides is part of a major effort to find new sustainable alternatives since only 10% of the applied pesticides come into direct contact with their target pests, the rest undergo bioaccumulation and biomagnification processes (Chaudhary et al., 2017). However, the essential oil's physicochemical characteristics such as lipophilicity and volatility make it challenging their commercial use. Volatile oils are liquid oils, generally aromatic, composed of terpenoids (e.g. monoterpenes and sesquiterpenes) and phenylpropanoids that can be obtained by distillation processes (e.g. hydrodistillation) that may be found in all plant parts (Assadpour et al., 2023).

Nanostructure is a valuable tool to enable these volatile oils in aqueous media since they encapsulate it with compatible substances forming a stable interface between them(Ansari, 2023). A nanoemulsion is a coherent approach that has been used for larvicidal essential oils since they are a dispersion of two immiscible liquids, generally stabilized by surfactants, to avoid gravitational phase separation tendency (Echeverría & Albuquerque, 2019; Machado, Folly, Salas Enriquez, et al., 2023; Moola et al., 2023; Viana et al., 2023; Vivekanandhan et al., 2023). They are kinetically stable and thermodynamically unstable systems, with reduced droplet diameter (e.g 20 to 200 nm) that can be prepared by low or high-energy techniques (Marhamati et al., 2021). However, low-energy methods are more appropriate for essential oil formulations, as the spontaneous generation uses internal chemical energy, and in that way does not affect the essential oil composition being more suitable for industrial scalement (Solans & Solé, 2012). In contrast, high-energy methods depend on mechanical energy that dissipates the excess as heat in the nanoemulsification system, which may induce the volatilization of the low-weight monoterpenoids from the essential oil (McClements & Jafari, 2018).

Myrciaria floribunda (H. West ex Willd.) O. Berg (Myrtaceae) is a native plant from Brazil, popularly known as "*Camboim*", "*Cambui*", or "*Camboim amarelo*" (de Oliveira et al., 2018). The plant's biological and chemical properties have been reported in the literature over the years probably due to its fruits since it can be eaten fresh, and also popularly consumed in alcoholic beverages (Santos, Santos Dos Santos, Oliveira, Costa, Zagmignan, Da Silva, et al., 2020). The plant can be found in the Atlantic Forest, near coastal areas in the north of the Rio de Janeiro state (Machado, Folly, Esteves, et al., 2023). Previous studies have described the biological properties of this plant species, within this, one can mention antioxidant, antiproliferative, anti-inflammatory, antinociceptive, molluscicidal, cercaricidal, and anti-fungal among others (de Azevedo et al., 2019; Duarte et al., 2023; Machado, Folly, Esteves, et al., 2023; Santos, Santos Dos Santos, Oliveira, Costa, Zagmignan, da Silva, et al., 2020; L. A. C. Tietbohl et al., 2017). The essential oil has already been described with insecticidal properties on *Rhodnius prolixus*, *Dysdercus peruvianus*, and *Oncopeltus fasciatus* (L. A. C. Tietbohl et al., 2014, 2020a). Thus, the objective of the work was to evaluate for the first time the effects of the essential oil nanoemulsion against *Aedes aegypti* larvae.

2. Methodology

2.1 Plant material

Fresh leaves of *Myrciaria floribunda* were collected at Restinga de Jurubatiba National Park (site 1: 22°12'98.6” S - 41°35'00.7” O; site 2: 22°12'99.8” S - 41°35'01.8” O). The plant material collection and research were authorized by SISBIO/ICMBio (13659-18) and SisGen/Brazil (A314288). A voucher specimen (RFFP: 13.789) was deposited at the Faculdade de Formação de Professores (FFP) herbarium of the Universidade Estadual do Rio de Janeiro (UERJ).

2.2 Essential oil extraction

The essential oil was obtained by hydrodistillation in a modified Clevenger apparatus for 4h. The fresh leaves (400.1 g) were crushed in a blender (model SPL-052, Spolu-benesse, Itajobi, SP, Brazil) in distilled water, placed in a 5 L round bottom flask, and submitted to extraction. After that, the essential oil was dried with anhydrous sodium sulfate (≥99%, Sigma-Aldrich, St. Louis, MO, USA), and stored in an amber glass vial at -4 ºC.

2.3 Essential oil characterization

The essential oil of fresh leaves from *M. floribunda* was analyzed using a GC–MS QP2010 (Shimadzu, Corp., Kyoto, Japan) gas chromatograph equipped with a mass spectrometer and a GC-2014 (Shimadzu, Corp., Kyoto, Japan) gas chromatograph equipped with a flame ionization detector (FID). Gas chromatographic (GC) conditions were as follows: injector temperature at 260 °C; flame ionization detector (FID) temperature at 290 °C; helium (White Martins Corp., Rio de Janeiro, Brazil) as carrier gas; flow rate of 1 ml/min and split injection with a split ratio of 1:40. The oven temperature was initially 60 °C then increased to 290 °C at a rate of 3 °C/min. One microliter of the sample, dissolved in dichloromethane (1:100 mg/µL), was injected into an RTX-5MS column (RESTEK Corp., Bellefonte, PA, 0.25 mm ID, 30 m in length, 0.25 µm, and film thickness). Mass spectrometry (MS) electron ionization was 70 eV, and the scan rate was 1 scan/s. The arithmetic index (AI) was calculated by interpolating the retention times of a mixture (C7-C40) of aliphatic hydrocarbons (Sigma-Aldrich, St. Louis, MO, USA) analyzed under the same conditions. Substances were identified by comparing their retention indices and mass spectra with those reported in the literature (Adams, 2007). The MS fragmentation pattern of compounds was also compared with NIST mass spectrum libraries. The relative abundance of the chemical constituents was determined by flame ionization gas chromatography (GC-FID) under the same GC–MS conditions. The FID peak area normalization method obtained these compounds' analysis and percentages.

2.4 Nanoemulsion preparation

The formulation of the nanoemulsion of *Myrciaria floribunda* essential oil was previously described by Machado et al. (2023) (Machado, Folly, Esteves, et al., 2023). The nanoemulsion was prepared by the low-energy by-phase inversion method. The oil phase consisted of 2% (w/w) of the essential oil, 2% (w/w) of the surfactants blend (polysorbate 20 and monooleate sorbitan 80, Sigma-Aldrich, St. Louis, MO, USA) at a 4:1 ratio, and the 96% aqueous phase (deionized water).

Initially, the oil phase was homogenized for 1 min in a vortex agitator model AP56 (Pheonix Luferco, Araraquara, SP, Brazil), then the aqueous phase (deionized water) was slowly dripped in continuous agitation until phase inversion. The nanoemulsion was stored in screw-top glass flasks at room temperature (25 ºC).

2.4.1 Nanoemulsion characterization

The characterization of the nanoemulsion was realized by dynamic light scattering (DLS) to determine the average size of the droplets (nm) and polydispersity index (PdI). To evaluate the zeta potential (ZP) was electrophoretic light scattering (ELS). The measures were realized in triplicate in a Zetasizer Advance Lab Blue device (Malvern Panalytical, UK). The nanoemulsion stored at room temperature (25 ºC) was diluted (1:50) with deionized water to avoid multiple scattering and then analyzed in triplicate.

2.4.2 Nanoemulsion stability

A thermal stability study was conducted after preparation to evaluate the nanoemulsion droplet size, PdI, and ZP behavior when exposed to rising temperatures (25 to 55 ºC) with a 10 ºC increase between analyses.

Also, a new batch of the nanoemulsion was prepared according to item 2.4. The average droplet size (nm), polydispersity index, and zeta potential of the nanoemulsion stored at room temperature (25 ºC) were evaluated in Zetasizer Advance Lab Blue (Malvern Panalytical, UK) after 0, 7, and 15 days after preparation. The nanoemulsions characterization was realized under the same condition described in item 2.4.1.

2.5 *Aedes aegypti* larvicidal assay

The *Ae. Aegypti* eggs were provided by the Instituto de Biologia de Exército (IBEx). The bioassay was realized as described by the WHO (2005) with modifications. Third instar larvae (L3) of *Ae. Aegypti* (n=10) were deposited in polypropylene plastic containers (30 mL) with 10 mL of the nanoemulsion at concentrations of 400 to 20 µg/mL (expressed in essential oil). The negative control was distilled water. Also, the blank nanoemulsion (without essential oil) at 400 µg/mL was evaluated. All assays were realized in triplicate. The estimation of the LC_{50} was realized by probit analysis in SAS software (SAS Institute Inc., 2018) and one-way analysis of variance (ANOVA), followed by Tuckey's post-test was performed in GraphPad Prism (ver. 8) with a significance level of $p < 0.05$.

3. Results and discussion

3.1 *Myrciaria floribunda* essential oil chemical characterization

The *Myrciaria floribunda* essential oil from fresh leaves yielded 0.74% and showed a translucent slightly yellowish-beige appearance with a pleasant odor with slight aromatic notes characteristic of the monoterpene 1,8-cineole. The gas chromatographic analysis allowed the identification of 91.59% of the oil, 23 substances of 30 substances, in the *M. floribunda* leaves essential oil chemical profile (Figure 1). Hydrocarbon sesquiterpenes were the major fraction of the oil (47.51 %). In addition, the oil presented 1,8 cineole (13.03 %), β-selinene (12.13 %), and (E)-nerolidol (13.48 %) as major components of the oil totalizing 38.64 % (Table 1).

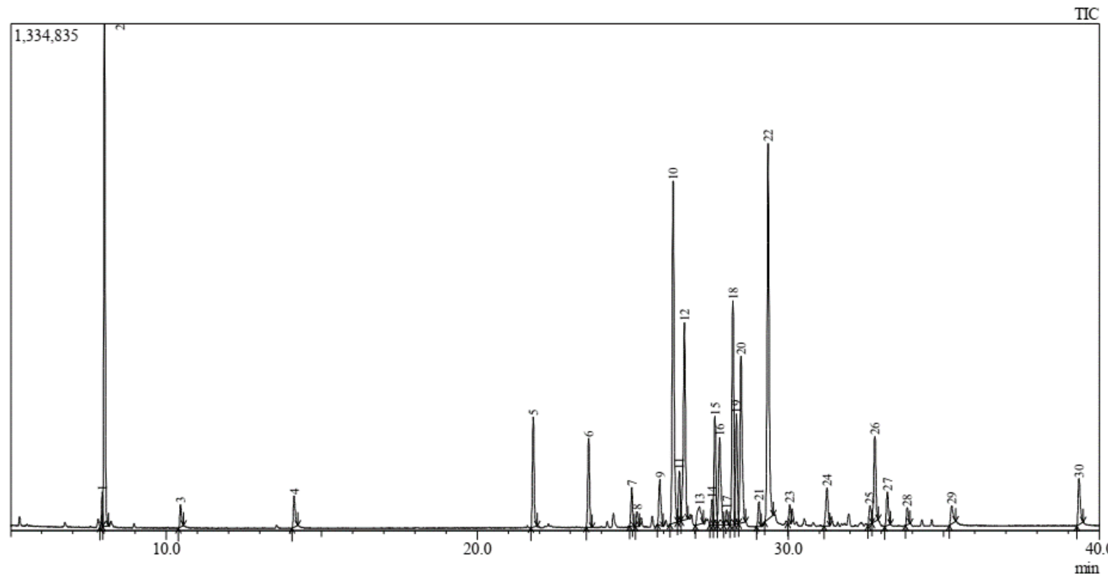

Figure 1: Chemical profile of the leaves essential oil from *Myrciaria floribunda*.

Table 1: Chemical characterization of the *M. floribunda* essential oil of leaves by GC-MS and GC-FID.

	RT	AI_{rep}	AI_{Lit}	Substances	Relative abundance (%)
1	7.944	1028	1024	Limonene	0.74
2	8.014	1030	1026	1,8-cineole	**13.03**
3	10.460	1101	1095	Linalool	0.69
4	14.113	1192	1186	α-terpineol	1.15
5	21.802	1375	1374	α-copaene	3.53
6	23.585	1419	1417	β-caryophyllene	2.92
7	24,970	1453	1452	α-humulene	1.24
8	25.140	1457	1454	(E)-β-farnesene	0.55
9	25.879	1475	1476	β-chamigrene	1.74
10	26.302	1486	1489	β-selinene	**12.13**
11	26.499	1491	1492	δ-selinene	1.67
12	26.666	1495	1498	α-selinene	7.62
13	27.145	1507	1511	δ-amorphene	1.3
15	27.646	1520	1522	δ-cadinene	3.65
16	27.798	1524	1528	Zonarene	3.73
18	28.225	1535	1566	Maaliol	7.73
20	28.485	1542	1545	Selina-3,7(11)-diene	6.58
21	29.066	1557	1559	Germacrene B	0.85
22	29.355	1564	1561	(E)-nerolidol	**13.48**
26	32.792	1655	1658	Neo-intermedeol	3.36
27	33.830	1666	1665	Intermedeol	1.25
29	35.264	1724	1713	Farnesol	0.83
30	39.352	1842	1829	(2Z,6E)-farnesyl acetate	1.82

TOTAL	91.59
Hydrocaron monoterpenes	0.74
Oxygenated monoterpenes	14.87
Sesquiterpene hydrocarbons	47.51
Oxygenated sesquiterpene	28.47

*RT: retention time; AI: arithmetic index from literature; AIrep: reported arithmetic index

The plant species *Myrciaria floribunda* has been gaining attention from Brazilian researchers due to the plant's chemical and biological properties, but especially because of the potential associated with its edible fruits, which represents economic interest for the national and international market of native fruits from the Brazilian biodiversity. Thus, recent studies have described the plant's chemical characteristics, especially the essential oil. Recently, Machado et. Al. (2023) described the *M. floribunda* essential oil chemical profile from a specimen collected in the same locality, showing the same major compounds (1,8-cineole, β-selinene, and nerolidol) in the leaves essential oil (Machado, Folly, Esteves, et al., 2023). Other studies with the plant collected in the same environment showed similar chemical results (L. A. Tietbohl et al., 2012; L. A. C. Tietbohl et al., 2020b).

3.2 Nanoemulsion preparation and characterization

The *M. floribunda* nanoemulsion showed a bluish reflection, characteristic of the diffraction of the light described in the Tyndall effect in colloidal systems. The nanoemulsion presented the collective main parameters, average droplet sizes of 88.34 nm, 0.276 PdI, and negative zeta potential (-34.70 mV). Several authors consider that a formulation must present a droplet size between 20-200 nm to be considered a nanoemulsion (Al-Hussaniy et al., 2023; Czerniel et al., 2023; Mushtaq et al., 2023). Also, must present a polydispersity index < 0.3 to be considered a monodisperse system (dos Santos Matos et al., 2020). In this context, the *M. floribunda* formulation prepared can be considered as a nanoemulsion.

The physicochemical stability of the *M. floribunda* nanoemulsion (NEMF) was also evaluated. The thermal stress stability study shows that the physicochemical parameters evaluated didn’t show relevant alterations in the function of the continuous temperature increase, maintaining the monomodal intensity and behavior (Figure 3), even showing a statistical significance ($p > 0.05$) between the droplet size before and after the thermal stress (Table 2). The polydispersity index (PdI) decreased by 6.3% of the initial value, which may suggest that more oxygenated terpenoids, which represent 43.34% of the *M. floribunda* essential oil, such as the major compounds 1,8-cineole (13.03%) and nerolidol (13.48%), may becoming more soluble and migrating from the nanodroplets to the external aqueous phase due to the increasing temperature (25-55 °C), and in that way, decreasing the PdI. In addition, showed the maintenance of the initial macroscopical aspects (bluish reflection, without homogeneity alteration) after the heating cycles. Within this context, is pertinent to consider, the NEMF as a stable system when briefly exposed the temperatures up to 55 ºC in a short period.

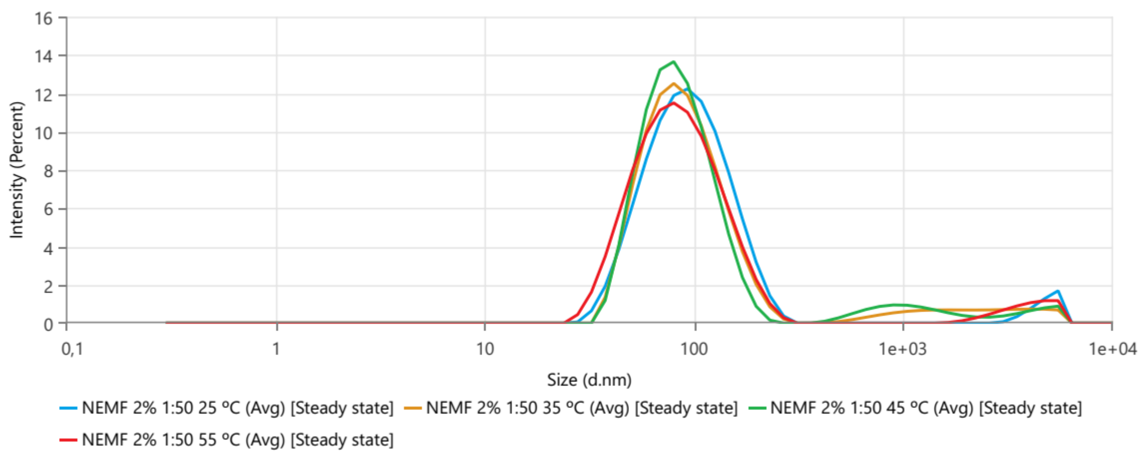

Figure 3: *Myrciaria floribunda* essential oil nanoemulsion average droplets size (nm) by intensity from the thermal stress (25-55 ºC). All analyses were realized in triplicate.

Table 2: *Myrciaria floribunda* nanoemulsion average size, polydispersity index, and zeta potential before and after the thermal stress (25 °C to 55 °C) stability.

Thermal stress stability			
Thermal stress	Average size (nm)	Polydispersity index	Zeta Potential (mV)
Before	88.34 ± 0.946*	0.276 ± 0.014	-34.70 ± 1.311
After	81.00 ± 0.281	0.258 ± 0.013	-33.17± 0.031

*95% confidence interval ($p < 0.0001$).

The zeta potential (ZP) did not show a relevant difference before and after the thermic stress ($p > 0.05$). However, the NEMF stability study stored at room temperature (25 ºC) for 15 days showed statistical significance ($p < 0.029$) between day 0 (-33.89 ± 0.5688), day 7 (-30.32 ± 0.3637), and day 15 (-28.66 ± 1.074). Alterations greater than ±5 mV in nanoemulsion ZP indicate system destabilization with possible nanodroplet aggregation. The surface charge of nanodroplets is an important stability parameter, since the electrostatic repulsion forces, related to the ZP, are directly related to the electric charge droplets have Brownian motion (Rai et al., 2018).

Table 3: *Myrciaria floribunda* nanoemulsion stability in 0, 7, and 15 days after preparation stored at room temperature (25 ºC).

Days	Average size (nm)	Average polydispersity index	Average zeta potential (mV)
0	89.13 ± 0.946	0.280 ± 0.022	-33.89 ± 0.5688*
7	88.42 ± 0.167	0.282 ± 0.028	-30.32 ± 0.3637
15	87.97 ± 1.512	0.257 ± 0.029	-28.66 ± 1.074

*95% confidence interval ($p < 0.029$) when compared with day 7.

3.3 *Aedes aegypti* Larvicidal assay

The *M. floribunda* nanoemulsion showed 80±4.47% and 70±8.94% of mortality in 3rd instar larvae at 400 µg/mL after 24 and 48h, respectively. The blank nanoemulsion (without essential oil) and negative control (distilled water) didn't show mortality after 48h. The blank nanoemulsion and negative control showed statistical differences when compared to the 400, 300, and 200 µg/mL ($p < 0.05$). However, regarding the groups at 100 and 50 µg/mL, there is no statistical difference ($p > 0.05$). Also, Figure 4 suggests that the observed larvicidal activity is directly proportional to the essential oil concentration in the nanoemulsion, as the mortality increases from 24h to 48h. The median lethal concentration (LC_{50}) estimated was 233.65 (199.9 - 272.5) µg/mL after 48h of larval exposure (Table 4).

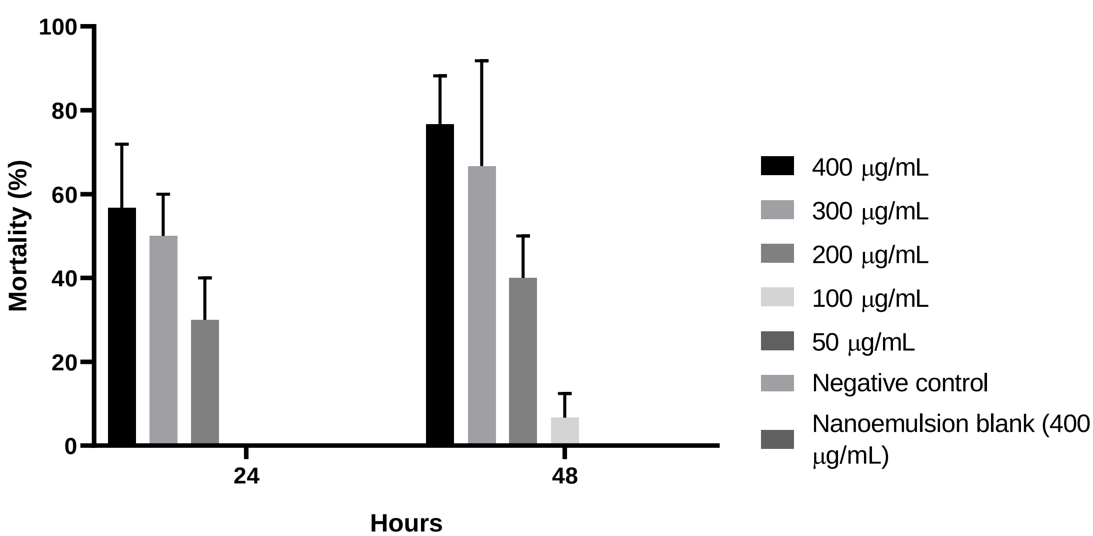

Figure 4: Mortality of the *Myrciaria floribunda* nanoemulsion in *Aedes aegypti* larvae after 24 and 48h of exposure.

Table 4: Median lethal concentration of *Aedes aegypti* larvae exposed to the *Myrciaria floribunda* nanoemulsion after 24 and 48h.

	LC_{50} (µg/mL)	DF	χ^2	*p*-value
24h	299.29 (259.1 – 356.8)	3	1.69	0.63
48h	233.65 (199.9 - 272.5)	3	0.42	0.93

Natural products, especially essential oils, are sources of bioactive substances that have been used for the development of novel plant-derived pesticides, in particular, the development of insecticides (Echeverría & Albuquerque, 2019). It is suggested that those phytocomplex may be environmentally less aggressive than synthetic substances (e.g. organophosphates, among others) traditionally used to control insect populations, such as *Aedes aegypti* (Gupta et al., 2023). Among the advantages that can be associated with these volatile oils is their biodegradability, avoiding long-lasting permanence in the environment, and consequently, the biomagnification at different trophic levels (da Silva Sá et al., 2023). It also has distinct pharmacological mechanisms, since it is composed of a mixture of organic molecules derived from secondary plant metabolism, which can act synergistically, thus reducing the emergence of mosquito resistance (Budiman et al., 2021; Gupta et al., 2023).

The actual study focused on nanoscaling as a recent concept to disperse the essential oil in aqueous matrices (Sharma et al., 2020). Traditional larvicidal approaches use organic solvents, such as dimethyl sulfoxide (DMSO), acetone, or others to enable those lipophilic terpenoids (e.g monoterpenoids and sesquiterpenoids) in water. Nanoemulsions are advantageous delivery systems to disperse essential oil in water (Esmaili et al., 2021; Sharma et al., 2020). They have nanosized droplets (20-200 nm), and consequently higher surface area. In addition, are conventionally physically stabilized by surfactants against gravitational forces. As well as water viability and physical stability, they may improve the biological properties due to raised membrane permeability, thus, rationalizing the active and reducing possible side effects (Echeverría & Albuquerque, 2019).

4. Conclusion

The study describes for the first time the mortality effects of the nanoemulsion from *Myrciaria floribunda* essential oil from leaves in *Aedes aegypti* larvae. In addition, the nanoemulsion showed desirable monodisperse characteristics with reduced droplet size, zeta potential, and stability when occasionally exposed to higher temperatures. In this way, this work enforces the development of products of public health interest to control the vector *Aedes aegypti* and consequential transmitting arboviruses.

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10. CAPÍTULO VIII – PATENTE 1

10.1 COMPOSIÇÃO MOLUSCICIDA E CERCARICIDA, PROCESSO PARA PRODUÇÃO DA MESMA E SEU USO

Pedido Nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e Entrada na Fase Nacional do PCT

Patente depositada no Instituto Nacional de Propriedade Industrial

Em 21 de dezembro de 2020

Número do Processo: BR 10 2020 026179 7

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2020 026179 7

Dados do Depositante (71)

Nome ou Razão Social: UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

Tipo de Pessoa: Pessoa Jurídica

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Título da Invenção ou Modelo de Utilidade (54):COMPOSIÇÃO MOLUSCICIDA E CERCARICIDA, PROCESSO PARA PRODUÇÃO DA MESMA E SEU USO

Resumo: A presente invenção apresenta uma composição moluscicida e cercaricida que compreende um óleo essencial rico em monoterpenos e sesquiterpenos de baixa solubilidade em meio aquoso, através de uma formulação estável, homogênea e de baixa viscosidade. A possibilidade de dispersar substâncias de baixa hidrossolubilidade em meio aquoso permite que a composição seja utilizada para liberação de substâncias moluscicidas e cercaricidas que possuam esta característica, como o óleo essencial oriundo de folhas da espécie *Myrciaria floribunda*, obtido em alto rendimento. Além disto, a incorporação do óleo essencial em uma composição em forma de nanoemulsão óleo em água aumenta a biodisponibilidade do mesmo e o protege contra deterioração. Ainda, a presente invenção apresenta o processo de produção desta composição e seu uso Figura a publicar: 1a

11. DISCUSSÃO GERAL E CONSIDERAÇÕES FINAIS

A análise química por cromatografia a gás do óleo essencial de folhas *Ocotea indecora* permitiu a identificação do sesquiterpeno sesquirosefurano como metabólito amplamente majoritário representando 81,4% do total. Em relação ao óleo essencial das folhas de *Myrciaria floribunda*, o perfil químico apresentou nerolidol, zonarano, β-selineno e 1,8-cineol como metabólitos majoritários totalizando 47,67%. Estas informações sobre os perfis químicos dos óleos essenciais são necessárias para auxiliar na diferenciação entre espécies, ser utilizado como parâmetro de controle da qualidade das plantas em análises futuras e descrever na literatura sobre estas plantas nativas e pouco conhecidas presentes no Parque Nacional da Restinga de Jurubatiba.

Os estudos de nanoemulsificação dos óleos essenciais de *Ocotea indecora* e *Myrciaria floribunda* por baixo aporte energético permitiram a obtenção de nanoemulsões de diâmetro de gotícula reduzido, monodispersas e com reflexo azulado característicos da dispersão da luz por gotículas em suspensão em sistemas nanoemulsionados. Além disso, a otimização por planejamento fatorial permitiu estabelecer que nas condições avaliadas a proporção 1:4 entre surfactantes e óleo essencial e 500 RPM para nanoemulsão de *Ocotea indecora*. Estudos de estabilidade permitiram avaliar a longo prazo a manutenção dos parâmetros coletivos tamanho de gotícula e índice de polidispersão das nanoemulsão de *Ocotea indecora* por 365 dias e 200 dias para nanoemulsão de *Myrciaria floribunda*.

Em relação ao potencial bioativo, a nanoemulsão de *Ocotea indecora*, induziu a mortalidade e alterações morfológicas em larvas de *Aedes aegypti* e moluscos *Biomphalaria glabrata*. Adicionalmente, o estudo de docagem molecular do metabólito majoritário sesquirosefurano na enzima acetilcolinesterase sugere uma possível ação inibitória. Entretanto, não foram observados letalidade em insetos não alvo *Apis mellifera*, sugerindo uma possível seletividade do nanoproduto.

No que diz respeito a *Myrciaria floribunda* a nanoemulsão induziu a mortalidade em larvas de *Aedes aegypti*, entretanto em menor intensidade do que em relação a *Ocotea indecora*. Para o controle da esquistossomose, a nanoemulsão de *Myrciaria floribunda* apresentou amplo espectro de atividade no ciclo de vida de moluscos *Biomphalaria glabrata*, tal como em cercarias, forma infectante humana da esquistossomose mansonica. Em adição, não apresentou letalidade significante em *Danio rerio*. Portanto, os dados descritos neste trabalho revelam o potencial biotecnológico de produtos naturais, mais especificamente óleos essenciais de plantas nativas da Mata Atlântica do Brasil, para o desenvolvimento sustentável de praguicidas alternativos, orgânicos, biodegradáveis, eficientes, menos tóxicos e seletivos para organismos não alvo.

Os resultados encontrados neste estudo levantam questões sobre o aumento de escala de produção para os pesticidas da biodiversidade. Foi observado uma constância na produção de sesquirosefurano no óleo essencial de *Ocotea indecora* de 2019 a 2022. Entretanto, novos estudos devem ser realizados de forma a esclarecer e padronizar o cultivo desta espécie em regiões de restinga e/ou fora dela, e por consequente, garantir a reprodutibilidade dos perfis químicos dos óleos essenciais das espécies vegetais *Myrciaria floribunda* e *Ocotea indecora* observados. Uma outra abordagem seria avaliar a possibilidade de síntese de sesquirosefurano em laboratório a partir da oxidação do precursor biossintético farnesol e possíveis funcionalizações da estrutura com novos grupamentos de forma a obter patente de processo, bem como das novas estruturas derivadas do furanoterpenoide supracitado.

Em relação a *Myrciaria floribunda*, devido ao óleo essencial ser fitocomplexo a avaliação moluscicida dos constituintes que compõem o óleo essencial devem ser observados separadamente e, posteriormente, misturados em proporções conhecidas, de forma a discriminar e justificar qual proporção, e de quais componentes, seriam mais adequados para representar a atividade moluscicida descrita. Esta abordagem permitirá a mimetização do óleo essencial em laboratório e desta forma, viabilizar a produção industrial do produto.

Por fim, deve ser levado em consideração os possíveis efeitos tóxicos do óleo essencial de *Ocotea indecora* e *Myrciaria floribunda* em linhagens celulares humanas, em modelos animais, e em outros organismos não alvo, de forma a estabelecer maiores evidências sobre a segurança humana e ambiental dos produtos. Estas propostas e perspectivas poderão direcionar e aperfeiçoar as informações descritas anteriormente.

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13. ANEXO 1

PRODUTIVIDADE ACADÊMICA 2019-2022

13.1 Insecticidal activity evaluation of *Persea venosa* Nees & Mart. essential oil and its nanoemulsion against the cotton stainer bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) and pollinator bees

Artigo publicado no periódico "Industrial Crops and Products"
Em 25 de janeiro de 2023
https://doi.org/10.1016/j.indcrop.2023.116348

13.2 Green Nanobioinsecticide of a Brazilian endemic plant for the *Aedes aegypti* control

Artigo publicado no periódico "Sustainable Chemistry and Pharmacy"
Em 11 de janeiro de 2023
https://doi.org/10.1016/j.scp.2023.100992

13.3 Glimpsing the chemical composition and the potential of Myrtaceae plant extracts against the food spoilage fungus *Thielaviopsis ethacetica*

Artigo publicado no periódico "Food Control"
Em 7 de novembro de 2022

https://doi.org/10.1016/j.foodcont.2022.109501

13.4 Potential of *Bursera graveolens* essential oil for controlling bean weevil infestations: Toxicity, repellence, and action targets

Artigo publicado no periódico "Industrial Crops and Products"
Em 24 de janeiro de 2022
https://doi.org/10.1016/j.indcrop.2022.114611

13.5 *Eugenia sulcata* (Myrtaceae) Nanoemulsion Enhances the Inhibitory Activity of the Essential oil on P2X7R and Inflammatory Response In Vivo

Artigo publicado no periódico "Pharmaceutics"
Em 21 de abril de 2022
https://doi.org/10.3390/pharmaceutics14050911

13.6 Characterization of the essential oil from *Annona acutiflora* and its nanoemulsion for the *Aedes aegypti* control

Artigo publicado no periódico "Journal of Essential Oil Research"
Em 6 de agosto de 2021
https://doi.org/10.1080/10412905.2021.1966847

13.7 Effects of nanoemulsion and essential oil from the leaves of *Ocotea elegans* against *Dysdercus peruvianus*

Artigo publicado no periódico "Research, Society and Development"
Em 19 de setembro de 2020
http://dx.doi.org/10.33448/rsd-v9i10.8424

13.8 Effects of *Zanthoxylum caribaeum* essential oil against cotton bug *Dysdercus peruvianus*

Artigo publicado no periódico "Research, Society and Development"
Em 15 de agosto de 2020
http://dx.doi.org/10.33448/rsd-v9i9.7152

13.9 Green Insecticide against Chagas disease: effects of essential oil from *Myrciaria floribunda* (Myrtaceae) on the development of *Rhodnius prolixus* nymphs

Artigo publicado no periódico "Journal of Essential Oil Research"
Em 27 de junho de 2019
https://doi.org/10.1080/10412905.2019.1631894